Glycosylphosphatidylinositol anchors regulate glycosphingolipid levels

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Abstract Glycosylphosphatidylinositol (GPI) anchor biosynthesis takes place in the endoplasmic reticulum (ER). After protein attachment, the GPI anchor is transported to the Golgi where it undergoes fatty acid remodeling. The ER exit of GPI-anchored proteins is controlled by glycan remodeling and p24 complexes act as cargo receptors for GPI anchor sorting into COP II vesicles. In this study, we have characterized the lipid profile of mammalian cell lines that have a defect in GPI anchor biosynthesis. Depending on which step of GPI anchor biosynthesis the cells were defective, we observed sphingolipid changes predominantly for very long chain monoglycosylated ceramides (HexCer). We found that the structure of the GPI anchor plays an important role in the control of HexCer levels. GPI anchor-deficient cells that generate short truncated GPI anchor intermediates showed a decrease in very long chain HexCer levels. Cells that synthesize GPI anchors but have a defect in GPI anchor remodeling in the ER have a general increase in HexCer levels. GPI-transamidase-deficient cells that produce no GPI-anchored proteins but generate complete free GPI anchors had unchanged levels of HexCer. In contrast, sphingomyelin levels were mostly unaffected.

Lipid anchoring of proteins to the outer leaflet of the plasma membrane is essential for cellular function and development (1). One prominent lipid anchor is a complex glycolipid called glycosylphosphatidylinositol (GPI). The GPI anchor has the core structure phosphatidylinositol (PI)-glucosamine (GlcN)-(Mannose)-γ-phosphoethanolamine (EtN-P), which is conserved among all species. After biosynthesis, the GPI anchor is attached posttranslationally to the newly generated C terminus of certain eukaryotic proteins destined for anchoring thereby tethering the protein to the membrane surface by the glycolipid moiety. GPI-anchored proteins can be released from the cell surface by phosphatidylinositol specific phospholipases and this cleavage event can induce major conformational changes on the GPI-anchored protein itself (2).

At least three organelles, the endoplasmic reticulum (ER), Golgi, and peroxisomes, are involved in the biosynthesis and remodeling of the GPI anchor. The biosynthesis is initiated on the outer side of the ER membrane. After the first two reactions, the GPI anchor precursor is flipped and biosynthesis continues on the luminal side of the ER where the diacyl chains of phosphatidylinositol are then replaced by alkyl-acyl chains. This step is impaired in mutants of the peroxisomal alkyl phospholipid biosynthesis pathway (3).

After protein attachment, the GPI anchor undergoes complex remodeling that begins in the ER with the removal of the inositol-linked acyl chain (4) and the remodeling of the GPI glycan part (5). Glycan remodeling is crucial for sorting GPI-anchored proteins into ER exit sites and their subsequent ER to Golgi transport (6). In mammalian cells, remodeling of the GPI anchor is then continued in the Golgi where the unsaturated fatty acid of the GPI anchor is replaced by a saturated fatty acid chain (7).

Supplementary key words ceramides • glycolipids • lipidomics • mass spectrometry • sphingolipid • transport • glucosylceramide
From the Golgi compartment, GPI-anchored proteins are transported to the plasma membrane where they are thought to associate preferentially with glycosphingolipids and cholesterol to be enriched in lipid-ordered microdomains. Lipid remodeling is likely to be important for this association because unremodeled GPI-anchored proteins, which carry unsaturated fatty acids, are no longer enriched in detergent resistant membrane fractions (7). Treatments that deplete either membrane cholesterol or sphingolipids also disrupt the association of GPI-anchored proteins with detergent-resistant membranes (DRMs) further supporting the notion that specialized domains are critical for the correct localization of this subset of proteins (8, 9).

Importantly, trafficking of GPI-anchored proteins is affected by alterations in sphingolipids and sterols. In yeast, the ER-to-Golgi transport of GPI-anchored proteins is rapidly reduced by inhibition of de novo sphingolipid biosynthesis without affecting the transport of soluble or transmembrane proteins (10, 11). However, GPI-anchored proteins might play an important role in the transport of membrane proteins such as Tat2 and Fur4p, which are no longer associated with DRMs and are retained in the ER in cells that are deficient at an early stage of GPI anchor biosynthesis (12). In mammalian cells, it was shown that inhibition of sphingolipid biosynthesis affects apical targeting of GPI-anchored proteins in Madin-Darby canine kidney (MDCK) cells (13) and sorting of the axonal GPI-anchored protein Thy-1 in primary hippocampal neurons (14). However unlike yeast, ER-to-Golgi transport of GPI-anchored proteins in mammalian cells does not depend on de novo sphingolipid biosynthesis (15).

An important characteristic of sphingolipid trafficking is the coexistence of at least two different ceramide transport pathways, a major ATP and cytosol-dependent pathway and a minor ATP or cytosol-independent pathway (16). Evidence for two different ceramide transport pathways was first obtained with the isolation of the Chinese hamster ovary (CHO) mutant cell line LY-A that shows a defect in SM but not in HexCer biosynthesis (17) and the subsequent identification of a ceramide transport protein called CERT (18). Two pathways for ceramide transport also exist in yeast (19).

As changes in lipid composition affect GPI-anchored proteins, we asked whether a lack of GPI-anchored proteins together with the abnormal accumulation of GPI anchor intermediates would affect the lipid profile of mammalian cells. To address this question, we made use of a series of mutant CHO cell lines (1) that have defects along the GPI anchor biosynthesis pathway. We determined the lipid profile of the C311 background express four proteins of the GPI anchor biosynthesis pathway in addition to the markers CD59 and DAF (21–23). Presence of those plasmids was verified by antibiotic resistance of cells to G418, hygromycin B and blastcidin S (F21 series) or resistance of cells to G418, hygromycin B and puromycin (C311 series). For lipid extraction, cells were maintained in Ham’s F-12 medium (Invitrogen) supplemented with 10% fetal calf serum (FCS) and 1% PS, 1% ST (streptomycin (50 U/ml) and penicillin (50 U/ml), Invitrogen). HeLa cells were cultured in DMEM (Invitrogen) with 10% FCS and 1% PS. All cells were grown at 37°C and 5% CO₂.

HeLa cells were transiently transfected with siRNA using Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer’s instructions. Human ON-TARGETplus SMARTpool siRNA for PIG-L (L-01195341-0005), DPM3 (L-01749202-0005), PIG-X (L-01378402-0005), PIG-F (L-01175303-0005), PIG-O (L-008728-01-0005), PIG-U (L-017428-00-0005), PGAP1 (L-008110-01-0005), PIG-X (L-013784-02-0005), CERT (L-012101-00-0005) and control scrambled siRNA (ON-TARGETplus nontargeting Pool D-001810-10-05) were purchased from Thermo Scientific. GeneSilencing siRNA against PGAP2 (1027416) was purchased from Qiagen.

Stable CHO transfectants expressing human PIG-F or PIG-U respectively together with the Venus-FLAG-CD59 construct were established by transfection with Lipofectamine 2000 (Invitrogen) followed by selection with Zeocin (300 μg/ml, Invitrogen) for 2 weeks. Single clones were isolated, expanded and analyzed for CD59-Venus surface expression as a marker for restored GPI anchor biosynthesis.

Plasmids

Using the EcoRI / NolI restriction sites human PIG-F and PIG-U were subcloned from pMEnvPIG-F and pME-PIG-U (20) into the pcDNA3.1/ZeoIII mammalian expression vector (Invitrogen). Constructs were verified by sequencing. Vector pME-puro-Venus-FLAG-CD59 (15) was obtained from the laboratory of Reika Watanabe (University of Geneva, Switzerland).

RNA isolation and quantitative RT-PCR

Total RNA was isolated from HeLa cells 72 h after transfection using the RNeasy MINI kit (Qiagen) according to the manufacturer’s instructions. RNA was converted into cDNA using random hexamers and Superscript II reverse transcriptase (Invitrogen). qRT-PCR
was carried out on a BIO-RAD iCycler machine (BioRad, Hercules, CA) with the ABsolute 3Q PCR SYBR Green reagent (Applied Biosystems). Results were normalized against TBP expression. All primers except for PIg-U, PIgA1, and PIgA5 were designed using the NCBI Primer BLAST web tool. The following primers were used: CHOP.For 5′-AGAACCA-GGAAAGCCCAGGCA-3′; CHOP.Rev 5′-TCTCCTTCATGCGCT-3′. For PIg-U, PIgA1, and PIgA5 predesigned primer sets from Qiagen (QuantiTect Primer Assay) were purchased.

**Lipid extraction protocols**

Lipid extracts were prepared using the MTBE protocol (25). Briefly, 2.5 × 10^6 cells were resuspended in 100 µl water. The cell suspension was transferred into a 2 ml Eppendorf tube. Three hundred and sixty microliters methanol and a mix of internal standards as described in (27) and then normalized to the total phosphate content of each total lipid extract to adjust for difference in cell size, membrane content, and extraction efficiency.

**Cellular ceramide glycosylation assay**

The cellular ceramide glycosylation assay was performed as described previously (28). Briefly, HeLa cells were treated with siRNA against PIg-F, PIg-O, or PIg-U for 70 h. Scrambled (SCR) siRNA was used as control. Cells were then switched to 950 µl of 1% BSA DMEM medium containing 10 µM of p23. Tubes were removed from the heat block and kept at RT for 5 min. Then 800 µl of freshly prepared water/1.25% NH4Molybdate (50 mg/4 ml water)/1.67% ascorbic acid (100 mg/6 ml water) in the ratio of 5:2:1 were added. Tubes were heated at 100°C for 5 min with a marble on each tube to prevent evaporation during heating. Tubes were removed from the block and cooled at RT for 5 min. One hundred microliters of each sample was then transferred into a 96-well microplate and the absorbance at 820 nm was measured.

**Phospho-and sphingo-lipid analysis by tandem mass spectrometry**

Tandem mass spectrometry for the identification and quantification of phospho- and sphingo-lipid molecular species was performed using multiple reaction monitoring with a TSQ Vantage Triple Stage Quadrupole Mass Spectrometer (Thermo Scientific) equipped with a robotic nanoflow ion source, Nanomate HD (Advion Biosciences, Ithaca, NY). Each individual ion dissociation pathway was optimized with regard to collision energy. Lipid concentrations were calculated relative to the relevant internal standards as described in (27) and then normalized to the total phosphate content of each total lipid extract to adjust for difference in cell size, membrane content, and extraction efficiency.

**Determination of GM3 levels**

Individual GM3 species were detected by high resolution mass spectrometry on the LTQ Orbitrap XL linear ion trap (Thermo Scientific). Sphingolipid-enriched extracts were infused at a low flow rate using the TriVersa NanoMate robotic ESI source (Advion Biosciences) equipped with a standard ESI chip (Advion Biosciences). Samples were analyzed in negative ion mode. Individual GM3 species were identified by their parental mass combined with fragmentation. Product ions of m/z 290 were obtained from HCD fragmentation of the GM3 precursor ions. These ions correspond to Neu5Ac fragments obtained after cleavage of the glycosidic bond.
Sterol analysis by GC-MS

Extracts were analyzed by GC-MS as described (29). Briefly, samples were injected into a VARIAN CP-3800 gas chromatograph equipped with a Factor Four Capillary Column VF-5ms 15 m x 0.32 mm i.d. DF = 0.10 and analyzed by a Varian 3900 MS triple quadrupole with electron energy set to –70 eV at 250°C. Samples were applied with the column oven at 45°C, held for 4 min, then raised to 195°C (20°C/min). Sterols were eluted with a linear gradient from 195 to 250°C (4°C/min), followed by raising to 320°C (10°C/min). Finally, the column temperature was raised to 350°C (6°C/min) to elute sterol esters. Cholesterol and cholesteryl esters were identified by their retention times (compared with standards) and fragmentation patterns, which were compared with the NIST library.

Statistical analyses

All results are representative of at least three independent experiments. Statistical analyses were performed using an unpaired Student’s t test. Differences were considered significant for P < 0.05 (*), P < 0.01 (**), and P < 0.005 (***)

RESULTS

The goal of this study was to establish the lipid profile of cells that have a defect in GPI anchor biosynthesis. CHO GPI anchor mutants have been very useful in the past to understand the GPI biosynthesis pathway and have allowed cloning of the majority of genes involved in this process (1). We focused on CHO mutants that had either the F21 or the C311 genetic background. The F21 series includes cell lines that are defective in Dol-P-Mannose synthase (DPM3), GPI mannosyltransferase I (PIG-X), ethanolamine phosphate transferase II and III (PIG-F), and GPI lipid remodeling (PGAP2). From the C311 series, we analyzed cells defective in ethanolamine phosphate transferase III (PIG-O) and GPI transamidase (PIG-U).

Defects in GPI anchor biosynthesis lead to changes in HexCer levels

As can be seen in Fig. 1 we observed changes in HexCer levels in CHO mutant cells that have a defect in GPI anchor biosynthesis. Because CHO cells do not possess endogenous GalCer (30) the changes in HexCer are due to a difference in their glucosylceramide (GlcCer) levels. In detail, we observed a downregulation of total GlcCer levels in a subset of GPI anchor mutants (DPM3, PIG-X, and PIG-F) that generate short truncated GPI anchor intermediates. To our surprise, we did not see an effect in GPI anchor mutant PIG-U cells (Fig. 1B). Because PIG-U mutant cells (PA16.1) have no expression of the GPI marker protein CD59 and only 1% remaining DAF expression (supplementary Table I), this indicates that the observed GlcCer changes are not due to a general absence of GPI-anchored proteins (23). Lipid remodeling in the Golgi also did not influence GlcCer levels as we did not observe a change in PGAP2 deficient cells (Fig. 1A), which have normal GPI anchor biosynthesis in the ER but greatly reduced surface expression levels of CD59 and DAF (31) due to rapid secretion (supplementary Table 1). In contrast, the GPI anchor mutant PIG-O (22), which shows a great reduction but not a complete deficiency in the surface expression of GPI-anchored proteins, displayed a strong increase in GlcCer levels (supplementary Table I, Fig. 1B).

When GlcCers were analyzed in detail, we observed an effect concerning the chain length of individual glucosylceramide species. Very long chain GlcCers with a fatty acid chain length of C22 and C24 were strongly decreased in the GPI anchor mutant PIG-U, whereas GlcCer species with a fatty acid chain length between C10 and C20 were mostly unaffected (Fig. 1C). In contrast, PIG-O mutant cells showed a general upregulation of all glucosylceramide species regardless of their chain length (Fig. 1D).

We next focused on the GPI anchor mutants PIG-F and PIG-U, which showed different phenotypes in our lipid analysis. Both GPI anchor mutants cannot generate GPI-anchored proteins and accumulate free GPI anchor intermediates that are similar in structure (Fig. 2A). However, PIG-U mutants are deficient in one subunit of the GPI transamidase and accumulate otherwise a functional free GPI anchor. PIG-F mutants on the other hand lack the regulatory subunit of the EtN-P transferases II and III and consequently have no EtN-P on both Man2 and Man3. The EtN-P on Man2 is later recognized and removed by PGAP5, a step which is necessary for efficient incorporation of GPI-anchored proteins into ER exit sites (5). PIG-F and PIG-U mutants belong to different series of CHO mutant cells (F21 and C311 respectively) and carry plasmids, which encode for several GPI anchor biosynthesis enzymes and GPI anchor marker proteins (20, 23). To test if the observed GlcCer changes are due to the defect in GPI anchor biosynthesis or if they are connected to their genetic background, we stably complemented both PIG-F and PIG-U mutant cells with the corresponding wild-type gene. In order to monitor restored GPI anchor biosynthesis we stably coexpressed Venus tagged CD59. As can be seen in Fig. 2B, the GPI-anchored protein CD59 localizes to the cell surface in PIG-F complemented cells whereas in the PIG-F deficient control cells, CD59 does not reach the cell surface. We then analyzed the lipid profile from both uncomplemented and complemented cells. As can be seen in Fig. 2C, GlcCer levels returned to wild-type in the complemented PIG-F cells indicating that the decrease in GlcCer levels was due to their defect in GPI anchor biosynthesis. As expected, we did not observe any changes in PIG-U cells after complementation (Fig. 2C).

We next wanted to see whether the observed sphingolipid changes are CHO cell specific or if they can be reproduced in another mammalian, preferentially human cell line. In addition, we were interested if a transient knockdown of GPI anchor biosynthesis leads to the same sphingolipid changes as a genetic mutation. To test these two parameters, we transiently silenced PIG-L, DPM3, PIG-X, PIG-F, PIG-O, PIG-U, and PGAP2 expression in HEK cells by siRNA. The gene silencing efficiency was confirmed by quantitative RT-PCR (supplementary Fig. 1). Hela cells possess ceramide galactosyltransferase activity (32) therefore their HexCer levels might be composed of GlcCer
therefore measured GM3 levels in the GPI anchor mutant CHO cells. When individual GM3 species were analyzed by high-resolution mass spectrometry, we found a decrease of the very long chain GM3 species for DPM3, PIG-X, and PIG-F but not for PGAP2 deficient cells (Fig. 4A). GM3 levels also did not change in PIG-O and PIG-U deficient cells indicating that the increase in GlcCer levels that was observed in PIG-O deficient cells did not translate into an upregulation of GM3 (Fig. 4B). HeLa cells predominantly synthesize Gb3 and have low amounts of GM3 (34), which were below our quantification level.

Ceramide levels are affected by defects in GPI anchor biosynthesis in a cell line dependent manner

Because ceramides are direct precursors of monoglycosylated ceramides we analyzed if the observed effect on HexCer levels was reflected by a decrease in ceramide. We measured ceramide levels in the same set of GPI anchor mutant CHO cells and found that DPM3, PIG-X, and PIG-F cells had reduced ceramide levels whereas no change was observed for PGAP2 deficient cells (Fig. 5A). On the individual level very long chain ceramides, in particular C24 ceramide, were decreased (Fig. 5C). In PIG-U and PIG-O mutants, ceramide levels were unaffected (Fig. 5B, D). In HeLa cells, however, total ceramide levels were not decreased upon knockdown of PIG-L, DPM3, PIG-X, or PIG-F (Fig. 5E) and also on the individual level, there was no significant change in very long chain ceramides (Fig. 5F).

Decrease in HexCer affects downstream glycosphingolipids

We next analyzed if the observed decrease in GlcCer levels in CHO cells also affects downstream glycosphingolipids such as GM3. The glycosphingolipid profile of CHO cells is rather simple as they only synthesize GlcCer, lactosylceramide and the ganglioside NeuAcα2-3Galβ1-4Glcβ1-1Cer (GM3) (30). CHO cells accumulate high amounts of GM3 because they are unable to synthesize GM2 and other complex gangliosides such as GM1 and GD1a (33). We therefore measured GM3 levels in the GPI anchor mutant CHO cells. When individual GM3 species were analyzed by high-resolution mass spectrometry, we found a decrease of the very long chain GM3 species for DPM3, PIG-X, and PIG-F but not for PGAP2 deficient cells (Fig. 4A). GM3 levels also did not change in PIG-O and PIG-U deficient cells indicating that the increase in GlcCer levels that was observed in PIG-O deficient cells did not translate into an upregulation of GM3 (Fig. 4B). HeLa cells predominantly synthesize Gb3 and have low amounts of GM3 (34), which were below our quantification level.
Lipidomic profile of GPI anchor-deficient cells

same set of GPI anchor mutant CHO cells (Fig. 6A, B). Total sphingomyelin levels also did not change in both the PIG-F and the PIG-U complemented cells (supplementary Fig. IV). However, CHO cells have predominantly C16 sphingomyelin, which accounts for 70% of the total sphingomyelin and very long chain sphingomyelins were slightly reduced in CHO mutants that have a decrease in GlcCer levels (Fig. 6C). Total sphingomyelin levels were also not significantly changed in HeLa cells (Fig. 6D) but the detailed analysis showed that defects in GPI anchor biosynthesis have an effect on very long chain sphingomyelins (Fig. 6E). As there is much more sphingomyelin than HexCer in both CHO and HeLa cells, the absolute amounts of sphingomyelin that are affected could be in fact more than HexCers.

The ceramide transport protein CERT mediates transport of ceramide for sphingomyelin biosynthesis. However, CERT deficiency does not affect total GlcCer levels in CHO cells, indicating that there is an alternative ceramide transport pathway for the biosynthesis of GlcCer (18). In vitro CERT has a strong preference for the transport of ceramides that are C20 or shorter and does not efficiently transport very long chain ceramides (37). We silenced CERT in HeLa cells and observed a decrease in total sphingomyelin as expected (Fig. 7A). HexCer levels did not

Very long chain sphingomyelin levels are partially affected in GPI anchor deficient cells

In contrast to the observed changes in HexCer and ceramide, total sphingomyelin levels were not affected in the same set of GPI anchor mutant CHO cells (Fig. 6A, B). Total sphingomyelin levels also did not change in both the PIG-F and the PIG-U complemented cells (supplementary Fig. IV). However, CHO cells have predominantly C16 sphingomyelin, which accounts for 70% of the total sphingomyelin and very long chain sphingomyelins were slightly reduced in CHO mutants that have a decrease in GlcCer levels (Fig. 6C). Total sphingomyelin levels were also not significantly changed in HeLa cells (Fig. 6D) but the detailed analysis showed that defects in GPI anchor biosynthesis have an effect on very long chain sphingomyelins (Fig. 6E). As there is much more sphingomyelin than HexCer in both CHO and HeLa cells, the absolute amounts of sphingomyelin that are affected could be in fact more than HexCers.

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This indicates that the decrease in very long chain ceramides could be cell line-specific or might be a compensatory effect of the genetic mutation. In addition, it also raises the possibility that reduced ceramide biosynthesis is not the only cause for the observed decrease in HexCer levels. We next tested if glycosylation of ceramide is affected in the GPI anchor deficient cells. It has been shown that glucosylceramide synthase localizes to the Golgi in HeLa cells (35) but it cannot be fully ruled out that some GlcCer synthase activity might also exist in the ER (36). To test if ceramide glycosylation is affected in the GPI anchor deficient cells, we performed an in vivo ceramide glycosylation assay (28). As can be seen in Fig. 5G, NBD-C6 ceramide was efficiently converted into GlcCer in PIG-F, PIG-O, and PIG-U depleted cells. There was, however, no synthesis of GalCer, which shows that ceramide galactosyltransferase activity is very low in HeLa cells (Fig. 5G). Relative to the total amount of input (NBD-C6 Cer) there was an increase in NBD-C6 GlcCer in the PIG-O depleted cells, which indicates more GlcCer synthase activity in PIG-O depleted cells.

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proteins associate with p24 family proteins (6, 39). We therefore analyzed the lipid profile of cells in which the GPI remodeling enzymes PGAP1, PGAP5, or the p24 family members p23 or p24 were silenced respectively. As can be seen in Fig. 8A, total HexCer levels increased upon knockdown of PGAP1, PGAP5, p23, and in particular for p24. Individual HexCer levels showed that all HexCer species were affected equally (Fig. 8B). This result is in line with the observed increase of HexCer levels in PIG-O deficient cells and indicates that an abnormal GPI structure or a delay in ER to Golgi transport can lead to an increase in total HexCer levels. We also observed some increase in ceramide levels in particular for PGAP1 but sphingomyelin levels were not changed (supplementary Figs. VI, VII). In yeast, it has been shown that a defect in GPI anchor remodeling or trafficking results in ER stress and induces the upregulation of the unfolded protein response (UPR) (40–42). We assessed ER stress by measuring transcriptional activation of the UPR. The C/EBP homology protein (CHOP), which is involved in ER stress-mediated apoptosis was upregulated in the GPI anchor mutant PIG-O, the remodeling mutants PGAP1 and PGAP5, as well as in p23 and p24 knockdowns but not in PIG-F deficient cells (Fig. 8C). The ER chaperone BiP, was upregulated in PIG-O, PGAP5, and p23 depleted cells.

decrease and even modestly increased, which confirms the existence of different ceramide transport pathways (Fig. 7A). As shown in Fig. 7B, knockdown of CERT affected both C₁₆ and C₂₄ sphingomyelins, indicating that in vivo CERT is also required for the efficient transport of very long chain ceramides. This might explain why very long chain sphingomyelins are only partially affected in GPI anchor deficient cells. Individual HexCer levels showed a stronger increase in C₁₆ than in C₂₂ glycosylceramides (supplementary Fig. V).

Defects in GPI glycan remodeling or GPI anchor trafficking affect HexCer levels

We had noticed a strong global increase in HexCer levels for PIG-O deficient CHO cells, which was reproduced in HeLa cells during a transient knockdown of PIG-O. In PIG-O deficient cells, ethanolamine phosphate transferase III (PIG-F/GPI7) is active and cells can attach EtN-P onto mannose 2, which is important for glycan remodeling. Interestingly, PIG-O mutants are not completely deficient in the surface expression of GPI-anchored proteins and accumulate an intermediate, called KO-2 (Fig. 2A), which is competent for protein attachment but has an abnormal glycan structure (38). The glycan structure plays an important role for sorting of GPI-anchored proteins into ER exit sites (5, 6) and only glycan remodeled GPI-anchored proteins associate with p24 family proteins (6, 39). We therefore analyzed the lipid profile of cells in which the GPI remodeling enzymes PGAP1, PGAP5, or the p24 family members p23 or p24 were silenced respectively. As can be seen in Fig. 8A, total HexCer levels increased upon knockdown of PGAP1, PGAP5, p23, and in particular for p24. Individual HexCer levels showed that all HexCer species were affected equally (Fig. 8B). This result is in line with the observed increase of HexCer levels in PIG-O deficient cells and indicates that an abnormal GPI structure or a delay in ER to Golgi transport can lead to an increase in total HexCer levels. We also observed some increase in ceramide levels in particular for PGAP1 but sphingomyelin levels were not changed (supplementary Figs. VI, VII). In yeast, it has been shown that a defect in GPI anchor remodeling or trafficking results in ER stress and induces the upregulation of the unfolded protein response (UPR) (40–42). We assessed ER stress by measuring transcriptional activation of the UPR. The C/EBP homology protein (CHOP), which is involved in ER stress-mediated apoptosis was upregulated in the GPI anchor mutant PIG-O, the remodeling mutants PGAP1 and PGAP5, as well as in p23 and p24 knockdowns but not in PIG-F deficient cells (Fig. 8C). The ER chaperone BiP, was upregulated in PIG-O, PGAP5, and p23 depleted cells.
Cholesterol ester levels are reduced in cells that do not synthesize GPI-anchored proteins

It has been previously reported that GPI-deficient CHO cells have cholesterol contents similar to wild-type CHO cells (43). Using our mass spectrometry approach, we measured the amount of free cholesterol and of cholesterol ester in the GPI anchor-deficient CHO and HeLa cells. As can be seen in Fig. 9A, free cholesterol levels are unchanged between wild-type and the GPI anchor mutants. We found, however, that all GPI anchor mutant cells including PIG-U had lower levels of cholesterol ester than the corresponding wild-type cells (Fig. 9A). PIG-O mutant cells, which are not completely deficient in the biosynthesis of GPI-anchored proteins, did not show a reduction in cholesterol esters. We then also analyzed the sterol composition of HeLa cells in which PIG-L, DPM3, PIG-F, PIG-O, or PIG-U had been silenced, respectively. Under these conditions, we observed a similar result: free cholesterol levels were mostly unchanged (Fig. 9B) whereas cholesterol ester levels were strongly reduced in all knockdowns except PIG-O (Fig. 9B). This result suggests that the observed effect on cholesterol ester is due to the inability to synthesize certain GPI-anchored proteins and is not caused by a specific GPI anchor intermediate that is generated.

In parallel to the observed changes in glycolipids and sterols we also analyzed the phospholipid content of each GPI anchor mutant by mass spectrometry. We measured the levels of phosphatidylethanolamine (PE), phosphatidylcholine (PC), phosphatidylinositol (PI), and phosphatidylserine (PS). Among those glycerophospholipids we did not observe any major changes except for a mild increase in total PE in the GPI-anchor mutants DPM3, PIG-X, and PIG-O (supplementary Fig. VIII).

DISCUSSION

In this study we show that GPI anchor biosynthesis mutants that generate incomplete GPI anchor intermediates have a strong decrease in very long chain C<sub>22</sub> and C<sub>24</sub> glucosylceramide levels, without greatly affecting long chain C<sub>16</sub> glucosylceramides. In contrast, GPI transamidase-deficient cells (PIG-U) that synthesize complete anchors but do not attach them to proteins do not show this effect. This suggests that the GPI anchor, not necessarily attached to proteins, regulates very long chain GlcCer levels. Complementation experiments showed that these changes are specific to the GPI anchor biosynthesis and are not due to the complex genetic background of the GPI anchor mutant cells. siRNA silencing of GPI anchor biosynthesis genes in HeLa cells demonstrated that the observed effects on GlcCers are not restricted to one cell type and that the strategy of siRNA gene downregulation can be used to see changes in lipid profiles in a relatively short time period.

In addition to the decrease in GlcCer, we also observed a decrease in very long chain ceramides in the CHO GPI anchor mutants. However, this decrease was not observed in HeLa cells following a transient knockdown of the same GPI anchor biosynthesis genes (Fig. 5E). This implies that the decrease in very long chain HexCer is probably not just the consequence of reduced ceramide levels.

Defects in GPI anchor biosynthesis lead to the ER accumulation of GPI anchor intermediates such as GlcNAc-PI...
mannose, suggesting that GlcN-(acyl)-PI is also elevated in those cells (23). Because GlcCer levels were unaffected in PIG-U deficient cells, it is therefore unlikely that the ER accumulation of GPI anchor intermediates is causing an inhibition of certain ceramide synthases or that GlcCer synthase activity would be affected. Another possible reason for reduced ceramide and HexCer levels could be that ER to Golgi transport of ceramide is linked to the transport

and GlcN-acyl-PI as well as the mannose containing GPI intermediates H2-H8 (1). Metabolic labeling with myo-[3H]inositol has shown that GPI anchor mutants such as DPM3 and PIG-X accumulate moderate amounts of the early GPI anchor intermediate GlcN-acyl-PI whereas PIG-U mutants show a high ER accumulation of all GPI anchor intermediates (20, 23). In particular, Man-GlcN-(acyl)-PI labels strongly in PIG-U deficient cells cultured with [3H]mannose, suggesting that GlcN-(acyl)-PI is also elevated in those cells (23). Because GlcCer levels were unaffected in PIG-U deficient cells, it is therefore unlikely that the ER accumulation of GPI anchor intermediates is causing an inhibition of certain ceramide synthases or that GlcCer synthase activity would be affected. Another possible reason for reduced ceramide and HexCer levels could be that ER to Golgi transport of ceramide is linked to the transport

**Fig. 6.** Sphingomyelin levels do not change in GPI anchor deficient cells. A: Total sphingomyelin (SM) levels of F21 wild-type and GPI anchor mutant cell lines DPM3, PIG-X, PIG-F, and PGAP2. B: Total SM levels of C311 wild-type and GPI anchor mutant cell lines PIG-O and PIG-U. C: Individual SM levels in the F21 series of GPI anchor mutants. D: Total SM levels of HeLa cells treated with siRNA against CERT. Scrambled (SCR) siRNA was used as control. E: Individual SM profile of GPI anchor-deficient HeLa cells.

**Fig. 7.** CERT knockdown does not lead to a decrease in HexCer levels. A: Total sphingomyelin and Hex-Cer levels of HeLa cells treated with siRNA against the ceramide transport protein CERT. Scrambled (SCR) siRNA was used as control. B: Individual sphingomyelin profile of HeLa cells treated with siRNA against CERT. **P < 0.01 SCR versus siRNA knockdown.
of GPI-anchored proteins and free GPI anchor molecules. If very long chain ceramides are not transported efficiently from the ER and are locally accumulating this could lead to a feedback inhibition of certain ceramide synthases and subsequently to a decrease in HexCers. The degree of feedback inhibition might be different in HeLa versus CHO cells.

In contrast to HexCer, sphingomyelin levels were mostly unaffected in the GPI anchor-deficient cells. When analyzed in detail, we found that CHO cells have primarily C_{16} sphingomyelin (Fig. 6C). In contrast, HeLa cells have a much higher content of very long chain sphingomyelins (C_{24} sphingomyelin is about 45% of total sphingomyelin) and very long chain sphingomyelin levels were mildly affected in HeLa cells (Fig. 6E). The major fraction of ceramide in mammalian cells is transported to the Golgi in a nonvesicular manner via the ceramide transport protein CERT and mutants in CERT affect mainly sphingomyelin levels, which suggested an alternative mechanism of transport of ceramide destined for GlcCer biosynthesis (18). CERT has a preference for ceramide species with an acyl chain length of C_{20} or less and transports C_{22} and C_{24} ceramide with greatly reduced efficiency in vitro (37). However, our results show that knockdown of CERT led to a global decrease of all sphingomyelin species and also greatly reduced C_{24} sphingomyelin (Fig. 7B). We therefore conclude that in vivo the transport of very long chain ceramides from ER to Golgi for sphingomyelin biosynthesis is regulated by CERT. Interestingly, ER to Golgi trafficking of GPI-anchored proteins is normal in CERT deficient cells (44). Taken together, these data support a model in which GPI anchor molecules regulate the transport of very long chain ceramides destined for GlcCer and to some degree also for sphingomyelin biosynthesis.

A common feature among the mutants that show decreased levels of very long chain GlcCer is the accumulation of GPI anchor precursors that have incomplete glycan structures. The GPI anchor mutants PIG-L, DPM3, and PIG-X produce very short truncated GPI anchor intermediates (GlcNAc-PI, GlcN-(acyl)-PI) that are unlikely to leave the ER. The mutant PIG-F however generates a GPI anchor intermediate that possesses an almost complete glycan structure but still has reduced GlcCer levels (Fig. 2A). PIG-F is the regulatory subunit of the GPI EtN-P transferase II (PIG-O/PIG-F) and III (GPI7/PIG-F) (45). Consequently, both EtN-P transferases are not active in PIG-F deficient cells (38) and mutant cells do not attach EtN-P onto mannose 2 or 3 (46). Because EtN-Ps are important

![Figure 8](image)

**Fig. 8.** HexCer levels are affected by GPI glycan remodeling and involve the p24 family members. A: Total HexCer levels of Hela cells treated with siRNA against PGAP1, PGAP5, p23 or p24, respectively. Scrambled (SCR) siRNA was used as control. B: Detailed HexCer profile of the corresponding cells. C: Real time RT-PCR analysis of genes involved in UPR induction. HeLa cells were treated with siRNA against PIG-F, PIG-O, PGAP1, PGAP5, p23 and p24. SCR was used as control. Cells were harvested 72 h after transfection and total RNA was isolated. Relative levels of CHOP and BiP were assessed by qRT-PCR.

![Figure 9](image)

**Fig. 9.** Sterol levels in the GPI anchor deficient cells. A: Free cholesterol and cholesterol ester levels in in the GPI anchor deficient CHO cell lines DPM3, PIG-X, PIG-F, PIG-O, and PIG-U. Values were calculated as percentage of wild-type (F21 or C311 respectively). B: Free cholesterol and cholesterol ester levels in HeLa cells after a transient knockdown of the GPI anchor biosynthesis genes PIG-L, DPM3, PIG-F, PIG-O, and PIG-U respectively. Scrambled (SCR) siRNA was used as control.
for glycan remodeling and ER to Golgi transport, we speculate that this abnormal glycan structure makes it impossible for the PIG-F GPI anchor intermediate to exit the ER.

In contrast, PIG-U deficient cells, which make a complete GPI anchor but lack the enzymatic activity to transfer the free GPI anchor to a protein, have unchanged levels of HexCer. Interestingly, it has been reported that cells generate large pools of free nonproteins linked GPI anchors that exit the ER and are transported to the cell surface (47, 48). Although there is no direct evidence that free GPI anchors can be remodeled in vivo, it has been shown that free GPI anchors are substrates for remodeling enzymes in vitro (5).

In contrast to the mutants PIG-L, DPM3, PIG-X, and PIG-F, we observed a strong increase in HexCer levels for the GPI anchor mutant PIG-O. This was observed in both the CHO cell lines as well as HeLa cells under siRNA silencing conditions. PIG-O mutants generate intermediates with a similar GPI structure as PIG-F. However, the ethanolamine phosphate transferase II, which is a complex of GPI7 and PIG-F is active in PIG-O deficient cells (45). Ethanolamine phosphate transferase II adds EtN-P onto mannose 2, which is important for glycan remodelling by PGAP5 (5). Interestingly, PIG-O mutants are not completely deficient in the surface expression of GPI-anchored proteins (38). The surface expression of PIG-O deficient cells is due to a minor GPI anchor intermediate called KO-2 that is competent for protein attachment. KO-2 has an abnormal glycan structure with most likely four mannoses and EtN-P on Man1 and Man3 (38). An additional EtN-P on mannose 2 might be transitory and is a potential substrate for PGAP5. Because of this abnormal glycan structure, we speculate that PIG-O deficient cells might have a defect in sorting of free or protein anchored GPIs into ER exit sites. The ER exit of GPI-anchored proteins is controlled by glycan remodeling and p24 complexes act as cargo receptors for GPI anchor sorting into COPII vesicles (6, 39, 49). In agreement with this model, we observed a global increase in HexCer levels in the remodeling mutants PGAP1 and PGAP5 as well as for the p24 family proteins. However, we did not detect an increase in GM3 levels in PIG-O depleted cells, which shows that a global increase in HexCer does not necessarily translate into an increase in GM3 and most likely follows a different mechanism. In yeast, it has been shown that the unfolded protein response is highly activated in the GPI remodeling mutants (40–42). Here, we show that ER stress markers such as CHOP are upregulated in the GPI anchor remodeling and trafficking mutants including PIG-O but not in PIG-F deficient cells (Fig. 8C). Recently, we have shown that ceramide levels are increased in response to induction of the unfolded protein response (27). Because HexCer synthesis protects against Cer-induced stress in mammalian cells (50, 51), we speculate that the ER stress response might cause the observed increase in HexCer levels in the GPI anchor remodeling mutants.

Finally, we did not observe any sphingolipid changes for the GPI anchor mutant PGAP2, which has normal GPI anchor biosynthesis in the ER but greatly reduced surface expression levels of GPI-anchored proteins due to secretion (31). Sorting of GPI-anchored proteins into ER exit sites and ER to Golgi trafficking is not affected in the PGAP2 deficient cells.

Concerning the sterol composition of the GPI anchore-deficient cells we observed no effect on free cholesterol levels but found a decrease in cholesterol ester (CE) levels in certain GPI anchor-deficient cells (Fig. 9). There was, however, no correlation between the sterol and the Hex-Cer profile since PIG-U deficient cells showed a similar reduction of CE levels as PIG-F deficient cells. In contrast, PGAP2 and PIG-O deficient cells, which synthesize GPI-anchored proteins had almost wild-type levels of CEs. We therefore speculate that the decrease in CE is due to the absence of certain GPI-anchored proteins that affect biosynthesis or degradation of CEs. In addition, uptake of cholesterol from the medium, which contains FCS, might be affected in cells that lack GPI-anchored proteins.

GPI-anchored proteins have been postulated to segregate into sphingolipid enriched lipid ordered microdomains (52). In yeast, it has been shown that ongoing ceramide synthesis is required for GPI-anchored protein transport from the ER to the Golgi compartment, linking the two processes (10, 11). In mammalian cells, however, de novo sphingolipid biosynthesis is not required to transport mammalian GPI-anchored proteins from the ER to the Golgi (15, 53). However, this does not exclude the possibility that also in mammalian cells ER to Golgi transport of ceramide is linked to the transport of GPI-anchored proteins. It might be possible that under sphingolipid depleted conditions GPI-anchored proteins are trafficked normally but that in the absence of GPI-anchored proteins very long chain ceramides are not transported properly.

We present here a model whereby the levels of very long chain GlcCer are correlated to the biosynthesis of GPI anchor molecules. We propose that very long chain GlcCer levels and to some extent also very long chain sphingomyelin levels are linked to GPI biosynthesis to ensure that proportional amounts of each lipid class are synthesized and transported.

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