Low-density lipoprotein (LDL) delivers cholesterol to mammalian cells through receptor-mediated endocytosis. The LDL cholesterol is liberated in lysosomes and transported to the plasma membrane (PM) and from there to the endoplasmic reticulum (ER). Excess ER cholesterol is esterified with a fatty acid for storage as cholesteryl esters. Recently, we showed that PM-to-ER transport of LDL cholesterol requires phosphatidylserine (PS). Others showed that PM-to-ER transport of cholesterol derived from other sources requires Aster(s) (also called GRAMD1s), a family of three ER proteins that bridge between the ER and PM by binding to PS. Here, we use a cholesterol esterification assay and other measures of cholesterol delivery to demonstrate that Aster(s) participate in PM-to-ER transport of LDL cholesterol in Chinese hamster ovary cells. Knockout of the gene encoding PTDS51, the major PS-synthesizing enzyme, lowered LDL-stimulated cholesterol esterification by 85%, whereas knockout of all three Aster genes lowered esterification by 65%. The reduction was even greater (94%) when the genes encoding PTDS51 and the three Aster genes were knocked out simultaneously. We conclude that Aster(s) participate in LDL cholesterol delivery from PM to ER, and their action depends in large part, but not exclusively, on PS. The data also indicate that PS participates in another delivery pathway, so far undefined, that is independent of Aster(s).

A nimal cells obtain cholesterol through the receptor-mediated endocytosis of cholesterol-carrying plasma low-density lipoprotein (LDL) (1). The LDL particle is digested in lysosomes and the liberated cholesterol is transported to the plasma membrane (PM), where it fills three pools: 1) an accessible pool that is available to bind bacterial cytolysins such as perfringolysin O; 2) a sequestered pool that is bound to sphingomyelin; and 3) an essential pool whose depletion leads to cell death (2–4). Once the three pools have been filled, excess cholesterol is transported from the PM to the endoplasmic reticulum (ER), where it inhibits the pro- teolytic activation of sterol regulatory element-binding protein-2 (SREBP-2), a transcription factor that activates genes encoding several proteins involved in cholesterol delivery to demonstrate that Aster(s) participate in PM-to-ER transport of LDL cholesterol in Chinese hamster ovary cells. Knockout of the gene encoding PTDS51, the major PS-synthesizing enzyme, lowered LDL-stimulated cholesterol esterification by 85%, whereas knockout of all three Aster genes lowered esterification by 65%. The reduction was even greater (94%) when the genes encoding PTDS51 and the three Aster genes were knocked out simultaneously. We conclude that Aster(s) participate in LDL cholesterol delivery from PM to ER, and their action depends in large part, but not exclusively, on PS. The data also indicate that PS participates in another delivery pathway, so far undefined, that is independent of Aster(s).

Mammalian cells express three Aster(s), designated A, B, and C. Aster(s) are anchored in the ER membrane by virtue of a membrane-spanning helix near the carboxy terminus. The cytosolic amino-terminal region contains two functional domains: a GRAM domain that binds anionic lipids and a START-like domain that binds cholesterol. When the accessible pool of PM cholesterol expands, the GRAM domain is triggered to bind to the PM, creating a bridge between the ER and the PM. Binding is postulated to be triggered by the known ability of the GRAM domain to bind anionic phospholipids, including phosphatidylserine (PS), that inhabit the inner leaflet of the PM. Binding to the PM has been demonstrated in vivo by total internal reflection fluorescence (TIRF) microscopy using EGFP-tagged GRAMD1s (10, 11) or Aster(s) (8, 9). Binding is triggered when the accessible pool of PM cholesterol is increased by incubation with cholesterol/cyclodextrin complexes (8, 11) or by treatment with sphingomyelinase (10, 11), which expands the accessible pool by releasing cholesterol from the sequestered pool (2).

Our laboratory has focused on cholesterol movement from the accessible pool in the PM to the ER, a key step that allows the ER to monitor and optimize the level of PM cholesterol.

Significance

Cholesterol constitutes 50% of lipids in the plasma membrane (PM) of animal cells. Sensors in the endoplasmic reticulum (ER) maintain this level by adjusting cholesterol uptake, synthesis, and storage. Uptake is mediated by LDL receptors, which deliver cholesterol-carrying LDL to lysosomes from which cholesterol moves to the PM and then to the ER. We report PM-to-ER transport of LDL cholesterol requires cholesterol-binding Aster proteins anchored to the ER and phosphatidylserine embedded in the PM. Aster(s) are known to bind phosphatidylserine, and this accounts for part of the phosphatidylserine requirement. However, the current data suggest an additional requirement for phosphatidylserine independent of Aster(s). These data advance our knowledge of PM cholesterol homeostasis, a control mechanism essential for cell growth and survival.
In this context, we recently discovered another requirement for PM-to-ER cholesterol transport, namely PS (12). We conducted a CRISPR-Cas9 knockout screen for genes required for transport of LDL cholesterol from the lysosome to the ER. The screen disclosed a requirement for PTDSS1, the major PS-synthesizing enzyme in mammalian cells (13). PS is reduced by 90% in human cells lacking PTDSS1 (12). When LDL is added to these cells, the particle is taken up and digested in lysosomes normally, and the LDL-derived cholesterol reaches the PM, but in the absence of PS the cholesterol fails to move from the PM to the ER. As a result, the accessible pool of cholesterol in the PM increases, cleavage of SREBP-2 is not suppressed normally, and excess cholesterol is not esterified. All of these deficits are reversed when PS is restored by incubating the Ptdss1−/− cells with PS liposomes. We hypothesized that the cholesterol transport defect in the PS-deficient cells results from the failure of Asters to transport cholesterol (12).

In the current study, we have tested this hypothesis by preparing hamster cells that lack all three Asters with or without PTDSS1. The results indicate that loss of Aster function accounts for some, but not all of the block in cholesterol transport in PS-deficient cells. The data also suggest the existence of a second PS-dependent pathway that is independent of Asters. This paper explores the interplay between these two pathways.

Results

The current experiments were performed with a line of Chinese hamster ovary (CHO) cells designated CHO-K1. Fig. 1A shows that CHO-K1 cells express mRNAs encoding all three Aster isoforms, with Aster-A expression being the highest. When we used CRISPR-Cas9 to knock out the Gramd1a gene encoding Aster-A, the mRNA for Aster-B increased (Fig. 1A). Therefore, to eliminate all Aster expression, we performed serial transfections with CRISPR-Cas9 plasmids to deplete both copies of all three Gramd1 genes (SI Appendix, Fig. S1). The resulting cells, designated Aster-abc−/−, did not express significant amounts of any of the Aster mRNAs (Fig. 1A). We also used CRISPR-Cas9 to eliminate the gene encoding PTDSS1, the enzyme that synthesizes PS. Fig. 1A shows that Aster-A and -B expression increased approximately twofold in the Ptdss1−/− cells.

The Aster-abc−/− and Ptdss1−/− cells exhibited normal uptake of LDL labeled with BODIPY-FL, a fluorescent lipid that integrates into the hydrophobic core of LDL (Fig. 1B). Uptake was blocked by an excess of unlabeled LDL, confirming that it was mediated by a saturable receptor. Indeed, uptake was absent in cells lacking the gene encoding the LDL receptor (Fig. 1B). We used two assays to measure the delivery of LDL cholesterol to the ER. One assay measures cholesterol esterification, and the other measures inhibition of the proteolytic processing of SREBP-2. Despite the normal uptake of LDL, the Aster-abc−/− cells and the Ptdss1−/− cells showed major deficits in the incorporation of [14C]oleate into cholesteryl esters (Fig. 1C). In wild-type (WT) cells, LDL inhibited the proteolytic processing of SREBP-2 by 82%, as reflected by a reduction in the ratio between the nuclear fragment and the membrane-bound precursor observed on SDS-PAGE (Fig. 1D). Inhibition by LDL was decreased in the Aster-abc−/− cells (<50%), and it was even less in the Ptdss1−/− cells (14%).

As shown previously for human cells (12), the level of PS was reduced by 90% in CHO-K1 cells lacking PTDSS1 (SI Appendix, Fig. S2). The level of phosphatidylethanolamine (PE) was also reduced since PE is formed from PS (13). In the previous studies, we also showed that addition of PS overcomes the block in cholesterol transport in human Ptdss1−/− cells (12). To determine whether PS would overcome the block in the hamster Aster-abc−/− cells, we incubated the cells with cholesterol complexed to methyl-β-cyclodextrin (MCD), which delivers cholesterol directly to the PM (14). To measure cholesterol delivery to the ER, we used the cholesterol esterification assay. In WT cells, addition of cholesterol/MCD led to an increase in the incorporation of [14C]oleate into cholesteryl esters, and addition of PS caused a slight further increase (Fig. 2A). In the Ptdss1−/− cells, cholesterol esterification was low and was restored nearly to normal with PS supplementation (Fig. 2B). Cholesterol esterification was also low in the Aster-abc−/− cells, and there was no restoration by PS (Fig. 2C). We also produced cells lacking all three Asters as well as PTDSS1 (SI Appendix, Fig. S1). In these cells cholesterol was not esterified, and there was a slight but consistent increase when PS was added (Fig. 2D).

In the same experiment, we also used human LDL to deliver cholesterol to the CHO-K1 cells (Fig. 2E–H). Whereas MCD delivers cholesterol directly to the PM, LDL delivers cholesterol to the lysosome, from which it is transported to the PM. When LDL was added, the results of the cholesterol esterification assay were directionally similar to the ones obtained with cholesterol/MCD, but the absolute amount of esterification was lower. In WT cells, LDL cholesterol reached the ER where it was esterified with [14C]oleate, and the addition of PS had no effect (Fig. 2E). Esterification was reduced in the Ptdss1−/− cells and was restored with PS addition (Fig. 2F). Esterification was reduced in the Aster-abc−/− cells and was not restored by PS (Fig. 2G). Esterification was even lower in the Ptdss1−/−/Aster-abc−/− cells, and there was a small but consistent increase when PS was added.

Fig. 2 I and J present the cholesterol esterification rates observed in five independent studies in which duplicate dishes from WT or mutant CHO-K1 cells were treated with cholesterol/MCD at 300 μM (Fig. 2I) or 100 μg/mL LDL (Fig. 2J). The results from each of the 10 dishes are expressed as a percentage of the esterification observed in WT cells on the same day. Independently of whether cholesterol was added in complex with MCD or contained in LDL, cholesterol esterification in Ptdss1−/−/Aster-abc−/− cells was lower than the rate in either Ptdss1−/− cells or the Aster-abc−/− cells. This observation indicates that the PS-dependent pathway and the Aster-dependent pathway (Fig. 1) are partially independent (Fig. 2).

In the experiment of Fig. 3A, we incubated cells with LDL and measured the relative amount of cholesterol in the PM by incubating the cells with AF488-PFO*, a fluorescently tagged, genetically optimized form of bacterial perfringolysin O that binds to accessible cholesterol in PMs (12, 15). AF488-PFO* binding was quantitated by flow cytometry (Fig. 3A), and the median cellular fluorescence was calculated (Fig. 3B). As we previously reported (12), after incubation with LDL, Ptdss1−/− cells showed an increase in PM cholesterol as reflected by increased AF488-PFO* binding. A similar increase was observed in the Aster-abc−/− cells (Fig. 3 A and B). The increase was greater when the two deficits were combined in the Ptdss1−/−/Aster-abc−/− cells. We also stained the cells with AF488-PFO* while grown on coverslips (Fig. 3C). Staining of the PM was increased in the Ptdss1−/− cells and in the Aster-abc−/− cells, and the intensity was increased further in the Ptdss1−/−/Aster-abc−/− cells. Sequencing of LDL-derived cholesterol in the PM led to a decrease in cholesterol esterification in the Aster-abc−/− cells and in the Ptdss1−/− cells (Fig. 3D). The decrease was even greater in the Ptdss1−/−/Aster-abc−/− cells, consistent with the greater trapping of LDL cholesterol in the PM.

The data thus far indicate that Asters and PS are both required for maximal transport of cholesterol from the PM to the ER. To further explore the relation between the two requirements, we incubated cells with varying concentrations of cholesterol/MCD and used the cholesterol esterification assay.
Asters and PTDSS1 required for delivery of LDL cholesterol to the ER in CHO-K1 cells. All cells were set up on day 0 in medium A with 10% fetal calf serum (FCS). (A) mRNA expression of Aster-A, -B, and -C in WT and mutant cell lines. On day 2, cells were switched to cholesterol-depletion medium A. After incubation for 16 h, cells received cholesterol-depletion medium B containing 50 μg protein/mL human LDL. After 6 h, cells were harvested for RNA extraction and quantitative RT-PCR as described in SI Appendix, Materials and Methods. Ct values for expression of Aster-A, -B, and -C in WT cells are shown. The mRNA levels for Aster-A, -B, and -C in the mutant cell lines are expressed relative to the Ct values in the WT cells. Mean and range of six replicates are shown. (B) BODIPY FL-LDL uptake. On day 2, cells were switched to cholesterol-depletion medium A. After incubation for 16 h, cells received cholesterol-depletion medium B containing 5 μg protein/mL BODIPY FL-LDL in absence (red) or presence (blue) of 150 μg protein/mL of unlabeled human LDL. After 6 h, cells were harvested for flow cytometry. The heights of the bars indicate the mean of triplicate incubations. (C) Stimulation of cholesteryl ester synthesis by LDL in WT, Aster-abc−/−, and Ptdss1+/− cells. On day 2, cells were refed with cholesterol-depletion medium A. After incubation for 16 h, cells received cholesterol-depletion medium B containing the indicated amount of LDL. After 4 h, cells were pulse labeled for 2 h with 0.1 mM sodium [14C]oleate (7,157 dpm/nmol), after which the cellular content of cholesteryl [14C]oleate was measured. The lines denote the average of duplicate incubations with individual values shown. (D) SREBP-2 processing. On day 2, cells were switched to cholesterol-depletion medium A containing 1% hydroxypropyl-β-cyclodextrin (HPCD). After incubation for 1 h, the cells received cholesterol-depletion medium A in absence or presence of 100 μg protein/mL LDL. After 6 h, cells were harvested for extraction, SDS-PAGE, and immunoblotting of SREBP-2 and Scap (SI Appendix, Materials and Methods). P, precursor. N, nuclear. SREBP cleavage was quantified using ImageJ (16). For each lane, the ratio of nuclear to total SREBP-2 (i.e., nuclear + precursor) was calculated and expressed relative to the value in WT cells without LDL.

Discussion

Previous studies from the laboratories of Tontonoz and coworkers (8, 9) and Saheki and coworkers (10, 11) demonstrated that Aster proteins are required for the transport of cholesterol from the PM to the ER when cholesterol is delivered directly to the PM by incubation with cholesterol/MCD complexes or when it is liberated within the membrane by sphingomyelinase treatment. Our previous study showed that PS is required for PM-to-ER cholesterol transport when cholesterol is delivered to the cell by LDL or cholesterol/MCD (12). Inasmuch as Aster proteins are known to bind to negatively charged phospholipids,
including PS, we hypothesized that the role of PS is to support Aster function. In the current study, we tested this hypothesis using genetically modified Chinese hamster cells. The data demonstrate that: 1) Aster proteins are indeed required for normal rates of PM-to-ER transport of LDL-derived cholesterol after it moves from lysosomes to the PM; 2) Aster-mediated transport is dependent on PS, but only partially; and 3) in addition to supporting Aster-mediated transport, PS is required for another PM-to-ER cholesterol transport pathway that is independent of Asters.

The requirement for Asters in transport of LDL-derived cholesterol from the PM to the ER is revealed by the data in Figs. 1–3. LDL uptake in the Aster-abc-/C0 cells was the same as in WT cells (Fig. 1B), but the rate of cholesterol ester synthesis...
was less (see the individual experiments in Figs. 1–3 and the averaged data from five experiments in Fig. 2). Moreover, LDL did not inhibit SREBP-2 processing as much in the Aster-abc/C0/C0 cells as it did in WT cells (Fig. 1D). Instead of reaching the ER in normal amounts, in the Aster-abc/C0/C0 cells LDL-derived cholesterol accumulated in the PM as it did in Ptdss1/C0/C0 cells (Fig. 3A–C). Finally, addition of PS liposomes restored transport of LDL-derived cholesterol nearly to normal in the Ptdss1/C0/C0 cells (Fig. 2F), but the restoration was much less when the Ptdss1/C0/C0 cells also lacked Asters (Fig. 2H). Considered together, all of these data indicate that Aster proteins and PS are both required for transport of LDL-derived cholesterol from the PM to the ER in CHO-K1 cells.

Although PS and Asters are both required for normal PM-to-ER cholesterol transport, their functions overlap only partially. This conclusion is based on a genetic epistasis analysis. If Aster proteins absolutely require PS for function, then the Ptdss1/C0/C0;Aster-abc/C0/C0 cells should lack Aster function, and the addition of Aster deficiency in the Ptdss1/C0/C0;Aster-abc/C0/C0 cells should produce no further reduction. However, this was not the case. As shown in Fig. 2I and J, cholesterol esterification in the Ptdss1/C0/C0;Aster-abc/C0/C0 cells was significantly lower than in the Ptdss1/C0/C0 cells whether...
The epistasis analysis also indicates that PS is required for a transport process that is independent of Asters. If PS were required only to support Aster function, then the Aster-abc−/− cells should lack all functions of PS, and the addition of PTDSS1 deficiency should produce no further reduction. Again this was not the case. As shown in Fig. 2 J and K, adding PTDSS1 deficiency to Aster deficiency caused a further reduction in cholesterol esterification, indicating that, in addition to supporting Aster function, PTDSS1 is required for another PM-to-ER cholesterol transport process that is independent of Aster.

Further evidence for the role of PS in Aster function is illustrated by the Upper panels in Fig. 2. As we showed previously (12), restoration of cellular PS by addition of PS-containing liposomes restored cholesterol delivery from the PM to the ER in Ptdss1−/− cells, as manifested by an increase in cholesterol esterification after addition of cholesterol/MCD (Fig. 2B). Similar restoration occurred when cholesterol was delivered in LDL (Fig. 2F). Restoration was much less when the Ptdss1−/− cells lacked Asters (Fig. 2 D and H).

The current data raise two questions for future study of PM-to-ER cholesterol transport. First, what is the substance that can support partial Aster function in the absence of PS? It is likely to be another negatively charged lipid, perhaps phosphatidylinositol, which is already known to bind to Asters (11). Second, what is the Aster-independent pathway that requires PS? Does it depend on contact sites between the PM and the ER or could it involve a vesicular mechanism through endocytosis of cholesterol-rich membranes? These questions should be answered now that the roles of Asters and PS have been distinguished.

Materials and Methods

Reagents, mutant cell lines and knockouts, plasmids, purification and labeling of PFO+, BODIPY FL-LDL uptake, PFO* binding, visualization of PFO* binding by fluorescence microscopy, flow cytometry, cholesterol ester synthesis, PS unilamellar liposomes, SREBP-2 processing, immunoblot analysis, measurement of phospholipids by liquid chromatography with tandem mass spectrometry (LC-MS/MS), and reproducibility can be found in SI Appendix, Materials and Methods.

Data Availability. All study data are included in the article and/or SI Appendix.

ACKNOWLEDGMENTS. We thank our colleagues Jaeil Han, Joshua Mendell, and Marcel Mettlen for helpful discussions; Erin Ruhlman, Alexa Smith, and William Salter for excellent technical assistance; and Lisa Beatty and Alexandra Hatton for invaluable help with tissue culture. This research was supported by grants from the NIH (HL20948 and GM096070) and the Welsh Foundation (I-1910). M.N.T. was supported by the NIH Medical Scientist Training Program (GM008014) and is a recipient of the Paul & Daisy Soros Fellowships for New Americans.

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