Lysophosphatidic acid acyltransferase 3 regulates Golgi complex structure and function

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Recent studies have suggested that the functional organization of the Golgi complex is dependent on phospholipid remodeling enzymes. Here, we report the identification of an integral membrane lysophosphatidic acid–specific acyltransferase, LPAAT3, which regulates Golgi membrane tubule formation, trafficking, and structure by altering phospholipids and lysophospholipids. Overexpression of LPAAT3 significantly inhibited the formation of Golgi membrane tubules in vivo and in vitro. Anterograde and retrograde protein trafficking was slower in cells overexpressing LPAAT3 and accelerated in cells with reduced expression (by siRNA). Golgi morphology was also dependent on LPAAT3 because its knockdown caused the Golgi to become fragmented. These data are the first to show a direct role for a specific phospholipid acyltransferase in regulating membrane trafficking and organelle structure.

Introduction

The functional organization of the Golgi complex is mediated by a wide array of proteins that interact with specific lipid components to facilitate membrane bending for vesicle formation and tubule fission (Bard and Malhotra, 2006; De Matteis and Luini, 2008; Jackson, 2009). Phosphoinositides serve as landmarks for the recruitment of proteins that regulate diverse trafficking events (Gallop and McMahon, 2005; Bashkirov et al., 2008; Frost et al., 2009). Lysophospholipids (LPLs), phosphatidic acid (PA), and diacylglycerol (DAG) have intrinsic curvature, which may aid in vesicle or tubule formation by altering membrane morphology and acting as a scaffold for downstream factors (Kooijman et al., 2003; van Meer and Sprong, 2004). Phospholipase C (PLC) and phospholipase D (PLD) may produce DAG or PA for Golgi membrane fission (Bard and Malhotra, 2006; Fernandez-Ulibarri et al., 2007; Yang et al., 2008; Asp et al., 2009). Additionally, phospholipase A_2 (PLA_2) activity, which generates LPLs, appears to be involved in endocytic recycling, Golgi retrograde trafficking, and Golgi cisternal structure by altering membrane tubule formation (de Figueiredo et al., 1998; Brown et al., 2003).

Pharmacological studies have suggested that LPL acyltransferases (LPATs) play a role in membrane trafficking by catalyzing the transfer of a fatty acid from an acyl-CoA donor to LPLs to generate PLs. The drug CI-976, originally identified as a weak cholesterol acyltransferase inhibitor, also inhibits a tightly associated Golgi membrane LPAT (Chambers and Brown, 2004), with a consequent stimulation of Golgi membrane tubules and retrograde trafficking (Drecktrah et al., 2003). CI-976 also inhibits COPI (Yang et al., 2005) and COPII vesicle formation (Brown et al., 2008) and endocytic recycling (Chambers et al., 2005), suggesting the role of multiple LPATs in membrane trafficking.

A family of nine transmembrane acyltransferases, variously named LPAATα–ι and AGPAT 1–9 (1-acylglycerol-3-phosphate-*O*-acyltransferase) (Lewin et al., 1999; Leung, 2001), but more recently LPAAT 1–9 (Shindou and Shimizu, 2009), has been identified. Lysophosphatidic acid (LPA) is the preferred acceptor for LPAAT enzymes including ER-localized LPAAT1 and 2, which are involved in de novo PL metabolism; however, little is known about the activity, localization, or function of LPAAT3–9 (Shindou and Shimizu, 2009). Recently, mouse LPAAT3 was shown to be widely expressed (Lu et al., 2005), but with an age-dependent elevation of levels in testis (Yuki et al., 2009).

Here, we show that LPAAT3 (LPAAT-γ/AGPAT3) is directly involved in regulating Golgi membrane trafficking events. LPAAT3 prefers LPA as a substrate, but surprisingly the protein itself is located in both the ER and Golgi complex. Overexpression

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Abbreviations used in this paper: BBC, bovine brain cytosol; DAG, diacylglycerol; LPA, lysophosphatidic acid; LPAAT, lysophosphatidic acid acyltransferase; LPAT, lysophospholipid acyltransferase; PA, phosphatidic acid; PLA_2, phospholipase A_2; ssHRP, soluble secreted horseradish peroxidase.

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of LPAAT3 increases Golgi levels of PA and inhibits membrane tubule formation and trafficking both in vivo and in vitro. Knockdown of LPAAT3 caused changes in Golgi morphology as well as accelerated trafficking. We conclude that by regulating PA levels, LPAAT3 contributes to Golgi structure and trafficking dynamics.

Results and discussion

LPAAT3 is an ER and Golgi-localized transmembrane protein

Annotated in the human genome are nine predicated LPAAT enzymes, all containing multiple putative transmembrane domains and two consensus sequences with similar amino acids that lie within a PLsC, or acyltransferase domain (Fig. 1 a). These conserved regions are likely involved in acid-base sn-2 addition of a fatty acid from acyl-CoA to an LPL acceptor, as well as substrate binding and specificity (Shindou and Shimizu, 2009).

To identify an LPAAT that might be involved in Golgi structure and function, we expressed GFP-tagged versions of four uncharacterized LPAATs in HeLa cells to see if any were located in the Golgi complex. One of these, LPAAT3, localized in a diffuse-ER and juxtanuclear Golgi-like pattern and was therefore chosen for further study (Fig. 1). Both expressed LPAAT3-GFP (or DsRed) and endogenous LPAAT3 detected by immunofluorescence, colocalized with resident cis, medial, and trans-Golgi and ER proteins (Fig. 1, b and c; Fig. S1, a–d). HeLa cells and Clone 9 rat hepatocytes stably expressing LPAAT3-GFP were also generated and displayed Golgi localization (Fig. S1 e). The other LPATs we examined were mostly ER-like (LPAAT4 and 6) or mitochondrial (LPAAT5) (Fig. S2).

LPAAT3 is a lysophosphatidic acid acyltransferase

To determine the enzymatic activity of LPAAT3, post-nuclear supernatants from cells overexpressing LPAAT3 were incubated with ^{14}C-palmitoyl Co-A and various LPL species to assay for the production of radiolabeled PL products. Post-nuclear supernatants from cells overexpressing LPAAT3 had elevated levels of LPA acyltransferase (LPAAT) activity compared with control supernatants (Fig. 1 d). Other exogenously added LPLs were tested, but there was no significant change in the production of the corresponding PL species. This increase in LPAAT activity was observed in cells stably or transiently transfected with wild-type LPAAT3 (LPAAT3 WT) in both HeLa cells (Fig. 1 e) and Clone 9 cells (not depicted). Cells stably expressing the ER-localized LPAAT6 (a known LPAAT) (Chen et al., 2008) were used as a positive control for LPAAT activity. Furthermore, the acyltransferase activity was not observed when acidic and basic residues in the two conserved regions of the acyltransferase motif (Fig. 1 a) were changed to alanines in the LPAAT3 mutant (MT) (Fig. 1 e). Despite these changes, the catalytically inactive LPAAT3 MT was still partially localized to the Golgi complex (Fig. S1 d). Finally, subcellular fractions enriched in Golgi membranes from cells stably expressing LPAAT3 also exhibited an increase in LPAAT activity when endogenous LPA was used as an acceptor (Fig. 1 e). Together, these data show that LPAAT3 has LPA acyltransferase activity and that this activity

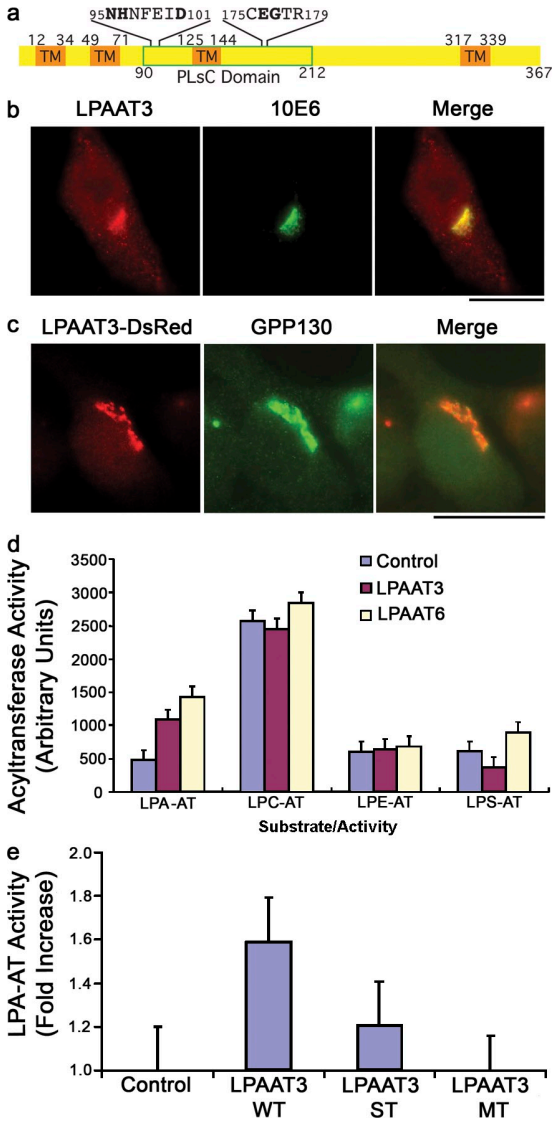

Figure 1. **LPAAT3 motifs, subcellular localization, and activity.** (a) LPAAT3 contains two regions in a conserved PLsC acyltransferase motif and multiple predicted transmembrane domains. Mutations made in the two consensus sequences are shown in bold (changing the residues to alanines). (b) The cis-Golgi marker 10E6 and rabbit anti-LPAAT3 colocalize. Bar = 20 µm. (c) LPAAT3-DsRed colocalizes with the medial Golgi marker GPP130. Bar = 20 µm. (d) Post-nuclear supernatants from HeLa cells transfected with LPAAT3 and LPAAT6 have elevated levels of LPA acyltransferase activity compared with control lysates. Lysophosphatidylcholine (LPC), lysophosphatidylethanolamine (LPE), and lysophosphatidylserine (LPS) were not significant acceptors of ^{14}C-palmitoyl-CoA in these cells. (e) Golgi from cells transiently (WT) or stably (ST) transfected with wild-type LPAAT3 have elevated LPAAT activity, whereas those with LPAAT3 containing mutations in residues required for catalytic activity do not (LPAAT3 MT). Isolated Golgi membranes from cells overexpressing wild-type LPAAT3 also exhibit elevated LPAAT when using endogenous LPA as a substrate (LPAAT3 WT Endog). Error bars are SD for three independent experiments.

can increase the amount of PA in Golgi membranes. These results are consistent with reports that other members of this family also exhibit LPA acyltransferase activity (Shindou and Shimizu, 2009). In addition, mouse LPAAT3 was recently shown to have highest activity with LPA, and weaker amounts with lysophosphatidylinositol (Yuki et al., 2009), which was not tested here.

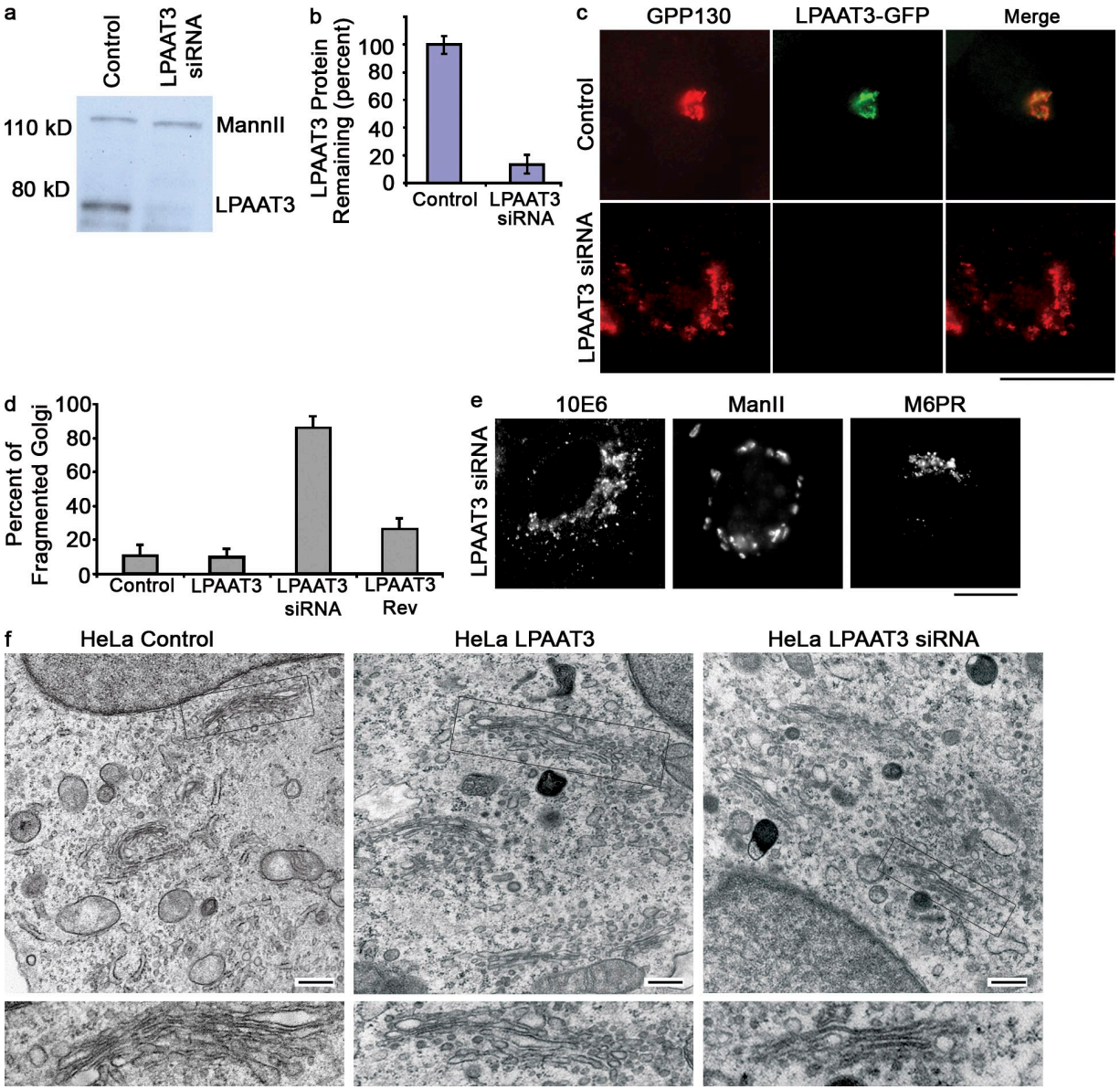

Figure 2. **The Golgi fragments in LPAAT3 knockdown cells.** (a) Cells stably overexpressing LPAAT3-GFP were transfected with a mixture of four siRNA molecules targeting LPAAT3 mRNA and analyzed by Western blot of cell lysates using anti-GFP and mannosidase II antibodies. (b) LPAAT3 expression was reduced by 85% (n = 3). (c) Immunofluorescence of GPP130 in LPAAT3 knockdown cells showed fragmented Golgi membranes. Bar = 20 µm. (d) The percentage of cells with fragmented Golgi ribbons as observed by immunofluorescence. (e) Golgi fragments contain markers for the cis-Golgi (10E6), the medial-Golgi (ManII) and the TGN (M6PR). Bar = 20 µm. (f) EM of thin sections from control, LPAAT3-GFP expressing, and knockdown cells. Higher magnification views of each boxed region are shown below. Bars = 500 nm.

LPAAT3 mediates Golgi structure

Cells stably expressing LPAAT3-GFP were used to examine Golgi morphology in siRNA knockdown experiments. Knockdown was verified by Western blot, which showed an average of 85% reduction in LPAAT3 expression (Fig. 2, a and b). In LPAAT3 knockdown cells, the Golgi complex was fragmented, forming numerous juxta-nuclear puncta (Fig. 2 c). Quantification showed that ~90% of LPAAT3 knockdown cells had fragmented Golgi ribbons (Fig. 2 d). Each of the fragments colocalized with cis-Golgi markers (10E6), medial-Golgi markers (mannosidase II), and TGN markers (M6PR) (Fig. 2 e). Golgi fragmentation caused by LPAAT3 knockdown was reversed by transfection with an siRNA-resistant LPAAT3 construct (Fig. 2 d). Electron microscopy (EM) revealed that knockdown cells had multiple Golgi stacks throughout the cytoplasm (Fig. 2 f). The combined fluorescence and EM studies show that knockdown of LPAAT3 results in the formation of Golgi mini-stacks, which did not colocalize with the ER protein PDI, or the ER exit site marker Sec24 (unpublished data).

LPAAT3 regulates the rate of Golgi tubule formation and retrograde trafficking in vivo

Pharmacological studies have suggested that Golgi membrane tubules are positively influenced by PLA_2 activity and negatively influenced by LPAT activity (Brown et al., 2003). Cells were all treated with the fungal metabolite brefeldin A (BFA), which

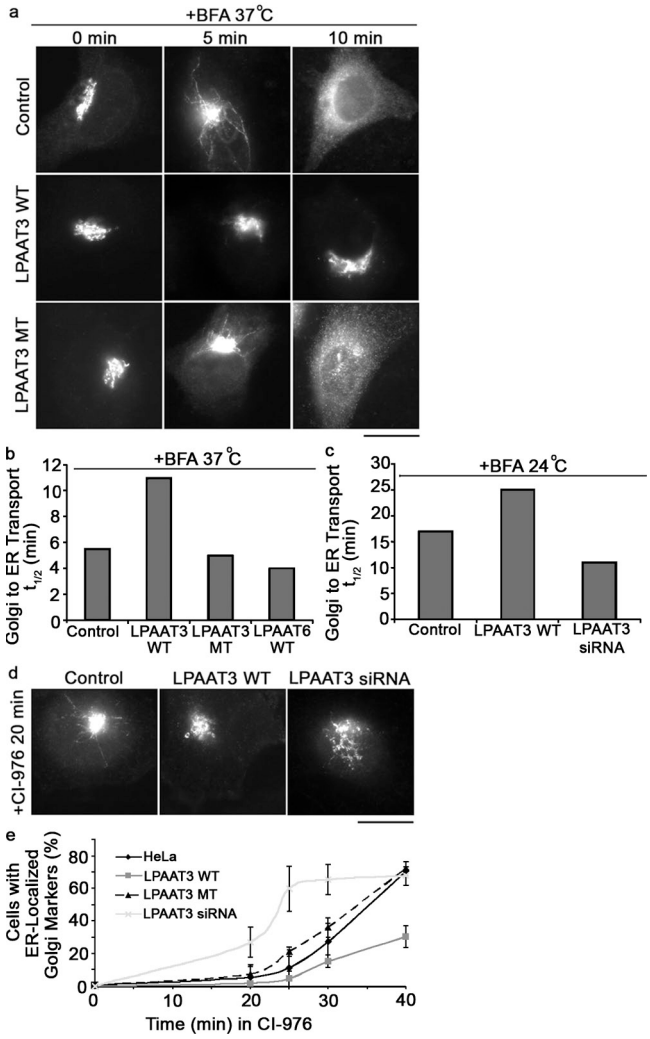

Figure 3. **LPAAT3 overexpression slows BFA and CI-976–stimulated Golgi retrograde trafficking to the ER.** (a) HeLa cells and cells transfected with LPAAT3 WT or LPAAT3 MT were treated with 10 µg/ml BFA and analyzed by immunofluorescence with GPP130 as a Golgi marker. Control cells, LPAAT3 MT cells, and LPAAT6 cells formed Golgi membrane tubules from 2 to 5 min after addition of BFA and were completely relocalized to ER membranes within 10 min, whereas LPAAT3 WT cells were slower. Bar = 20 µm. (b) The $t_{1/2}$ of Golgi loss due to BFA tubulation was 5.5, 5.0, and 4.0 min for HeLa cells, LPAAT3 MT cells, and LPAAT6 cells, respectively, and 11 min for LPAAT3 WT cells. (c) To measure LPAAT3 knockdown cells treated with BFA, the experiment above was repeated at 24°C where the $t_{1/2}$ values for cells was 18 min for control cells, 25 min for cells overexpressing LPAAT3 WT, and 11.2 min for LPAAT3 knockdown cells. Data are representative of three independent experiments. (d) Control cells, LPAAT3 WT cells, LPAAT3 MT cells, and LPAAT3 siRNA knockdown cells were treated with 50 µM of the LPAT inhibitor CI-976 for 30 min. Cells expressing LPAAT3 WT were slower at forming Golgi membrane tubules, whereas knockdown cells were considerably faster. Bar = 20 µm. (e) Quantification of the CI-976 results. All error bars are SD for three independent experiments (n = 300).

generates numerous membrane tubules that redistribute Golgi membranes to the ER (Lippincott-Schwartz et al., 1989). Cells overexpressing LPAAT3 form Golgi tubules much slower than control and LPAAT3 MT cells (Fig. 3, a and b). Moreover, the Golgi tubules that did form in LPAAT3 WT overexpressing cells were far fewer in number and shorter in length than untransfected cells, as shown by Sholl analyses (Fig. S3, a and b; Sholl, 1953). The inhibition of BFA-stimulated tubule formation by LPAAT3 overexpression was not due to ineffective COPI disruption (Fig. S3 c). In contrast to overexpression, LPAAT3 knockdown cells exhibited a very rapid loss of the Golgi in BFA, so the experiment was repeated at 24°C to obtain quantitative data. The results showed that the half-time for loss of the Golgi in siRNA-treated cells was 11 min compared with 25 min for overexpressing cells (17 min in control cells) (Fig. 3 c).

The LPAT inhibitor CI-976 also stimulates Golgi membrane tubules (Drecktrah et al., 2003), presumably by increasing the levels of positive curve-inducing LPLs. Thus, a prediction is that cells overexpressing or knocked down for LPAAT3 should become, respectively, more resistant or sensitive to CI-976. Indeed, similar to BFA, cells overexpressing LPAAT3 and treated with CI-976 formed tubules slower than controls, whereas tubule formation in LPAAT3 knockdown was much faster (Fig. 3, d and e). These results show that overexpressing LPAAT3 significantly reduces the rate, the number, and the length of induced Golgi membrane tubules.

LPAAT3 overexpression inhibits Golgi tubule formation in vitro

Golgi membrane tubulation is attenuated in cells overexpressing LPAAT3 in vivo, so we tested the effects of overexpression on Golgi tubulation using an in vitro reconstitution assay. In brief, Golgi complexes from tissue culture cells stably expressing LPAAT3-GFP were isolated, incubated with a fraction of bovine brain cytosol (BBC) to generate membrane tubules, and analyzed by negative stain EM (Cluett et al., 1993; Banta et al., 1995). In reactions without BBC, no significant Golgi tubulation was observed regardless of the cells. With the addition of BBC, concentration-dependent tubulation was observed (Fig. 4 a); however, tubule formation was reduced ~50% in Golgi membranes from cells overexpressing LPAAT3 compared with control (Fig. 4 b). Thus, increases in LPAAT3 significantly decrease Golgi membrane tubules both in vivo and in vitro.

LPAAT3 alters ERGIC-53 distribution

Overexpression of LPAAT3 dramatically decreases the rate of Golgi tubulation in vivo from BFA and CI-976 and in vitro from BBC, so we examined overexpression on the cellular localization of an endogenous membrane marker involved in retrograde trafficking. The p58 receptor (hERGIC-53) is a lectin receptor that traffics between the cis-Golgi and the ER. When cells are incubated at 15°C, the p58 receptor accumulates in the cis-Golgi and ERGIC compartments. When the cells are shifted from 15 to 37°C, p58 returns to the ER via membrane tubules (Saraste and Svensson, 1991; Klumperman et al., 1998). In control cells following the shift to 37°C, most the p58 receptor returned from the cis-Golgi to the ER in 5 min, whereas the LPAAT3-overexpressing cells showed a delay in the loss of Golgi and gain in ER localization of p58 (Fig. 4, c–e). Conversely, cells knocked down for LPAAT3 exhibited faster retrograde movement of p58 (Fig. 4, c and d). This shows that LPAAT3 also affects the retrograde recycling of endogenous p58 from the cis-Golgi to the ER.

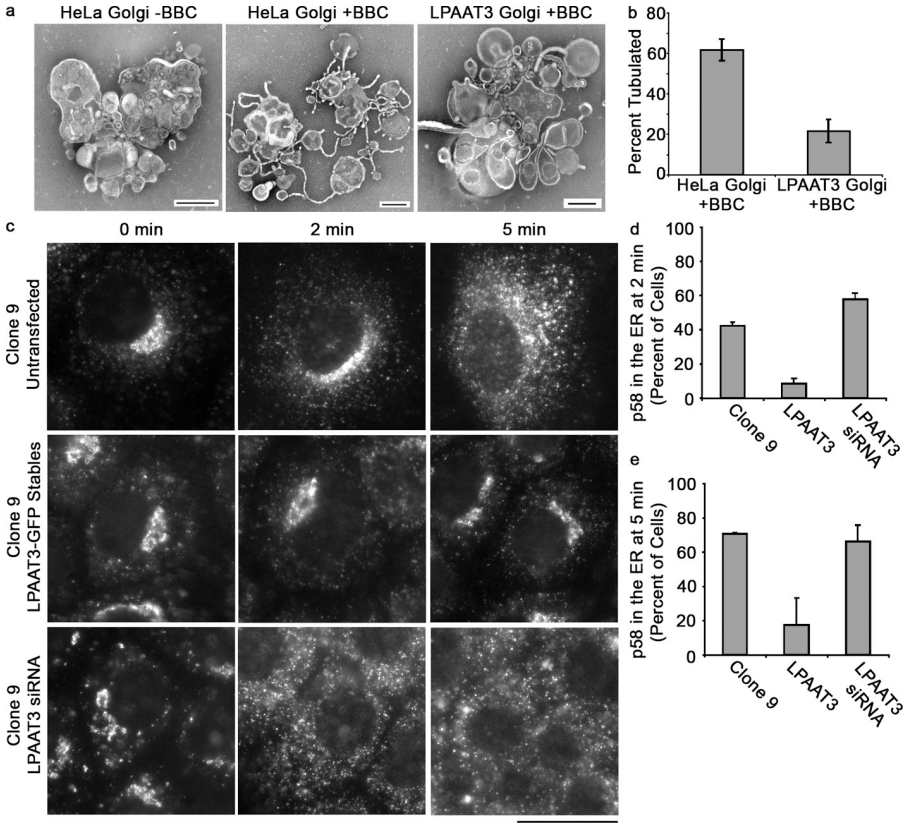

Figure 4. **In vitro tubulation of Golgi membranes and ERGIC-53 trafficking.** (a) Isolated Golgi membranes from control cells and cells stably expressing LPAAT3 WT were incubated with bovine brain cytosol (BBC) to form membrane tubules. Bars = 500 nm. (b) In the LPAAT3 cells, the percentage of tubulated membranes was only ~20% compared with ~60% of control Golgi membranes. (c) Control Clone 9 rat hepatocytes, Clone 9 cells stably expressing LPAAT3-GFP, and Clone 9 cells knocked down for LPAAT3 were incubated at 15°C for 3 h, then shifted to 37°C and fixed after 0, 2, 5, 7, and 10 min and visualized for endogenous p58 (ERGIC-53). Bar = 20 µm. (d) Quantification of cells with Golgi-localized p58 after 2 min at 37°C. (e) Quantification of cells with Golgi-localized p58 after 5 min at 37°C. Cells overexpressing LPAAT3-GFP retained p58 in the Golgi, whereas cells knocked down for LPAAT3 transported p58 to the ER more rapidly than control cells. Error bars are SD for three independent experiments n = 300.

LPAAT3 mediates anterograde trafficking

Golgi tubules have been documented to play an important role in anterograde membrane trafficking, particularly from the TGN (Bard and Malhotra, 2006), and perhaps in anterograde intercisternal movement under certain conditions (Trucco et al., 2004). Cells transfected with LPAAT3 or siRNA for LPAAT3 were cotransfected with ts045 VSV-G-DsRed to examine trafficking through the secretory pathway. At 40°C VSV-G-DsRed was located in the ER as expected, and within 20–30 min after shift to 32°C, it was transported to the Golgi complex (Fig. 5 a), with no significant difference between control and modified cells (unpublished data). However, we observed that in cells either overexpressing or siRNA treated for LPAAT3, VSV-G-DsRed remained in the Golgi longer and did not reach the plasma membrane until 40–50 min after temperature shift (Fig. 5, a and b).

To examine anterograde trafficking of soluble cargo, cells were cotransfected with soluble secreted horseradish peroxidase (ssHRP) (Bard et al., 2006), and HRP activity was measured in the media. The results showed that secretion of ssHRP was inhibited in cells overexpressing LPAAT3, but increased in LPAAT3 knockdown cells by twofold when compared with mock-transfected cells (Fig. 5 c). When LPAAT3 knockdown cells were rescued using the LPAAT3 reversion construct (LPAAT3 Rev), ssHRP secretion was similar to cells overexpressing LPAAT3 WT.

Conclusions

We have identified a transmembrane LPA acyltransferase, LPAAT3, which localizes to the Golgi and ER, and consumes LPA to produce PA. Previous pharmacological studies have shown that altering the LPL and PL species in membranes can alter membrane tubule formation and Golgi structure (de Figueiredo et al., 2000; Drecktrah et al., 2003; Chambers and Brown, 2004), and here we show that overexpression of LPAAT3 significantly slows the formation of Golgi membrane tubules both in vivo and in vitro. These data agree with previous observations that PLA_2 activity, which increases LPLs, is needed for membrane tubules to form (de Figueiredo et al., 2000). When LPAAT3 levels were reduced using siRNA transfection, the Golgi became hyper-sensitive to CI-976 and BFA and rapidly formed Golgi-to-ER retrograde membrane tubules, possibly due to an increase in LPA.

Membrane tubules also readily form at the TGN for the transport of cargo to the plasma membrane and endosomes (Bard and Malhotra, 2006; De Matteis and Luini, 2008), and LPAAT3 similarly affects trafficking kinetics in the TGN. Overexpression of LPAAT3 slowed export of ts045 VSV-G from the TGN and secretion of ssHRP. Conversely, LPAAT3 knockdown accelerated secretion of ssHRP, but curiously not ts045 VSV-G. It is not clear why ssHRP and not ts045 VSV-G was accelerated. TGN cargoes exist by several independent pathways/carriers, and the differential effects of LPAAT3 knockdown on export of VSV-G and secretion of ssHRP may reflect different phospholipid requirements for these carriers. Secretion may also be compromised indirectly through effects on retrograde trafficking, which were also influenced by LPAAT3 levels.

Results here and elsewhere suggest that LPL/PL has a critical role in generating membrane curvature, which is required for the formation of membrane tubules and vesicles, the latter either from flattened cisternal membranes or from tubular

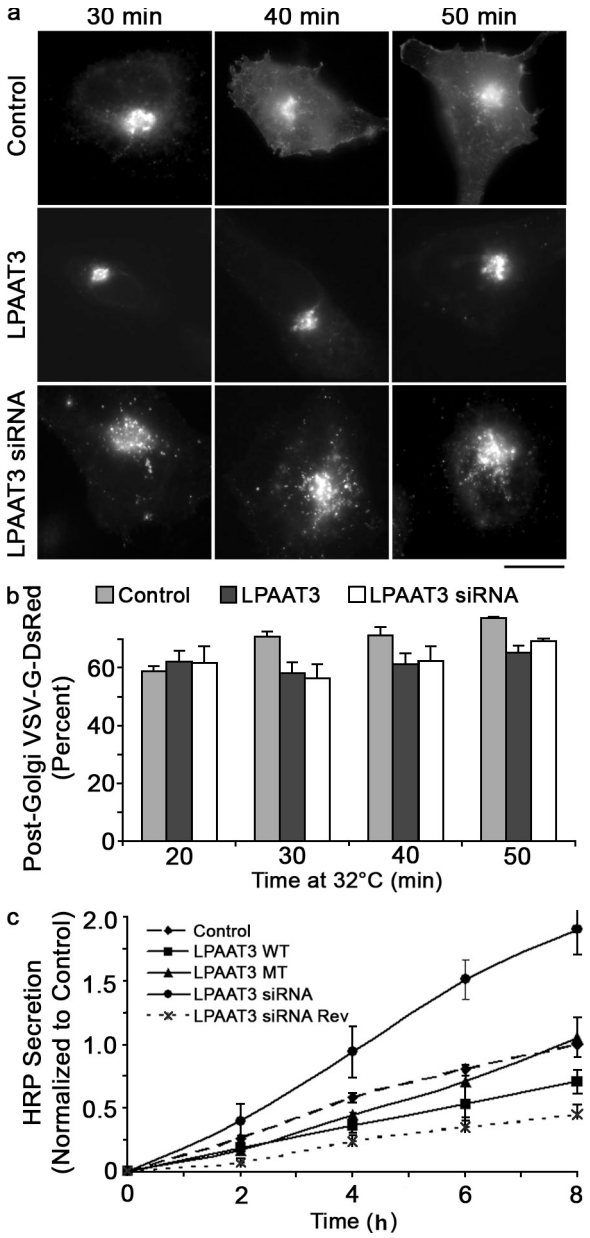

Figure 5. **LPAAT3 and TGN trafficking.** (a) Cells overexpressing LPAAT3 or knocked down for LPAAT3 were transfected with ts045 VSV-G-DsRed and incubated at 40°C for 18 h, then released to 32°C for various time points. Bar = 20 µm. (b) The amount of ts045 VSV-G-DsRed fluorescence in the Golgi complex was subtracted from the total fluorescence to determine the amount of post-Golgi transport of ts045 VSV-G. Error bars represent SD for three independent experiments (n = 100). (c) Cells were transfected with a secretable form of horseradish peroxidase (ssHRP). Media and lysates were tested every 2 h for HRP activity. Error bars represent three independent experiments.

precursors. In addition, however, PA is thought to have important roles in CtBP3/BARS50-mediated vesicle fission and/or fusion by generating negative membrane curvature and forming microdomains for protein docking (Kooijman et al., 2003; Yang et al., 2008). Moreover PLD, which also generates PA, is important for establishing transport carriers at the TGN (Bard and Malhotra, 2006; Yang et al., 2008). DAG may be an indirect product of LPAAT3 as increased PA levels may drive DAG levels via PA phosphatase. Importantly, PKD, which binds to DAG, is also required for forming post-TGN trafficking intermediates (Maeda et al., 2001; Baron and Malhotra, 2002). DAG is also associated with multiple trafficking events from the Golgi (Fernandez-Ulibarri et al., 2007; Asp et al., 2009).

In addition to changes in membrane trafficking, reduced expression of LPAAT3 caused the Golgi ribbon to fragment into mini-stacks. The results suggest that by altering the LPL/PL ratio in Golgi membranes, changes in either positive or negative curve can alter the stability of the compact cisternal morphology. Of course, any significant change in the LPL/PL ratio could have multiple direct and indirect effects.

Aside from phosphoinositides, the roles of specific PLs in membrane trafficking are still elusive because multiple pathways exist for the metabolism of PLs. PL levels are regulated by cellular needs for de novo PL synthesis via the Kennedy pathway but also by downstream organelle trafficking events (Gaspar et al., 2008). Membrane lipids such as LPLs and DAG may also be short-lived and localized to specific domains where cargo is concentrated for tubule or vesicle formation. In addition, it is becoming increasingly clear that continual PL remodeling via Lands' cycle enzymes such as LPAAT3 and PLA_2 plays important roles in the formation of membrane trafficking intermediates and in the maintenance of the Golgi complex.

Materials and methods

Materials

Antibodies against the following antigens were used: GPP130 (Adam Linstead, Carnegie Mellon University), hManII (Kelly Moreman, University of Georgia), 10E6 (Wood et al., 1991), M6PR (Brown and Farquhar, 1987), βCOP (ABR), p58 (Jaakko Saraste, Bergen University), and FITC/TRITC conjugated secondary antibodies (Jackson ImmunoResearch Laboratories). Peptide antibody against LPAAT3 was designed and generated by Pacific Immunology. All phospholipids were from Avanti Polar Lipids, chromatography reagents were from Alltech Chromatography and radiolabeled Palmitoyl-CoA [palmitoyl-1-^{14}C] was from PerkinElmer Life Sciences.

DNA constructs and transfection

LPAAT3 (AGPAT3) cDNA was obtained in pCMV-SPORT6 from the IMAGE Human library (Invitrogen). Using PCR and the multiple cloning site of pEGFP N-1 (Clontech Laboratories, Inc.) or pDsRed N-1, LPAAT3 was inserted with EGFP or DsRed at the C terminus. To make the catalytic mutant of LPAAT3, Quick Changell (Stratagene) was used. For secretion measurements, the pEGFP N-1 SS-HRP-Flag plasmid was from Vivek Malhotra. To generate ts045 VSV-G-DsRed, the VSV-G sequence was ligated into the vector from pEGFP N-1 ts045 VSV-G, which was from Brian Storrie (University of Arkansas). All constructs were verified by DNA sequencing.

For PCR cloning of LPAAT3, the following forward and reverse primers were used: GCAATTATCTCGAGCATGGGCCTGCTGGGC and CGGCGGTACCGTTTCCTTTTTCTTAAACTCTTGGTTTCCGTAGCTGGA which contain XHOI and KPNI restriction digest sites. A mixture of four siRNA duplexes designed from the human AGPAT3 cDNA sequence (SMARTpool; Dharmacon) was transfected into cells stably expressing LPAAT3-GFP using lipofectamine RNAi MAX (Invitrogen) for 72 h. The following siRNA sequences were transfected with their antisense compliments: GAGACCAAGCACCGCGUUAUU, CGUCUUUGCCAGCGGAUCAUU, GCUCCAAGGUCCUGCUAAUU, and GGAAAUAGAAUGACGGGAAUU.

DNA constructs were transfected using Fugene (Roche) and processed for immunofluorescence experiments within 24–48 h unless otherwise indicated. For cells transfected with ts045 VSV-G-DsRed, cells were transfected and grown at 40°C for 18 h before temperature shift.

Tissue culture and immunofluorescence

HeLa cells and Clone 9 rat hepatocytes were grown and maintained at 37°C, 100% humidity, and 5% CO_2. Cells stably-expressing LPAAT3-GFP were selected with 500 µg/ml G418 (Mediatech).

For inhibitor assays, cells were washed twice with MEM and treated with 10 µg/ml brefeldin A (Sigma-Aldrich) or 50 µM CI-976 (Tocris, Ellisville, MO). Cells were fixed in 3.7% formaldehyde in PBS (pH 7.4) for 10 min at room temperature, washed three times for 5 min each in PBS, and permeabilized for 10 min in 0.1% Triton X-100 in PBS. Cells were incubated with primary antibody for 1 h at room temperature, washed three times in PBS for 5 min each, followed by secondary antibody incubation (same conditions as primary), and mounted on slides using Vectashield (Vector Laboratories). Imaging and cell quantification was done using the Zeiss Axioscope2. Images were obtained with a 100X Plan-Apochromat oil objective lens (NA 1.4) and acquired with a Hamamatsu Orca digital camera run by OpenLab software (Improvision). All confocal images were generated using the Perkin-Elmer Ultraview spinning disc microscope using a Nikon 100X Plan-Apochromat oil objective lens (NA 1.4).

HRP and VSV-G secretion assays

To measure secretion of ssHRP, cells were transfected with pEGFP N-1 ssHRP-Flag for 24 h, and cell lysates and media samples were analyzed for HRP activity (Bard et al., 2006). To measure ts045 VSV-G-DsRed localization, 100 images from fixed cells were processed using ImageJ (NIH) to measure the pixel intensity in the Golgi and total cell regions of interest (ROI). Background values of pixel intensity per unit area were measured and subtracted from each ROI.

Online supplemental material

Fig. S1 shows the localization of the ER and Golgi markers with LPAAT3-GFP WT and LPAAT3 –GFP MT in HeLa cells and Clone 9 rat hepatocytes. Fig. S2 shows the localization of LPAAT4-GFP, LPAAT5-GFP, and LPAAT6-GFP as part of our LPAAT screen. Fig. S3 shows that BFA-stimulated Golgi membrane tubules are shorter and less numerous in cells overexpressing LPAAT3-GFP when measured by the Sholl Analysis and that expression of LPAAT3-GFP does not alter β-COP localization in untreated cells or in cells treated with BFA. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200904147/DC1.

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