Fatty Acid Remodeling of GPI-anchored Proteins Is Required for Their Raft Association

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Whereas most of the cellular phosphatidylinositol (PI) contain unsaturated fatty chains and are excluded from rafts, GPI-anchored proteins (APs) usually contain two saturated fatty chains in their PI moiety, and they are typically found within lipid rafts. However, the origin of the saturated chains and whether they are essential for raft association are unclear. Here, we report that GPI-APs, with two saturated fatty chains, are generated from those bearing an unsaturated chain by fatty acid remodeling that occurs most likely in the Golgi and requires post-GPI-attachment to proteins (PGAP)2 and PGAP3. The surface GPI-APs isolated from the PGAP2 and -3 double-mutant Chinese hamster ovary (CHO) cells had unsaturated chains, such as oleic, arachidonic, and docosatetraenoic acids in the sn-2 position, whereas those from wild-type CHO cells had exclusively stearic acid, a saturated chain, indicating that the sn-2 chain is exchanged to a saturated chain. We then assessed the association of GPI-APs with lipid rafts. Recovery of unremodeled GPI-APs from the double-mutant cells in the detergent-resistant membrane fraction was very low, indicating that GPI-APs become competent to be incorporated into lipid rafts by PGAP3- and PGAP2-mediated fatty acid remodeling. We also show that the remodeling requires the preceding PGAP1-mediated deacylation from inositol of GPI-APs in the endoplasmic reticulum.

INTRODUCTION

GPI is a glycolipid widely found among eukaryotes that anchors many proteins to the outer leaflet of the plasma membrane (McConville and Ferguson, 1993; Ferguson, 1999; Ikezawa, 2002). The carboxy termini of precursor proteins are processed and covalently attached with GPI in the endoplasmic reticulum (ER), resulting in GPI-anchored proteins (APs). The common core structure of GPI is conserved among all species. GPI-APs are transported to the plasma membrane, and they are associated with lipid rafts, consisting mainly of sphingolipids and cholesterol (Simons and Ikonen, 1997), although there is still some controversy about the definition and existence of physiologically relevant lipid rafts (Simons and Toomre, 2000; Hancock, 2006). The lipid rafts modulate various biological functions of GPI-APs, such as signal transduction, endocytosis, and apical sorting (Brown and Rose, 1992; Harder and Simons, 1997; Tansey et al., 2000; Marquardt et al., 2005). Whereas most of the cellular phosphatidylinositol (PI) contains unsaturated fatty chains at sn-2 position (Kerwin et al., 1994), the PI moiety of mammalian GPI-APs consists mainly of saturated chains (McConville and Ferguson, 1993; Redman et al., 1994; Brewis et al., 1995; Treumann et al., 1995; Benting et al., 1999), although GPI-APs with unsaturated fatty chain at sn-2 position were also reported on some cells and molecules (Roberts et al., 1988; Wong and Low, 1992; Redman et al., 1994; Treumann et al., 1995). The saturated fatty chains in GPI are implied in the association with the liquid-ordered raft membrane by analogy of other phospholipids (Schoeder et al., 1994; Raza Shaikh et al., 2003) and palmitoylated proteins (Melkonian et al., 1999; Moffett et al., 2000; Zhang et al., 2000). However, the origin of saturated chains in GPI is unclear along with whether they are essential for raft association.

One idea to explain how saturated chains in mammalian GPI are synthesized is that GPI may use lipid remodeling. In lower microorganisms, processing of lipid moieties of GPI, called lipid remodeling, is well known, but the physiological significance is still an outstanding issue. In Saccharomyces cerevisiae, sn-2–linked acyl chain in PI moiety is changed to C26:0 chain and/or diacylglycerol moiety is replaced with ceramide after GPI is transferred to proteins and inositol-linked palmitic acid is removed (Sipos et al., 1997; Bosson et al., 2006). Bloodstream form of Trypanosoma brucei, a causative agent of African sleeping sickness, exchanges both acyl...
chains in PI of GPI strictly to myristate (C14:0) in the ER before attachment of GPI to variant surface glycoproteins (Morita et al., 2000). In contrast, very little is known so far in mammalian cells except for the presence of deacylation of inositol-linked acyl chain after conjugation of GPI with proteins in the ER. We previously reported Chinese hamster ovary (CHO) cells with defects in Golgi/ER-resident post-mammalian cells except for the presence of deacylation of lipase A (PLA) activity by the time when newly synthesized sequential cleavages of GPI took place in the Golgi and cell GPI-APs into the culture medium. We found that two sequential cleavages of GPI took place in the Golgi and cell surface in PGAP2-deficient CHO cells (Tashima et al., 2006). First, GPI-APs were converted to lyso-GPI-APs by phospholipase A (PLA) activity by the time newly synthesized GPI-APs exit from the trans-Golgi network. Second, this event was followed by cleavage of lyso-GPI-APs by phospholipase D (PLD) activity on the cell surface, resulting in secretion of GPI-APs. Although we have not identified the surface PLD yet, it seemed different from GPI-PLD, a well-known PLD abundant in serum, and might be specific for lyso-form GPI-APs because normal GPI-APs carrying two saturated chains are resistant to this PLD activity. We hypothesized that lyso-GPI-AP was an intermediate in the process of fatty acid remodeling, which ought to be reacylated with a saturated chain in the presence of PGAP2. We expected that the PLA activity involved in the fatty acid remodeling is lost in PGAP2-deficient cells, GPI-APs would remain unremodeled and have two lipid chains as normal GPI-APs, resulting in recovery of the surface expressions due to the resistant to the surface PLD. To prove our hypothesis, here we report establishment and characterization of double mutant cells from PGAP2-deficient CHO cells, in which surface expression of GPI-APs was indeed restored, and we reveal the significance of fatty acid remodeling on the raft association of GPI-APs.

MATERIALS AND METHODS

Cells, Antibodies, and Materials

All cell lines used in this study were derivatives of CHO cells, and they are summarized in Table 1. Wild-type 3B2A and GD3S-C37, PGAP2-deficient C84 and AM-B (Tashima et al., 2006), and PGAP1-deficient C10 (Tanaka et al., 2004) cells were described previously. All cell lines were cultured in Ham’s F-12 medium (Sigma-Aldrich, St. Louis, MO) supplemented with 10% fetal calf serum and 600 μg/ml Geneticin (G-418; Invitrogen, Carlsbad, CA) with various combinations of 6 μg/ml puromycin, 600 μg/ml hygromycin, 6 μg/ml blasticidin, and 250 μg/ml zeocin for maintenance of transfected plasmids. 3B2A and DM2&3-C2 cells stably expressing HFGF-CD59 were established by transfection of linearized pME-pgkpuro-HGF-CD59 and pME-pkgkhyg-HGF-CD59, respectively. After selection with antibiotics, cells expressing high levels of HFGF-CD59 were sorted in FACS Vantage BD Biosciences, San Jose, CA) after staining with anti-FLAG antibody and limiting-dilution to obtain clones. DM2&3-C2 cells restored with rPGAP2 and rPGAP3 were established under the selection with zeocin by transient expression of mCAT1, a receptor for ectropic retroviruses, by using pME-KmCAT1 (a gift from Dr. K. Ohishi, Osaka University, Osaka, Japan), followed by infection of retrovirus that was produced in FLAT-E packaging cells (a gift from Dr. T. Kitamura, University of Tokyo, Tokyo, Japan) transduced with pMSCV-rPGAP2-zeo-hPGAP3.

The commercial antibodies used here were mouse monoclonal antibodies against transferrin receptor (Zymed Laboratories, South San Francisco, CA), caveolin1, flotillin1 and syndecan, (BD Biosciences, San Jose, CA) andFLAG-tagged mouse monoclonal antibody against fibronectin II (Santa Cruz Biotechnology, Santa Cruz, CA). Secondary antibodies were used horse serum-peroxidase (HRP)- conjugated anti-mouse IgG, anti-rabbit IgG (GE Healthcare, Little Chalfont, Buckinghamshire, United Kingdom), and anti-goat IgG (Promega, Madison, WI), phycocerythrin (PE)-conjugated anti-mouse IgG (BD Biosciences PharMingen, San Diego, CA), and Alexa488-conjugated anti-mouse IgG and Alexa594-conjugated goat anti-rabbit IgG (Invitrogen). Mouse IgG1 monoclonal antibody against hamster urokinase-type plasminogen activator receptor (uPAR) (clone 5D6) was made in this laboratory by immunizing detergent-resistant membrane fragments obtained from CHO cells and by conventional method. The reactive protein to this antibody was purified by antibody-coupled affinity column by using Affi-Gel Hz (BioRad, Hercules, CA), sequenced by tandem mass spectrometry (MS/MS), and identified as hamster uPAR. Mouse monoclonal antibodies against decay accelerating factor (DAF) (clone 1A10) and CD59 (clone 5H8) were described previously (Tashima et al., 2006). Phosphatidylinositol-specific phospholipase C (PI-PLC) was purchased from Invitrogen.

Plasmid Construction

The expression vector of human PGAP3/PLERD1, constructed in pCMV-SPORT6, was purchased from Mammalian Gene Collection (clone ID 3855206: National Center for Biotechnology Information accession no. BC010652). Yeast PER1, amplified by polymerase chain reaction (PCR) (primers CTCTCGTCCCCGCTGTAGACGCGTGTCCAGCTTGAACTTGTCCTCT) and GCTGACGCGTGTCCAGCTTGAACTTGTCCTCT) into EcoRI and MluI sites of pME-3HA (a gift from Dr. K. Ohishi). All fragments obtained by PCR were confirmed by sequencing. pMSCV-rPGAP2-zeo-hPGAP3 was constructed by ligation of EcoRI-XbaI fragment of rat PGAP2 from pME-PyrPGAP2 (Tashima et al., 2006), XbaI-BstZ17I fragment of SV40-zeo from pcDNA3.1-zeo (Invitrogen), and SspI–BamHI fragment of CMV-hPGAP3 (Tashima et al., 2004). PGAP3 were established under the selection with zeocin by transient expression-diluted to obtain clones. DM2&3-C2 cells restored with PGAP2 and PGAP3 were established by transfection of linealized pME-pgkpuro-HGF-CD59 and pME-pkgkhyg-HGF-CD59 were established by subcloning EcoRI-NotI fragment of HFGF-CD59 into pME-pgkpuro and pME-pkgkhyg (gifts from Dr. K. Ohishi), respectively.

Table 1. CHO cell lines used in this study

<table>
<thead>
<tr>
<th>Cells</th>
<th>Parent cells</th>
<th>Derivation</th>
<th>Phenotype</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>CHO-K1</td>
<td>CHO-K1</td>
<td>Stably expressing CD59 and DAF</td>
<td>Wild type</td>
<td>Tanaka et al. (2004)</td>
</tr>
<tr>
<td>GD3S-C37</td>
<td>3B2A</td>
<td>Stably expressing GD3 synthase (and GD3 as the result)</td>
<td>PGAP1-deficient</td>
<td>Tashima et al. (2006)</td>
</tr>
<tr>
<td>C84</td>
<td>GD3S-C37</td>
<td>Treated with EMS</td>
<td>PGAP1-deficient</td>
<td>Tashima et al. (2006)</td>
</tr>
<tr>
<td>DM2&amp;3-C2</td>
<td>C84</td>
<td>Treated with EMS</td>
<td>PGAP2-deficient</td>
<td>Tashima et al. (2006)</td>
</tr>
<tr>
<td>DM1&amp;2-C17</td>
<td>C84</td>
<td>Treated with EMS</td>
<td>PGAP2&amp;3-deficient</td>
<td>This study</td>
</tr>
<tr>
<td>F21.3.8.10</td>
<td>CHO-K1</td>
<td>Stably expressing CD59, DAF and many PIG genes involved in GPI biosynthesis</td>
<td>Wild type</td>
<td>Tashima et al. (2006)</td>
</tr>
<tr>
<td>AM-B</td>
<td>F21.3.8.10</td>
<td>Treated with EMS</td>
<td>PGAP2-deficient</td>
<td>This study</td>
</tr>
<tr>
<td>DM1&amp;2-C14</td>
<td>AM-B</td>
<td>Treated with EMS</td>
<td>PGAP1&amp;2-deficient</td>
<td>This study</td>
</tr>
</tbody>
</table>
Cloning of Chinese Hamster PGAP3

A partial cDNA of hamster PGAP3 was obtained from CHO cDNA library by nested PCR with two degenerated primer sets of de1U (5'-CARTTYCA- GGNAARTGGCCTNT-3') and de3L (5'-ARRTANRSWTRTCTYTC-3'), and de2U (5'-TTTCAAYACMNMGYAYACNGA-3') and de3L (5'-ATRTEC- CADD) only by using the sequence of obtained partial hamster PGAP3 cDNA, several new primers were designed, and PCRs with these primers and those corresponding to vector portion were done to obtained longer cDNA containing full coding region.

Analysis of PGAP3 mRNA by Sequencing and Real-Time Reverse Transcription (RT)-PCR

Total RNAs were obtained from GD3S-C7 (wild-type cells) and DM2&3-C2 (double-mutant cells) by using TRIZol (Invitrogen). First strand cDNAs were synthesized by PrimeScript RTase (TaKaRa, Tokyo, Japan) with mixtures of oligo(dT) and random primers as manufacturer's protocol. Real-time PCR was performed with SYBR Premix Ex Taq (TaKara) and Prism 7900HT (Applied Biosystems, Foster City, CA). Two sets of primer, set A (5'-AGCTAGAC- CAATCTCGGTCTCCT-3' and 5'-GGGACGAAGTCTGCTGTGAT-3' and set B (5'-CATACAGCAGCATTCACTG-3' and 5'-AGGAAAGACCGA- GAACCC-3') were used for hamster PGAP3, and 5'-GCTTGGCCTGAT- GGCTTGGC-3' and 5'-TTCGGGCTGTGCTGGTGAAG-3' were cloned into pBluescriptII vector. The sequence of six independent clones revealed that two of six clones had 19 base pairs insertion (TTCCTGCTGCTGTTCACG) between 60th and 61th amino acids. To examine whether this insertion was specific to DM2&3-C2 cells, the region of 190 base pairs containing this site from both GD3S-C7 and DM2&3-C2 RNAs was amplified by PCR (35 cycles) with primer set A (above) and amplified DNA fragments were applied to 3% agarose gel.

Fluorescence-activated Cell Sorting (FACS) Analysis

Cells (5 x 10^6) were suspended in 0.4 ml of Opti-MEM I (Invitrogen) with 20–25 μg of the indicated plasmids and electroporated at 260 V and 1000 μF by using a Gene Pulser (Bio-Rad). Two days after transfection, cells were stained with antibodies against CD59, transferrin receptor (TfR), syntaxin6, and ribosome inactivating protein (RIP) using a Gene Pulser (Bio-Rad). Two days after transfection, cells were treated with PI-PLC, and PI-PLC–sensitive clones were retained longer cDNA containing full coding region.

Establishing Mutant Cells

C84 and AM-B cells were treated with 0.24 mg/ml ethyl methanesulfonate (EMS) for 24 h, cultured for 1 wk, and CD59-positive cells were enriched twice by auto MACS (Miltenyi) Biotec, Auburn, CA) with biotinylated 5H8 anti-CD59 and streptavidin microbeads and sorted in FACS Vantage after staining with 5H8 anti-CD59 and PE-conjugated anti-mouse IgG, and limiting-diluted clones were obtained. Two days after transfection, cells were treated with PI-PLC, and PI-PLC–sensitive clones were retained longer cDNA containing full coding region.

Electrospray Ionization Mass Spectrometric (ESI-MS) Analysis of Phospholipids

The ESI-MS analyses were performed using a 4000Q TRAP, quadrupole-linear ion trap hybrid MS (Applied Biosystems/MD Sciex, Foster City, CA) with a LC-10AD VP high-performance liquid chromatography system combined with SIL-10AD VP autosampler (Shimadzu, Tokyo, Japan). Phospholipids were subjected directly to ESI-MS analysis without LC separation by flow injection sample: typically, 4 μl of sample was applied. The mobile phase composition was acetonitrile/methanol (4:1; M:4:1), and immediately applied to a column (200-μl bed) of S10 silica ( Sigma-Aldrich) packed in a glass Pasteur pipette plugged with glass wool and washed with 1 ml of C/M1:4 three times followed by 1 ml of C/M4:1 distilled water. After washing with 0.1 ml of C/M:4:1 five times, PI was eluted with 0.2 ml of C/M:4:1 five times, dried up under N2 stream, and redissolved in 100 μl of C/M:2.3 for mass spectrometry.

Immunofluorescence Microscopy

CHO cells transfected with mPE-PGAP3/PERLD1-3HA by using Lipopectame 2000 (Invitrogen) were fixed with 4% paraformaldehyde in PBS for 20 min at room temperature. After washing with 40 μm ammonium chloride in PBS, the cells were permeabilized with PBS containing 0.1% TX-100 and 2.5% goat serum for 1 h at room temperature and stained with rabbit anti-GPAP3 and mouse anti-HA antibodies followed by Alexa 594-conjugated goat anti-rabbit IgG and Alexa 488-conjugated goat anti-mouse IgG antibodies. The pictures were taken by BX50 microscope (Olympus, Tokyo, Japan) and Vi-6010 charge-coupled device (CCD) camera (Keyence, Osaka, Japan).

Detergent-resistant Membrane (DRM) Fractionation

Cells were detached from the plate by using PBS containing 2.5 mM EDTA and 0.5% bovine serum albumin. After centrifugation, the cell pellet was resuspended in 50 mM Tris–HCl buffer (pH 7.4) containing 1% Nonidet P-40 and digitonin, and incubated with DIG oligonucleotide probe at 4°C for 16–18 h. After washing the column, 1 ml of 100 mM NaCl, and 5 mM EDTA containing protease inhibitors supplemented with 1% TX-100, incubated for 20 min on ice, and homogenized by a potter-type Teflon homogenizer. The volume of incubation was ∼20 ml times the weight of cell pellet (typically 25–30 million cells/ml lysis buffer). One milliliter of lysate was mixed with 1 ml of 80% (wt/vol) sucrose in MBS-E, transferred to a centrifuge tube for SW41, overlaid with 7 ml of 30% and 2 ml of 5% sucrose in MBS-E, ultracentrifuged at 38,000 rpm for 16–18 h at 4°C, and...
Figure 1. PGAP2&3 double-mutant cells are defective in fatty acid remodeling of GPI-APs. (A) Restored surface expression of CD59 on double-mutant DM2&3-C2 cells derived from C84 PGAP2-deficient cells. C84 PGAP2-single-mutant (parent for DM2&3-C2; top), DM2&3-C2 double-mutant (middle), and GD3S-C37 wild-type (parent for C84; bottom) cells were stained with antibody 5H8 against human CD59 and analyzed by FACS. A dotted line in C84 cells represents background staining without anti-CD59 antibody. (B) MS analysis of PI of epitope-tagged CD59 isolated from the plasma membrane. Top (A) and bottom (B), wild-type 3B2A and DM2&3-C2 cells, respectively. Alk and acyl represent alkyl-acyl (or alkenyl-acyl) and diacyl glycerols, respectively, and figures before and after colon represent the number of total carbons and unsaturated bonds, respectively. Neutral loss scanning of 277 Da (phosphorylinositol/H+11001NH4) was used for the detection of positive molecular weight-related ions. (C) Summary of the MS/MS analysis (Supplemental Figure S1) of various precursor PI species of...
fractionated from the top using Pison Gradient Fractionator (BioComp Sys-
tems) with each fraction of 1 ml (total 11 fractions). Fractions 2–4, 5–8, and 9–11 were combined as top, middle, and bottom fractions, respectively. Aliquots of each fraction were mixed with 6× sample buffer, without a reducing reagent, and applied to 4–20% gradient SDS-PAGE.

**Fractionation of GPI-APs with Octyl-Sepharose**

3B2A, C10 (PGAP1 single-deficient), and DM1&2-C17 (PGAP1 and PGAP2
double-mutant) cells (5 × 10⁷) were transfected with pME-Neo2dH-VSVGs-
FF-mEGFP-GPI (Tashima et al., 2006). Cells were cultured for half a day at 37°C followed by 1.5 d at 32°C and harvested. The cell pellets were lysed in 5 ml of lysis buffer (60 mM 1-oxyl-β-γ-glucoside and protease inhibitors in TBS-E) for 1 h at 4°C. After centrifugation at 15,000 rpm at 4°C for 15 min, the supernatants were incubated with M2 anti-FLAG beads overnight. The im-
munoprecipitates were washed twice with 0.5 ml of buffer (30 mM 1-oxyl-
β-n-glucoside in TBS-E), twice with 0.5 ml of buffer (20 mM Tris- HCl, pH 7.4, and 0.1% Nonidet P-40), and twice with 0.5 ml of buffer (20 mM Tris- HCl, pH 7.4, and 0.03% Nonidet P-40). FLAG-mEGFP-GPI was eluted with 100 µl of 0.03 M ammonium acetate containing 1 mg/ml FLAG-peptide (Sigma-Alrich) and 0.03% Nonidet P-40 four times (total volume 400 µl). One hundred microliters of the eluate was mixed with 100 µl of buffer (0.1 M ammonium acetate, 0.03% Nonidet P-40, and 1% triton X-100), loaded onto an octyl-FF column (GE Healthcare), and chromatographed in AKTA (GE Healthcare) at a flow rate of 0.5 ml/min by using a 5–40% gradient of 1-propanol. Fractions of 0.7 ml were collected, dried, and subjected to SDSPAGE/Western blotting by using M2 antibody against FLAG-tag followed by HRP-conjugated anti-
mouse IgG antibody.

**RESULTS**

**PGAP2 and PGAP3 Double-Mutant Cells Are Defective in Fatty Acid Remodeling of GPI-APs**

After a new round of mutagenesis of PGAP2 single-mutant CHO cells with ethylmethane sulfonate, we obtained cells with restored GPI-APs expression, such as CD59, DAF (CD55), and uPAR, and termed one clone of the double-
mutant cell, DM2&3-C2 (Figure 1A). We termed the gene defective in DM2&3-C2 as PGAP3. Using this double-mutant and wild-type 3B2A cells, we compared the compo-
sition of PI moiety of the surface GPI-APs. For this, we established cells with stable expression of epitope-tagged CD59, HFGF-CD59. HFGF-CD59 was extracted from the plasma membrane, purified with glutathione-beads, applied to SDS-
PAGE, transferred to PVDF membrane, and subjected to sodium nitrite treatment to release PI from GPI by means of deamination of glucosamine residue (Fontaine et al., 2003). The released PI was analyzed by MS in positive ion mode with neutral loss scanning of 321 Da (phosphorylinositol

**PGAP3, the Gene Responsible for the Second Defect in DM2&3-C2, Encodes a Protein Mainly Expressed in the Golgi**

Some of us recently showed that yeast PER1 (Ng et al., 2000) is involved in lipid remodeling of GPI-APs, and we identi-
fied per1-like domain containing 1 (PERLD1) as the mamm-
alian homologue (Fujita et al., 2006a). Mammalian PERLD1 encodes a protein of 320 amino acids, which has 28% iden-
tity with yeast PER1. Transfection of either human PERLD1 or yeast PER1 decreased the surface expression of CD59 and uPAR on DM2&3-C2 to the levels on PGAP2 single-mutant cells (Figure 3A), indicating that PERLD1 was the PGAP3. The expression of PGAP3/PERLD1 together with PGAP2 in DM2&3-C2 restored the association of GPI-APs with lipid rafts (Figure 3B and C), indicating that the weak association of GPI-APs with lipid rafts in DM2&3-C2 was in fact due to a lack of fatty acid remodeling.

To confirm that PGAP3 is the gene mutated in DM2&3-C2 double-mutant cells, we first cloned the normal Chinese hamster PGAP3 cDNA and sequenced it (GenBank accession no. AB288236). Hamster PGAP3 encodes a protein of 320 amino acids, which has 86, 91, and 91% identities with human, mouse, and rat PGAP3, respectively. Based on the sequence, we performed real-time RT-PCR using two different sets of primers to evaluate the amount of PGAP3 transcript in the double-mutant cells. After normalization by β-actin as an internal control, the amount of PGAP3 tran-
script in DM2&3-C2 was assessed to be <20% of that in GD3S-C37 (Figure 3D). By sequencing full-length RT-PCR

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**Figure 1 (cont.)**  epitope-tagged CD59 detected in the first negative ion mode MS (precursor ion scanning of m/z 241). Stearic acid (C18:0) was exclusively used at sn-2 position of PI in wild-type cells (left), whereas oleic (18:1), arachidonic (20:4) and docosatetraenoic (C22:4) acids were used at the same position in DM2&3-C2 cells (right). Alkyl chains are underlined.

**Fatty Acid Remodeling Is Critical for the Raft Association of GPI-APs**

It is thought that saturated fatty chains in GPI-APs are important for integration into the liquid-ordered raft membrane (Schroeder et al., 1994; Melkonian et al., 1999; Moffett et al., 2000; Zhang et al., 2000; Raza Shaikh et al., 2003). However, clear experimental evidence has not been presented, because GPI-APs with unsaturated fatty chains at sn-2 po-
sition are rare in the plasma membrane of normal cells, except for GPI-APs with inositol-linked fatty acid (Roberts et al., 1988; Redman et al., 1994; Brewis et al., 1995; Treumann et al., 1995; Benting et al., 1999). As DM2&3-C2 cells expressed GPI-APs with unsaturated fatty chains, they are useful to assess requirement of the saturated chains for raft association of GPI-APs. Through this study, we defined raft asso-
ciation as partitioning into DRMs, which is the sole biochemical method widely used and accepted; yet, we noticed that DRM can be somewhat different from physiologically relev-
ant lipid rafts (Lichtenberg et al., 2005). We examined whether CD59, uPAR and DAF were fractionated into DRM by sucrose density gradient ultracentrifugation, after solu-
obilization with 1% TX-100 (Figure 2A) (Brown and Rose, 1992). Caveolin-1 and flotillin-1, and TfR were used as raft and nonraft markers, respectively. These markers (flotillin-1 not shown) were similarly fractionated in both wild-type GD3S-C37 and DM2&3-C2 cells. In wild-type cells, most of the GPI-APs were present in the DRM fractions (U), whereas in DM2&3-C2 cells <20% of GPI-APs were in the DRM fraction (Figure 2B). The differences between wild-type and double-mutant cells were statistically significant (two-sided p value = 0.0004 for DAF, 0.0002 for uPAR and <0.0001 for CD59 by t test). These results clearly indicated that the saturated lipid chain at sn-2 is critical for the raft association of GPI-APs.
products, we found a 19-base pair insertion (TCCTGTCT-GTCGTTTCAG) after the 60th amino acid that caused frame shift followed by appearance of immature stop codon closely downstream of this site. The last 15 base pairs of this insertion were almost the same as sequence of mouse PGAP3 intron 1 acceptor site (GTGGTGATGTTTCAG; Evidence Viewer, National Center for Biotechnology Information, Bethesda, MD), indicating the presence of splicing defect between exons 1 and 2. Moreover, we detected other minor but abnormal PGAP3 transcripts in DM2&3-C2 by RT-PCR around exons 1 and 2 junction with primer set A, which may reflect aberrant splicing (Figure 3E, asterisks). Thus, these results indicated that PGAP3 was the gene responsible for the second mutation.

Immunofluorescent microscopic analysis of HA-tagged PGAP3 expressed in CHO cells revealed its main localization in the Golgi with weaker staining of punctate unidentified organelle and the ER (Figure 3F). Together with the previous report that PGAP2 is localized mainly to the Golgi (Tashima et al., 2006), it is suggested that the fatty acid remodeling most likely occurs in the Golgi. This is in contrast to two other examples of fatty acid remodeling of GPI known to occur in the ER in bloodstream form of Trypanosoma brucei (Morita et al., 2000) and S. cerevisiae (Sipos et al., 1997; Bosson et al., 2006). Two proteins, Per1p (Fujita et al., 1997, 1999) and Gup1p (Sipos et al., 1997; Bosson et al., 2006), in yeast, which are involved in removal of sn-2 chain and incorporation of C26:0 chain, respectively, are localized to the ER.

A Defect in PGAP1 Inhibits Fatty Acid Remodeling Mediated by PGAP3 and PGAP2

We also obtained a different kind of double-mutant cell line with restored surface expression of GPI-APs from C84 and another line from PGAP2-deficient AM-B cells (Tashima et al., 2006). We found that the surface GPI-APs on these double-mutant cells were resistant to PI-PLC (Figure 4Ad), indicating that the PGAP1 gene (Tanaka et al., 2004) was defective, in addition to PGAP2. Moreover, these double-mutant clones, termed DM1&2-C14 and -C17 (data not shown), became like a PGAP2-single mutant after transfection of PGAP1 (Figure 4Ae). PGAP1 is the inositol-deacylase that removes palmitic acid linked to inositol in GPI before GPI-APs exit from the ER (Tanaka et al., 2004). The palmitylated inositol renders GPI-APs resistant to PI-PLC. That the defect in PGAP1 restored the surface expression of GPI-APs indicated either that elimination of palmitic acid from inositol is required for the subsequent removal of unsaturated sn-2 chain (three lipid chains remained); that removal of sn-2 chain occurs, but the presence of inositol-linked palmitoyl chain inhibited the action of the PLD that cleaves off the proteins (two lipid chains remained); or that PLD cleaves GPI, but the proteins are still attached to the membrane via palmitic acid (one lipid chain remained). To determine the number of chains linked to GPI-APs in DM1&2-C17 cells, we used hydrophobic chromatography with octyl-Sepharose, with which lyso GPI-AP (one lipid chain) was nicely separated from two-lipid-chain GPI-AP (Tashima et al., 2006). With a gradient concentration of 1-propanol, FLAG-mEGFP-GPI, a model GPI-AP from DM1&2-C17 cells, was reproducibly eluted in fractions 16–20 (Figure 4Bc) like three-chained FLAG-mEGFP-GPI from PGAP1-deficient cells (Figure 4Bb), whereas the same GPI-APs from wild-type cells bearing two lipid chains was eluted earlier in fractions 15–18 (Figure 4Ba), indicating that surface GPI-APs expressed on DM1&2-C17 had three lipid chains. Our attempt to directly analyze the lipid moiety by liquid chromatography-MS/MS has not been successful due to inability to recover inositol-acylated PI after nitrous acid deamination of GPI-AP. There are reports that inositol-acylated acetylated esterase (Roberts et al., 1988) and CD59 from human erythrocytes (Rudd et al., 1997), and CD52 from spleen cells (Treumann et al., 1995) had a highly unsaturated fatty acid at sn-2 position. Together, we conclude that inositol-deacylation is an essential step in fatty acid remodeling.

DISCUSSION

In this article, we report several new findings: 1) replacement of unsaturated fatty chain at sn-2 with saturated stearic acid in GPI-APs, fatty acid remodeling, occurs in mammalian cells; 2) PGAP2 and PGAP3 are involved in this process; 3) fatty acid remodeling is critical for the integration of GPI-APs into lipid rafts; and 4) inositol-deacylation by PGAP1 is an essential step in fatty acid remodeling. Figure 5 is a schematic of the current model of fatty acid remodeling of mammalian GPI-APs.

The modification of proteins by GPI is observed in eukaryotes from yeast to mammals and also plants (McConville and Ferguson, 1993; Ferguson, 1999; Ikezawa, 2002). GPI-APs are synthesized in the ER, transported to the plasma membrane via the secretory pathway through the Golgi, and incorporated into specialized microdomains, so-called lipid rafts.
rafts (Brown and Rose, 1992; Simons and Ikonen, 1997). The raft association is major and a common characteristic of GPI-APs in mammalian cells, and it is related to functions of GPI-APs in various ways. Cross-linking of GPI-APs or the ligand binding evokes the activation or association of Src-type nonreceptor tyrosine kinases (Shenoy-Scaria et al., 1992; Tansey et al., 2000). Folate receptor, a GPI-AP, is endocytosed via unique compartments, GPI-AP enriched early endosomes.

Figure 3. PGAP3, the gene responsible for the second defect in DM2&3-C2, encodes a protein mainly expressed in the Golgi. (A) FACS analysis of the surface expression of CD59 (a–c and g) and uPAR (d–f) on DM2&3-C2 cells transiently transfected with control (a and d), human PGAP3 (b and e), or yeast PER1 (g) expression vector and on C84 PGAP2-single-mutant cells transfected with control vector (c and f). Dotted lines (a and d) represent background staining without antibodies. (B) Proteins in DM2&3-C2 cells stably transfected with PGAP2 and PGAP3 were fractionated as described in Figure 2. Fractions obtained from two independent experiments showed a quite similar pattern to fractions obtained from wild-type cells, as shown in Figure 2. (C) The percentages of proteins in rafts were quantified as described in Figure 2. Results are means and standard deviations from two independent experiments. (D) The amounts of PGAP3 mRNA expressed in GD3S-C37 (white bars) and DM2&3-C2 (black bars) were evaluated by real-time RT-PCR. Two different primer sets (set A, left and set B, right) were used for PCR of PGAP3, and the amount was normalized by the amount of β-actin. The relative ratio was calculated with the amount in GD3S-C37 being set as 1. Bars represent SD of n = 3. (E) PGAP3 cDNAs obtained from GD3S-C37 (lane 1) and DM2&3-C2 (lane 2) were amplified by PCR with primer set A. Twenty base-pair ladder maker DNA was used as reference (lanes M). Asterisks, two abnormal bands in lane 2. (F) Expression of PGAP3 in the Golgi. DM2&3-C2 cells transiently transfected with HA-tagged PGAP3 were fixed, permeabilized with TX-100, and stained for GPP130, a Golgi protein, (left) and HA (middle). (Right) Merged profile showing colocalization of GPP130 and PGAP3.
dosome compartments (Sabharanjak et al., 2002), and the replacement of GPI with transmembrane and cytoplasmic portions of low-density lipoprotein receptor causes much less efficient uptake of 5-methyl tetrahydrofolate (Ritter et al., 1995). The compartmentalization of ephrin-A, a GPI-anchored ligand, is essential for the normal response of growth cone to its receptor, EphA (Marquardt et al., 2005). GPI-APs are selectively transported to apical surface in polarized cells (Brown and Rose, 1992). Although these characteristics are thought to be dependent upon their raft association, little is known about how GPI-APs are associated with lipid rafts.

From the studies of raft-associated phospholipids (Schroeder et al., 1994; Raza Shaikh et al., 2003) and palmitoylated proteins (Melkonian et al., 1999; Moffett et al., 2000; Zhang et al., 2000), straight acyl chains are strongly proposed to be important for their raft integrations. The present study showed for the first time that it is also true for GPI-APs, because GPI-APs with unsaturated fatty chains brought by the defect of the remodeling were associated with lipid rafts much weaker than normal rafts. Association of GPI-APs with lipid rafts was assessed by their recovery into DRM after sucrose density gradient centrifugation. Clearly, fatty chains are major determinants for DRM association of GPI-APs. Whether the decreased DRM association of unremodeled GPI-APs has any effect in various functions of GPI-APs discussed above is very interesting and is now experimentally testable.

Recent several reports, including the present report, have revealed components and their functions in the lipid remodeling in yeast and mammalian cells. There are similarity and difference between two systems. First, homologous components are used. PGAP3 in mammalian system and PER1p (Fujita et al., 2006a) in yeast are involved in generation of lyso-GPI-APs. PGAP3 and PER1p are functionally interchangeable between both species. PGAP3 does not have a lipase-like motif but has several serine and histidine residues that are conserved in active sites of PLA2 family (Fujita et al., 2006a). It is clear that PGAP3 is required for PLA2 activity; however, we still do not know whether PGAP3 is an

Figure 5. Current model of fatty acid remodeling of GPI-APs in mammalian cells. GPI is synthesized from PI with an unsaturated fatty acid at sn-2 position and has an acylated (usually palmitoylated) inositol. Immediately after attachment of GPI to newly synthesized proteins, inositol-linked palmitate is removed by PGAP1, the deacylase, in the ER (step a). The inositol-deacylation is essential for subsequent steps in fatty acid remodeling in the Golgi. An unsaturated fatty chain at sn-2 is removed, resulting in lyso-GPI-APs. PGAP3 is involved in this step (step b). Reacylation of sn-2 with stearic acid follows and PGAP2 is involved in this step (step c). A donor of stearoyl chain is yet to be determined. GPI-APs that have undergone fatty acid remodeling are competent for the integration into lipid rafts (step d). In PGAP1-deficient cells, GPI-APs before inositol deacylation are expressed on the cell surface without any further modification (step e). In PGAP3-deficient cells, GPI-APs carrying an unsaturated fatty chain at sn-2 are expressed on the cell surface (step f). In PGAP2-deficient cells, lyso-form GPI-APs are transported to the cell surface (step g), rapidly cleaved by surface (lyso-) PLD, and secreted (step h). PGAP1, PGAP3, and PGAP2 are epistatic in this order.

Figure 4. A defect in PGAP1 inhibits fatty acid remodeling mediated by PGAP3 and PGAP2. (A) Surface expression of CD59 was examined by FACS in AM-B, another PGAP2-single-mutant (a), wild-type 3B2A (b), C10 PGAP1-single mutant (c), and DM1&2-C14 (d–f) cells. DM1&2-C14 cells were either nontransfected (d), transiently transfected with PGAP1 expression vector (e), or cotransfected with PGAP1 and PGAP2 expression vectors (f). A dotted line (a) represents background staining. Thick gray lines (b–f) indicate the cells treated with PI-PLC. (B) 3B2A wild-type, C10 PGAP1-single-mutant, and DM1&2-C17 cells were transiently transfected with VSVGts-FF-mEGFP-GPI expression vector and the processed FLAG-mEGFP-GPI was collected from the cell lysates with anti-FLAG beads and eluted with a FLAG-peptide (Tashima et al., 2006). FLAG-mEGFP-GPIs were chromatographed in an octyl-FF column with a 5–40% gradient of 1-propanol. Fractions were subjected to SDS-PAGE and analyzed by Western blotting with an anti-FLAG antibody.
enough amount of ER-form of GPI-APs for lipid chain analysis is necessary for more precise localization of the site of the synthesis of PI structure in mammalian GPI-APs still in the ER (Bosson et al., 2006) whereas in yeast the ER is the main site of the remodeling. Although mammalian acyltransferase for the remodeling has not been identified, several mammalian proteins belong to this family, and one of them may function in the remodeling.

Second, both systems use the particular fatty acid for the replacement, i.e., C18:0 fatty acid in CHO cells and C26:0 in yeast, indicating that acyltransferases have narrow substrate specificities, yet the possibility that only particular lipids are available in the lumen of the ER/Golgi has not been excluded.

Third, in CHO cells the remodeling is thought to take place mainly in the Golgi, based on the localization of PGAP2 (Tashima et al., 2006) and PGAP3, although we cannot exclude the possibility that it dose occur in the ER, whereas in yeast the ER is the main site of the remodeling (Bosson et al., 2006; Fujita et al., 2006b). Although determination of PI structure in mammalian GPI-APs still in the ER is necessary for more precise localization of the site of the remodeling, it is not practical at the moment to collect an enough amount of ER-form of GPI-APs for lipid chain analysis. It is interesting to notice that the integration of GPI-APs into rafts also takes place in the same compartments where GPI-APs undergo the remodeling, the ER in yeast (Bagnat et al., 2000) and the Golgi in mammals (Brown and Rose, 1992). The observation that GPI-APs before the remodeling associate very weakly with rafts puts a concern about the theory that lipid rafts are formed in the Golgi in mammalian cells, because the theory is based upon both the fact that GPI-APs become competent for the recovery into detergent resistant membranes in the Golgi during their transports and an assumption that GPI-APs are always integrated into rafts if rafts are present there. Now, we have another possibility that lipid rafts are formed in earlier organelles such as ER, but that only GPI-APs that undergo the remodeling in the Golgi are able to associate with rafts. Our separate study demonstrated that GPI-APs expressed in PGAP1 mutants have considerable affinity for lipid rafts, although weaker, compared with those expressed in wild-type cells, as judged by conventional DRM fractionation method (Maeda and Kinoshita, unpublished observation). As the lipid structure of GPI-APs in PGAP1 mutant cells are unchanged along all the way from the ER to the cell surface, PGAP1 mutant cells will be useful to reevaluate where lipid rafts are formed.

Fourth, the transport of Gas1p from the ER to the Golgi was substantially delayed in perl-deleted yeast (Ng et al., 2000; Fujita et al., 2006b), whereas we did not observe significnt transport delay of GPI-APs from the ER to the surface in CHO DM2&3-C2 cells (Maeda and Kinoshita, our unpublished observation). It is suggested that the raft association may be required for the efficient exit from the ER in yeast but may not be required for exit from the Golgi in mammalian cells. Because the defect of either PGAP1 (Tanaka et al., 2004) or Bst1p (Elrod-Erickson and Kaiser, 1996; Fujita et al., 2006b) similarly caused the delayed transport of GPI-APs from the ER to the Golgi, the ER may more strictly regulate the sorting to exit site or transport vesicles than the Golgi.

GPI-APs on human erythrocytes, such as acetylcholine esterase (Roberts et al., 1988) and CD59 (Rudd et al., 1997), and CD52 on a population of spleen cells (Treumann et al., 1995), maintain inositol-linked palmitate on the cell surface, presumably due to an insufficient activity of PGAP1. Interestingly, such GPI-APs had a highly unsaturated lipid chain at the sn-2 position, indicating that fatty acid remodeling was also affected in these molecules. The results obtained from the analysis of PGAP1&2 double-mutant cells in this study was compatible with these known GPI structures, indicating that inositol deacylation by PGAP1 is an essential step in fatty acid remodeling.

Finally, raft disrupting reagents have been commonly used to study biological significance of the raft association of GPI-APs, but such reagents may exert side effects. Genetic manipulation of fatty acid remodeling should be useful in studying the function of rafts on sorting of and signaling from GPI-APs.

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Figure legend

Figure S1. MS/MS analysis of PI moiety of GPI-APs reveals the exclusive usage of stearoyl chain as a donor of fatty acid remodeling. (A and C) MS analysis of PI released from purified surface tagged CD59 of 3B2A (A) and DM2&3-C2 (C) cells (performed independently from the experiment shown in Figure 1). Upper and lower panels indicated positive ion mode (neutral loss scanning of 277 Dalton) and negative ion mode (precursor ion scanning of m/z 241), respectively. (B and D) MS/MS analysis of five precursor PI species separated in the first negative ion mode MS of 3B2A (B) and DM2&3-C2 (D) cells. Alk represents molecules with an alkyl (or alkenyl) chain at sn-1 of glycerol backbone. cPA; cyclic phosphatidic acid. FA; fatty acid. Stearic acid (C18:0) was exclusively released by the collision of PI from wild-type cells (B), whereas oleic (C18:1), arachidonic (C20:4) and docosatetraenoic (C22:4) acids were released from DM2&3-C2 cells (D).