Lipid droplets are arrested in the ER membrane by tight binding of lipidated apolipoprotein B-100

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Summary

Apolipoprotein B-100 (ApoB) is a major component of very-low-density lipoproteins, and is deposited in a region around lipid droplets (LDs) called the 'ApoB-crescent'. The ApoB-crescent is thought to be related to ApoB degradation because it drastically increases when proteasome or autophagy is inhibited. In the present study, we found that ApoB-crescents were significantly reduced when ApoB lipidation was suppressed by either the inhibition or knockdown of the microsomal triglyceride-transfer protein. By contrast, ApoB-crescents increased under conditions that are presumed to cause lipidated ApoB abnormalities in secretory compartments. By electron microscopic analyses, we identified the ApoB-crescent as a thin cholesterol-rich ER cistern fused to an LD, and – topologically – this structure is equivalent to a lipid-ester globule between the two leaflets of the ER membrane. ApoB localized in the thin cisternal lumen, and its binding to LDs was resistant to alkaline treatment. Overexpression of ADRP or TIP47 suppressed the increase in the number of ApoB-crescents, whereas knockdown of these proteins had the opposite effect. From these results, we inferred that the ApoB-crescent is formed by an LD that is arrested in the ER membrane by tight binding of lipidated ApoB to its luminal surface. We suggest that ApoB processing and LD formation are closely linked.

Supplementary material available online at
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Key words: Lipid droplet, ER, Apolipoprotein B, Microsomal triglyceride transfer protein (MTP), Adipose differentiation-related protein (ADFP, ADRP), TIP47

Introduction

Lipid droplets (LDs) are ubiquitous cytoplasmic structures that consist of a neutral lipid core surrounded by a unique phospholipid monolayer (Murphy and Vance, 1999; Tauchi-Sato et al., 2002). LDs have been regarded as a reservoir to store excess lipids as non-hazardous ester forms. The stored lipids are not only used as a source of energy, but they are also mobilized for membrane biogenesis, lipoprotein formation and eicosanoid synthesis. Recent proteomic studies revealed that many new proteins are located in isolated LDs, and LDs have been proposed to participate in a multitude of functions, including membrane trafficking, signal transduction and temporal protein storage (Fujimoto and Ohsaki, 2006; Martin and Parton, 2006; Welte, 2007).

LDs in hepatocytes are physiologically important as a lipid source for the production of very-low-density lipoprotein (VLDL) (Gibbons et al., 2000), but the excessive accumulation of LDs in hepatocytes is a hallmark of steatosis – or fatty liver – which may lead to liver cirrhosis or carcinoma (Ginsberg, 2006). Apolipoprotein B-100 (ApoB) is the principal protein component of VLDL, and the regulatory mechanism of ApoB expression has been extensively studied (Fisher and Ginsberg, 2002; Shelness and Ledford, 2005). ApoB is co-translationally lipidated by the microsomal triglyceride-transfer protein (MTP) (Hussain et al., 2003) and, when this lipidation process is impaired, ApoB is subjected to ER-associated degradation (ERAD). During ERAD, ApoB is retrotranslocated to the cytoplasm through the translocon, ubiquitylated, and degraded by the proteasome (Zhou et al., 1998). The lipidation process may be facilitated by the addition of fatty acids, and when this occurs ApoB and VLDL secretion increases. It is thought, however, that other mechanisms to degrade lipidated ApoB in secretory compartments must also exist, although these molecular mechanisms have not been defined as yet (Liao et al., 1998; Fisher et al., 2001; Olofsson and Boren, 2005).

In a previous study, we found that ApoB accumulates in a crescent-shaped area that surrounds LDs in cultured hepatocytes, and we named this structure the 'ApoB-crescent' (Ohsaki et al., 2006a). The occurrence of ApoB-crescents drastically increased upon inhibition of proteasomes or autophagy. Moreover, ApoB found in LDs was highly ubiquitylated, and proteasomal subunits were distributed around LDs. These results led us to propose that the ApoB-crescent is a site where ApoB is stored when the intracellular protein degradation mechanisms are downregulated (Fujimoto and Ohsaki, 2006; Ohsaki et al., 2006a). This study did not reveal, however, where in the ApoB-processing mechanism the ApoB-crescent is involved.

Here, we studied the effects of various reagents and manipulations that are known to influence ApoB and/or LD to investigate the function of the ApoB-crescent. As a result, we found that ApoB molecules that accumulate in the ApoB-crescent are lipidated by the action of MTP. This result suggested that ApoB in the ApoB-crescent is not processed by the canonical ERAD pathway. Consistent with this, morphological studies revealed that the ApoB-crescent is an LD fused to a thin ER cistern, and that ApoB is localized in the cisternal lumen. The structure of the ApoB-crescent is equivalent to a lipid-ester deposit between the two membrane leaflets of the ER membrane, or the putative intermediate stage of the prevailing LD biogenesis model. This kind of structure has never been observed in animal cells and is probably caused by the tight

binding of anomalous lipidated ApoB to the lumenal surface of the ER membrane. Altogether the results of these experiments suggest that there is a close link between the processes of intracellular ApoB processing and LD formation.

Results and Discussion

ApoB-crescents are formed by lipidated ApoB

ApoB is co-translationally lipidated to form pre-VLDL and is secreted as mature VLDL after acquiring more lipids in the ER or post-ER compartments (Fisher and Ginsberg, 2002; Shelness and Ledford, 2005). When lipidation is suppressed by inhibition of MTP activity or a shortage of lipids, ApoB is subjected to ERAD and degraded by proteasomes. Since the formation of ApoB-crescents was significantly increased by proteasomal inhibitors, we hypothesized that MTP inhibition should have the same effects.

In contrast to our hypothesis, we found that the basic level of ApoB-crescents in Huh7 cells was reduced following treatment with MTP inhibitors, and that the increase in the number of ApoB-crescents induced by the proteasomal inhibitor *N*-acetyl-L-leucinyl-L-leucinyl-L-norleucinal (ALLN) was also significantly suppressed (Fig. 1A). The increase in ApoB-crescents caused by other proteasomal inhibitors, such as lactacystin and MG132, was also suppressed by MTP inhibitors (data not shown). These results were not due to a decrease in the expression of the ApoB protein because treatment with an MTP inhibitor did not significantly change the cellular ApoB content (supplementary material Fig. S1A), and ApoB was still labeled in the ER network after treatment (supplementary material Fig. S1B). A similar reduction in ApoB-crescents was observed when MTP was knocked down by small interfering RNA (siRNA; Fig. 1B). Knockdown of ApoB naturally reduced the number of ApoB-crescents (Fig. 1B). These results indicated that MTP-mediated ApoB lipidation is necessary for the formation of ApoB-crescents. Consistent with these results, ApoB-crescents were not observed in HeLa cells that express ApoB but lack MTP (Dixon et al., 2002), even when the formation of LDs was induced by loading with oleic acid. By contrast, ApoB-crescents were observed in Caco-2 cells that express both ApoB and MTP, and produce lipoproteins (supplementary material Fig. S2).

Since MTP is a soluble protein in the ER, ApoB lipidation is believed to occur co-translationally at the luminal surface of the ER membrane. The above results suggested that, even after protein translation is stopped, ApoB-crescent formation will still proceed for some time, due to the deposition of ApoB molecules that are already lipidated and present in the ER lumen. We found that the number of ApoB-crescents increased following treatment with proteasomal inhibitors in cells that had been pretreated with cycloheximide (Fig. 1C). These results showed that the ApoB-crescent is formed by lipidated ApoB in the ER lumen and not by non-lipidated nascent ApoB that retrotranslocated to the cytoplasm.

A prominent increase in the number of ApoB-crescents was observed when cells were treated with docosahexaenoic acid (DHA), and this chemical has been reported to induce ApoB proteolysis in the secretory pathway (Fisher et al., 2001) (Fig. 1D). Cyclosporin A, a specific inhibitor of cyclophilin B, also increased the number of ApoB-crescents (Fig. 1D). Cyclophilin B is one of the ER chaperones that bind to lipoprotein particles in the secretory pathway and is necessary for correct ApoB folding (Zhang and Herscovitz, 2003). These results suggest that abnormalities in the process after lipidation lead to the formation of ApoB-crescents.

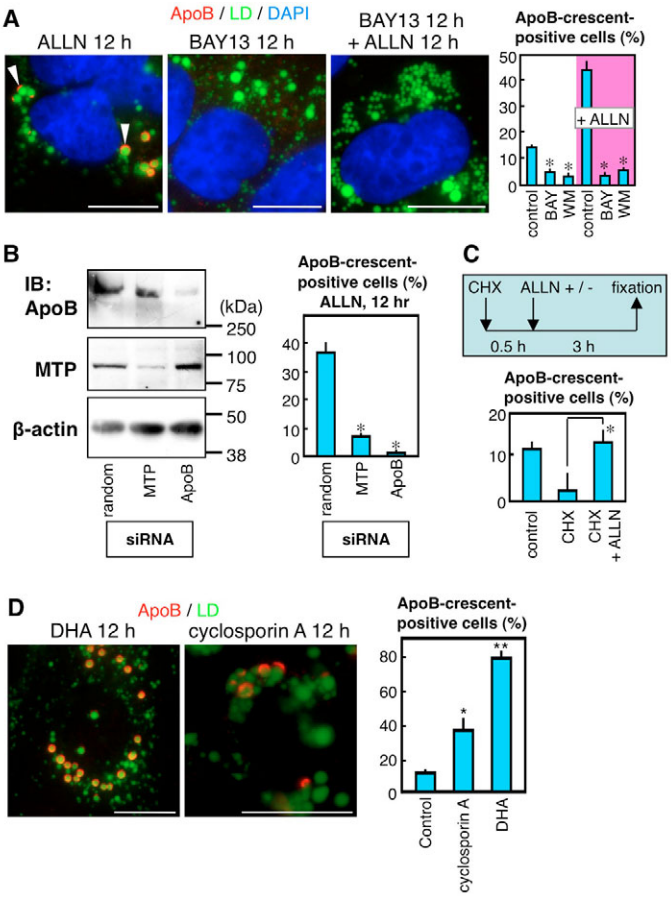

Fig. 1. ApoB lipidated by MTP activity is necessary for ApoB-crescent formation. (A) Incubation with MTP inhibitors (100 nM BAY13-9952 or WM1159) for 12 hours significantly decreased the number of ApoB-crescents in Huh7 cells. Triple labeling for ApoB (red), LD (green), and nucleus (blue) is shown. ApoB was labeled by a mouse anti-ApoB (6H12) antibody. The same result was obtained in HepG2 cells (data not shown). The number of ApoB-crescent-positive cells was reduced by the MTP inhibitors (left half of the bar graph), and it did not increase even after further treatment with 10 μM ALLN for 12 hours (right half of the bar graph). More than ten pictures were randomly taken, and the ratio of ApoB-crescent-positive cells was quantified. The results from three independent experiments were averaged, and the statistical difference from the control was examined by the Student's *t*-test (**P*<0.001). Bars, 10 μm. (B) Knockdown of MTP or ApoB by RNAi reduced the number of ApoB-crescents in Huh7 cells. The ratio of ApoB-crescent-positive cells was measured three days after RNAi, and after treatment with 10 μM ALLN for the final 12 hours. For western blotting, an equal amount (30 μg) of each cell lysate was electrophoresed. The results from three independent experiments were averaged, and the statistical difference from the control was examined using the Student's *t*-test (**P*<0.001). (C) The ApoB-crescent was increased by ALLN treatment even after protein translation was inhibited using 5 μg/ml cycloheximide (CHX). The results from three independent experiments were averaged, and the statistical difference was examined by the Student's *t*-test (**P*<0.01). (D) Treatment with either 0.4 mM DHA complexed to BSA or 1 μg/ml cyclosporin A for 12 hours considerably increased ApoB-crescent formation. Representative immunofluorescence micrographs using a mouse anti-ApoB (6H12) antibody are presented. The ratio of ApoB-crescent-positive cells is shown in the bar graph. The results from three independent experiments were averaged, and the statistical difference from the controls was examined by a Student's *t*-test (**P*<0.01, ***P*<0.001).

Several studies have shown that lipidated ApoB – or pre-VLDL – is degraded by using a mechanism that is different from the ERAD pathway that disposes of non-lipidated ApoB (Liao et al., 1998;

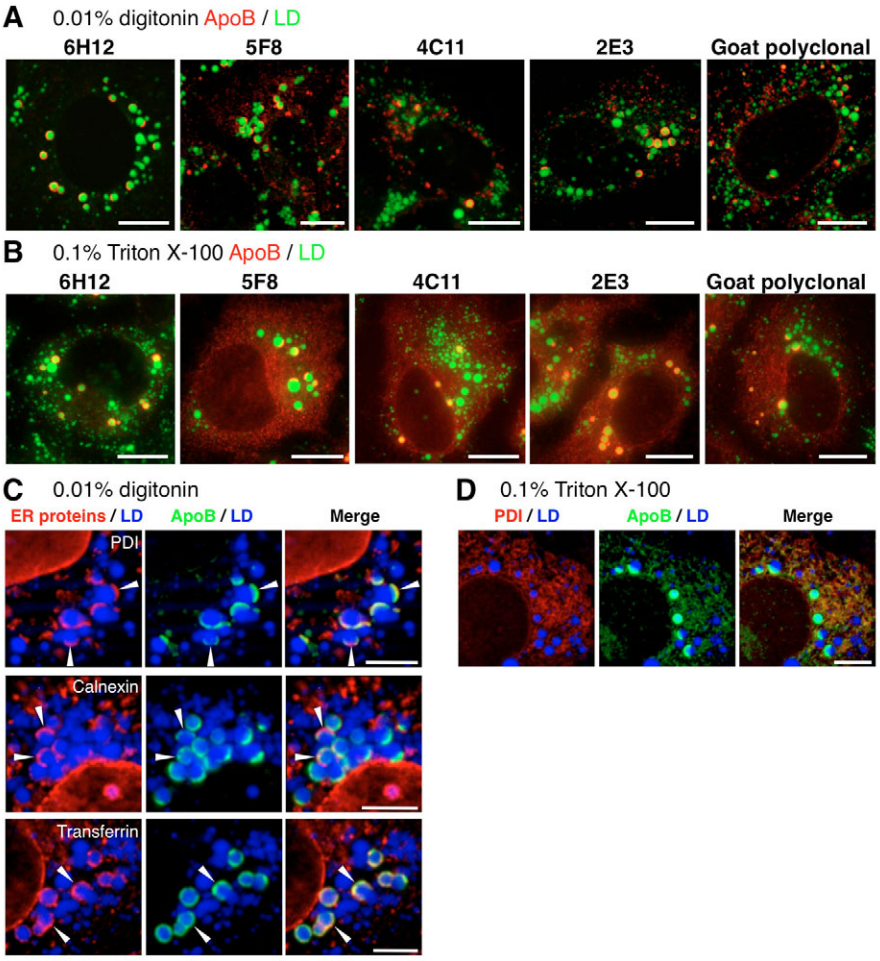

Fig. 2. Immunofluorescence microscopy of ApoB. (A) Labeling of ApoB with four monoclonal antibodies and one polyclonal antibody. Huh7 cells treated with 10 µM ALLN for 12 hours were fixed and permeabilized with 0.01% digitonin for 30 minutes. ApoB (red) and LD (green) are doubly labeled. One monoclonal antibody (6H12) labeled only the ApoB-crescent, whereas the other four antibodies labeled both ApoB-crescents and lysosomes (Ohsaki et al., 2006a) but did not label the ER. Bars, 10 µm. (B) Labeling of ApoB in ALLN-treated Huh7 cells that were fixed and permeabilized with 0.1% Triton X-100 for 5 minutes before labeling. One monoclonal antibody (6H12) exclusively labeled the ApoB-crescent, whereas the other four antibodies also labeled the ER network in addition to the ApoB-crescent. Bars, 10 µm. (C) Huh7 cells treated with 10 µM ALLN for 12 hours were fixed, permeabilized with 0.01% digitonin for 30 minutes, and subjected to triple labeling for ER proteins (red), ApoB (green), and LD (blue). PDI, calnexin, and transferrin showed a marked colocalization with ApoB around LDs (arrowheads). ApoB was labeled by either a goat polyclonal or mouse monoclonal (6H12) antibody. The nuclear membrane was also labeled for the ER proteins. Bars, 5 µm. (D) Huh7 cells incubated with 10 µM ALLN for 12 hours were fixed and permeabilized with 0.1% Triton X-100 for 5 minutes and labeled by PDI (red) and ApoB (green; goat polyclonal), and LD (blue). PDI was found in a network pattern throughout the cytoplasm, and labeling around LDs was not conspicuous. Bars, 5 µm.

Fisher et al., 2001; Olofsson and Boren, 2005). ApoB-crescents might be involved in this ill-defined pathway, but further studies are necessary to test whether this is the case.

The ApoB-crescent is a special ER sub-compartment fused to LD

The ApoB-crescent was identified by immunofluorescence labeling of ApoB in cells that had been fixed and permeabilized using digitonin at a low concentration (0.01%). Immunolabeling of ApoB by four monoclonal antibodies and one polyclonal antibody was confined to the ApoB-crescent and the late endosome/lysosome (Fig. 2A). Digitonin preferentially acts upon membranes that are enriched with free cholesterol (Elias et al., 1978), so the labeling of the late endosome/lysosome was expected. By contrast, the pattern of labeling in the ApoB-crescents was surprising, considering that lipidated ApoB should be generated in the ER lumen, and the ER membrane is deficient in free cholesterol.

In cells fixed and permeabilized with Triton X-100, three monoclonal antibodies and one polyclonal antibody against ApoB labeled the ER network as well as the ApoB-crescent, which was consistent with the presence of maturing VLDL in the ER lumen (Fig. 2B). A notable finding in the cell preparations treated with Triton X-100 was that one of the monoclonal antibodies (6H12) labeled only the ApoB-crescent but not the ER network (Fig. 2B). The specificity of all the anti-ApoB antibodies was verified by western blotting. All antibodies yielded a single band over 500 kDa, and the intensity of this band was significantly reduced in RNA interference (RNAi) experiments (Fig. 1B; data not shown). These results indicated that an ApoB epitope that is recognized by 6H12 is exposed only in the ApoB-crescent. In the ApoB-crescent ApoB might adopt a different conformation from ApoB in the secretory pathway or, alternatively, the 6H12 epitope might be masked by other molecules when ApoB is in other subcellular locations.

Further characterization of the ApoB-crescent was performed using various organelle markers. Using double labeling, markers for the Golgi complex, lysosomes and endosomes did not overlap with ApoB-crescents (Ohsaki et al., 2006a), whereas ER chaperones, PDI, cyclophilin B, ERp72 and BiP, were obviously concentrated within ApoB-crescents (Fig. 2C; supplementary material Fig. S3). Antibodies against the lumenal epitope of calnexin, a transmembrane ER protein and to transferrin (a secretory protein) also showed a similar distribution (Fig. 2C). The labeling of the ER proteins in the ApoB-crescent was conspicuous when cells were permeabilized with digitonin, whereas there was intense labeling throughout the ER network in cells that were permeabilized with Triton X-100 (Fig. 2D).

From these results, we inferred that the ApoB-crescent is a part of the ER or a structure connected to ER. To characterize the ApoB-crescent structure at the ultrastructural level, pre-embedding immunoelectron microscopy (immunoEM) was performed, using a nanogold-conjugated antibody and gold enhancement. The

cells were fixed and permeabilized by digitonin as for immunofluorescence microscopy and labeled for ApoB and PDI. The labeling of both proteins was consistently found in the lumen of a thin membrane cistern directly associated with LDs (Fig. 3A). It appeared that the thin membrane cistern was not simply apposed to LDs but fused with LDs.

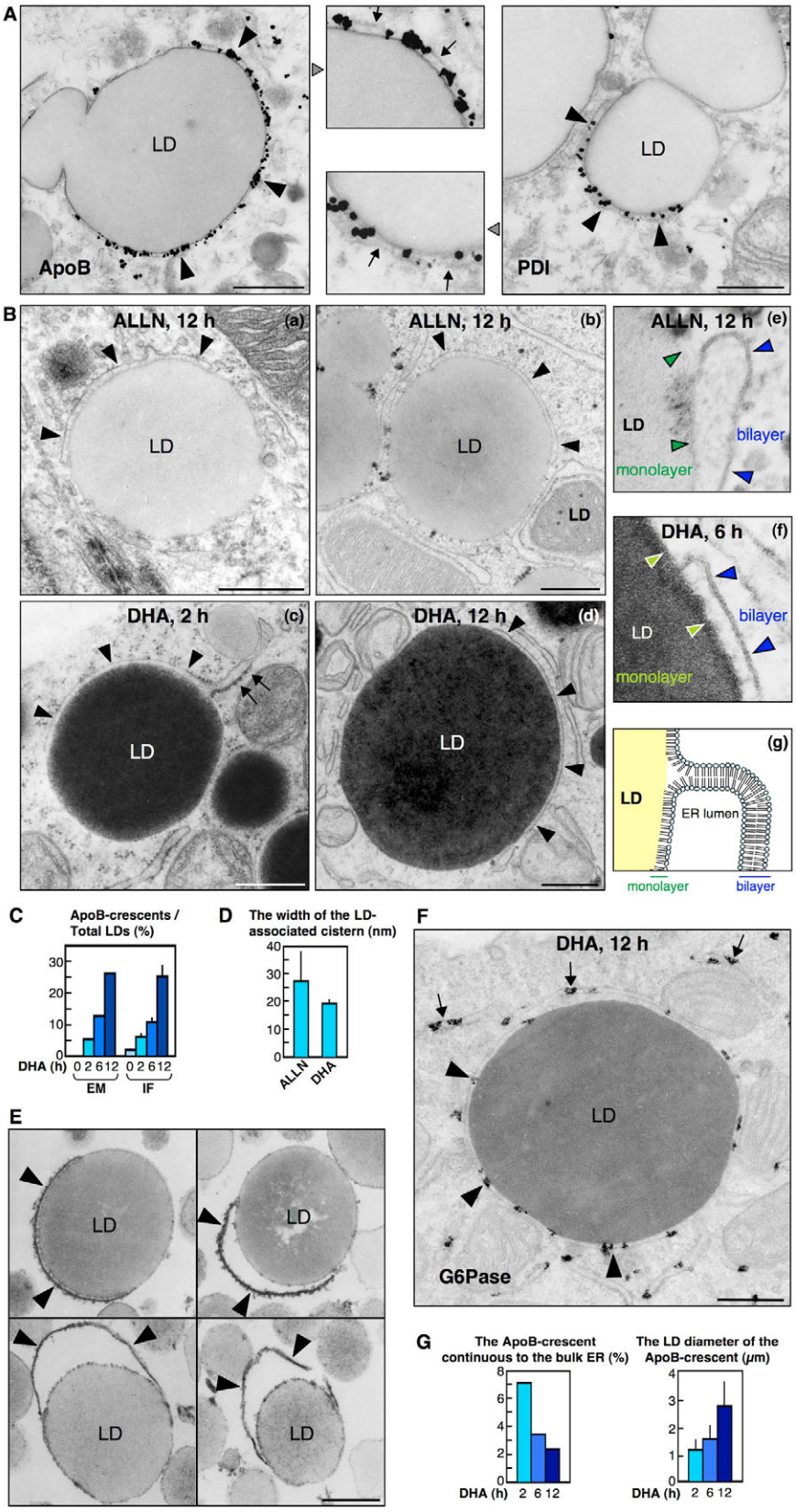

Fig. 3. EM revealed that the ApoB-crescent is a flat ER cistern fused to an LD. (A) Pre-embedding immunoEM of Huh7 cells treated with 0.4 mM DHA. The labeling for both ApoB and PDI was localized to the thin cisternal lumen (arrowheads) adhering to the LD. ApoB was labeled by a goat anti-ApoB antibody. High magnification pictures clearly show the presence of the cisternal membrane (arrows). Bars, 500 nm. (B) Conventional EM of Huh7 cells treated with 10 μM ALLN (a,b,e) or 0.4 mM DHA (c,d,f) for 2-12 hours. (a-d) A flat membrane cistern (arrowheads) was observed along the LD perimeter. Some of the cistern was continuous with the rough ER (arrows) or decorated with ribosomes (c). The electron density of the LD is high in DHA-treated samples because osmium tetroxide reacts with unsaturated bonds of DHA. (e,f) High magnification micrographs delineated that the cisternal membrane with a unit membrane appearance (blue arrowheads) ends on the LD surface that lacks the visible membrane (green arrowheads). (g) A scheme of the membrane leaflet continuity between the cisternal membrane and the LD surface. Bars, 500 nm. (C) The ratio of cells with an LD-associated membrane cistern was quantified in EM pictures and compared with the ratio of cells with ApoB-crescents by immunofluorescence microscopy. The two ratios showed a good correlation in Huh7 cells treated with 0.4 mM DHA for 2, 6, and 12 hours. (D) The width of the LD-associated membrane cistern. Ten cisterns were randomly selected from Huh7 cell samples treated with 10 μM ALLN or 0.4 mM DHA for 12 hours. The width of each cistern was measured at four different points. (E) Direct continuity of the cisternal membrane and the LD surface was maintained in the isolated LD preparation. The cisternal membrane (arrowheads) was disrupted in most samples. Bar, 500 nm. (F) Glucose-6-phosphatase activity was visualized by enzyme histochemistry using lead nitrate. The reaction product was found in the LD-fused membrane cistern (arrowheads) and in the ER (arrows). Bar, 500 nm. (G) The ApoB-crescents in cells treated with 0.4 mM DHA for 2, 6, and 12 hours were photographed by EM, and the ratio of crescents that were directly continuous with the ER was quantified. The diameter of LD that contributed to the ApoB-crescent was also measured for the same sample and averaged.

Since the relatively weak fixation that was used for immunoEM did not preserve fine structures to a satisfactory level, cells with a large number of ApoB-crescents were treated with an optimal fixative to delineate structural detail. LD-associated membrane cisterns were frequently observed (Fig. 3Ba-d), and the ratio of cells with that structure (obtained using EM) correlated well with that of the ApoB-crescent-positive cells (observed using immunofluorescence microscopy) (Fig. 3C). The cisternal membrane ended on the LD surface at an obtuse angle, and the lumen had a relatively constant width of about 20-40 nm in most cases (Fig. 3D). The surface of the thin cistern opposite the LD was lined with a trilaminar-unit membrane, whereas its surface on the LD side had no apparent membrane (Fig. 3Be,f). Direct continuity of the cisternal membrane to the LD surface was retained in isolated preparations and unambiguously observed (Fig. 3E). These results indicated that the luminal and cytoplasmic leaflets of the cisternal membrane were continuous with the phospholipid monolayer of the LD surface (see diagram in Fig. 3Bg). The LD-associated membrane cistern was clearly delineated when a mixture of osmium tetroxide and potassium ferrocyanide was used as a secondary fixative (White et al., 1979) or in rapid-frozen and freeze-substituted specimens (data not shown). The membrane was not clearly observed, however, when samples were treated with a secondary fixative containing osmium tetroxide alone.

The presence of PDI and other soluble ER chaperones suggested that the thin membrane cistern fused to LD is a part of ER. The cistern was occasionally continuous with the rough ER or the cisternal membrane itself was studded with ribosomes, especially at early stages after induction (Fig. 3Bc). Furthermore, the reaction product of glucose-6-phosphatase, an authentic ER marker, was detected in the cistern by enzyme histochemistry (Fig. 3F). These results confirmed that the ApoB-crescent is made of an LD, and an ER compartment fused to it. The direct structural continuity of the LD-associated ER cistern with the rest of ER was seen in 7.1% of the ApoB-crescents in cells treated with DHA for 2 hours, but this ratio decreased with longer DHA treatments (Fig. 3G). This decrease was probably caused because the conduit between the LD-associated cistern and the bulk ER is rather thin, so the chance of its being observed in ultrathin sections decreases as the LDs that form the ApoB-crescent grow larger. Owing to this technical difficulty, it was not possible to determine whether the lumen of the LD-fused cistern is always continuous with the rest of ER. For simplicity, the LD-ER fusion structure will also be called the ApoB-crescent or ApoB-crescent structure, and the membrane of the LD-associated cistern will be referred to as the ApoB-crescent membrane.

The ApoB-crescent membrane is enriched with free cholesterol

ER proteins in the ApoB-crescent lumen were labeled even when fixed cells were permeabilized with digitonin, whereas those in the ER network were not (Fig. 2C). This result suggested that the cisternal membrane of the ApoB-crescent was enriched with free cholesterol, which was confirmed by using streptolysin O – which acts more specifically upon cholesterol than digitonin (Rosenqvist et al., 1980) (supplementary material Fig. S4). Furthermore, filipin, which binds to free cholesterol specifically, stained areas around LDs of the ApoB-crescent and colocalized with ApoB on one side of LDs, whereas adipose differentiation-related protein (ADFP, hereafter referred to as ADRP) was found on the opposite side of the LDs (Fig. 4A). The characteristic wavy deformation caused by the filipin-cholesterol complex (Thyberg, 2002) was also observed in the ApoB-crescent membrane by EM (Fig. 4B). These results suggested that the ApoB-crescent membrane contains more free cholesterol than the bulk ER membrane.

The occurrence of the ApoB-crescents identified by immunofluorescence microscopy was, as described above (Fig. 1A), reduced in cells treated with MTP inhibitors. Under these conditions, the labeling of ER proteins around LDs was also decreased (data not shown), suggesting that the formation of the LD-ER fusion structure depends on the presence of lipidated ApoB. By contrast, positive labeling of ER proteins after permeabilization with digitonin or streptolysin O was also observed in other cells that do not produce lipoproteins (J.C. and T.F., unpublished data). These results suggested that an ER region that is enriched with free cholesterol exists in normal cells; but how this region is generated and related to the ApoB-crescent formation remains to be studied.

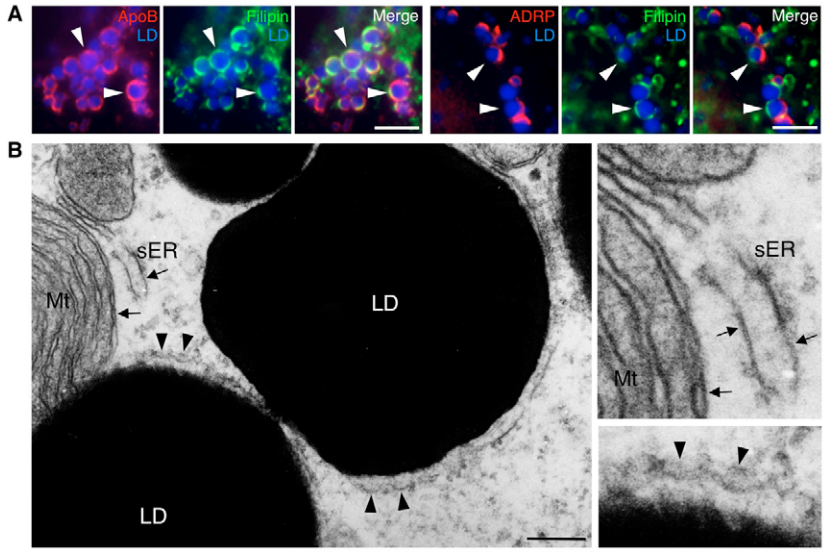

Fig. 4. The ApoB-crescent membrane is enriched with free cholesterol. (A) Huh7 cells treated with 10 μM ALLN for 12 hours were incubated with filipin (green) to label free cholesterol-enriched membrane. Filipin stained a crescent-shaped area around LDs (arrowheads). In most LDs, filipin staining was found on the same side as ApoB (red, goat polyclonal; the left panel), but on the opposite side from ADRP (red; the right panel). LDs were stained by BODIPY493/503 (blue). Bars, 5 μm. (B) The ALLN-treated Huh7 cells were treated with filipin and observed by EM. High-magnification images are shown in the right. Note that the membrane of the ApoB-crescent shows wavy deformation (arrowheads in the lower right figure), indicating the presence of filipin-cholesterol complexes. The membranes of mitochondria (Mt) and sER were smooth and not deformed (arrows in the upper right figure). Bar, 200 nm.

ADRP and TIP47 suppress ApoB-crescent formation

Deposition of lipid esters between the two leaflets of the ER membrane has been considered to be a plausible mechanism of LD biogenesis in

eukaryotic cells (Wanner et al., 1981; Murphy and Vance, 1999; Martin and Parton, 2006). Although this model has prevailed, little evidence has supported it. ^{1}H-NMR analysis indicated that there are isotropically mobile lipids in membrane samples (Ferretti et al., 1999; Lacey et al., 1999), but the origins of the signals have been debated (Hakumaki and Kauppinen, 2000). The accumulation of lipid esters in the ER-like membrane has been observed in some plant cells (Wanner et al., 1981) but not in other plant cells or in animal cells (Murphy, 2001). The EM results from our study showed that the ApoB-crescent is topologically equivalent to a lipid-ester globule intercalated between the two leaflets of the ER membrane (Murphy and Vance, 1999; Fujimoto and Ohsaki, 2006; Martin and Parton, 2006). We speculated that this peculiar structure was formed because the normal LD formation was suppressed.

To test this hypothesis, expression of ADRP and TIP47 (also known as M6PBP) was manipulated by transfection of cDNA or siRNA, and the effects upon ApoB-crescents were examined. ADRP promotes LD formation in non-adipose cells (Magnusson et al., 2006) and decreases the triglyceride content of the microsome (Chang et al., 2006). These results suggest that ADRP promotes LD formation, although this has not been shown directly. TIP47 is likely to share this function at least partially because it can replace ADRP under certain circumstances (Sztalryd et al., 2006). According to this model, overexpression of ADRP or TIP47 might be expected to decrease ApoB-crescent formation by facilitating LD formation. After transfection of ADRP or TIP47 cDNA, the frequency of ApoB-crescents after ALLN treatment decreased significantly (Fig. 5A). The amount of ADRP could be upregulated by proteasomal inhibition (Xu et al., 2005), but this did not occur in Huh7 cells under the present experimental conditions (data not shown). The suppressive effects upon the ApoB-crescent were more evident upon introduction of ADRP than of TIP47, probably because only a small fraction of TIP47 is localized to LDs under normal conditions (Ohsaki et al., 2006b). Consistent with the results of the overexpression experiments, downregulation of either ADRP or TIP47 using siRNA significantly increased the frequency of ApoB-crescents (Fig. 5B). These results were consistent with our inference that the ApoB-crescent represents an intermediate stage of LD formation in the ER membrane.

Lipidated ApoB binds tightly to the LD surface

We wished to determine why LD formation is stalled in the ApoB-crescent. Lipidated ApoB is thought to exist either as a soluble particle in the ER lumen or by anchoring to the ER membrane, and it develops into mature VLDL particles after incorporating more lipids. The results, using DHA and cyclosporin A, suggested that abnormalities in the VLDL maturation process are involved in ApoB-crescent formation (Fig. 1D). The exclusive labeling of the ApoB-crescent by a monoclonal antibody (6H12; Fig. 2B) also implied that ApoB in the ApoB-crescent adopts a different structure from ApoB in the ER. It was also of note that ApoB labeling appeared to be concentrated around LDs when compared with labeling in the ER network, even after indiscriminate permeabilization using Triton X-100 (Fig. 2B). On the basis of these results, we hypothesized that anomalous binding of lipidated ApoB to the ER membrane disturbs LD formation and induces formation of the ApoB-crescent structure.

In support of this hypothesis, LD isolated from cells that carry large numbers of ApoB-crescents labeled positively for ApoB, although the cisternal membrane was – according to EM analysis – largely disrupted (Fig. 3E). ADRP was also labeled in the same LD preparation, and ApoB and ADRP were seen in complementary areas in most cases (Fig. 6A). By contrast, labeling for PDI was scarcely detected in the LD sample, despite its presence in the ApoB-crescent (data not shown). Furthermore, when the LD preparation was treated with 0.1 M sodium carbonate for 30 minutes and subjected to a second sucrose-density-gradient centrifugation, both ApoB and ADRP were again recovered in the top-floating fraction, whereas a residual amount of cyclophilin B (present in the first LD fraction) was solubilized and moved to the bottom fractions (Fig. 6B). The results showed that ApoB in the ApoB-crescent tightly adheres to the LD through non-ionic interactions.

Altogether, these results suggested that the LD in the ApoB-crescent is a giant lipid-ester globule that is intercalated between the two leaflets of the ER membrane. This structure is probably unstable and might only exist transiently in normal cells. That is, when de novo LDs are formed, the lipid ester is likely to leave the membrane as LDs before reaching a certain size (Fig. 7). This explains why it has not been captured previously by conventional methods. In the ApoB-crescent, the LD in the membrane has probably been observed because the binding of lipidated ApoB

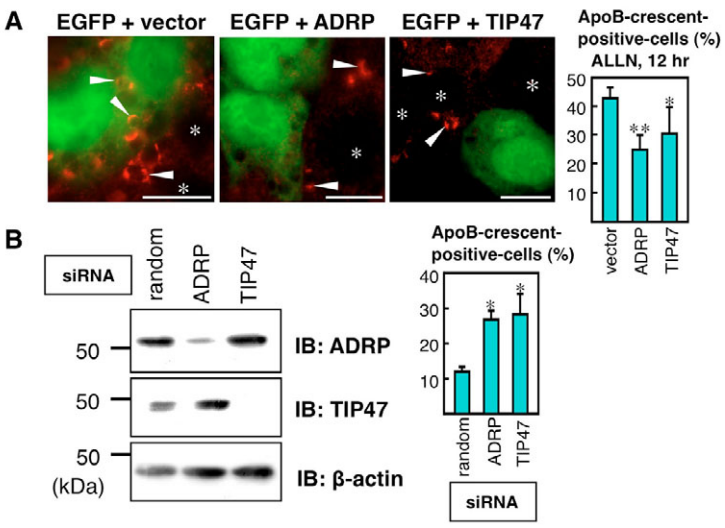

Fig. 5. PAT proteins suppress ApoB-crescent formation. (A) Overexpression of ADRP and TIP47 suppressed the formation of ApoB-crescents in Huh7 cells. To identify transfected cells, EGFP cDNA was introduced together with empty vector, ADRP cDNA, or TIP47 cDNA. Two days after transfection, cells were treated by 10 μM ALLN for 12 hours and labeled by mouse anti-ApoB (6H12) antibody (red, arrowheads). Asterisks in images indicate the nuclei of EGFP-negative cells. The results from three independent experiments were averaged, and the statistical difference from the control that was transfected with empty vector was examined by the Student's *t*-test (*P<0.05, **P<0.01). Bars, 10 μm. (B) Knockdown of ADRP and TIP47 by RNAi increased the number of ApoB-crescents. Huh7 cells were examined three days after RNAi without any further treatment. Western blotting of cell lysates (10 μg) confirmed that there was a significant decrease in the levels of the targeted proteins. The results from three independent experiments were averaged, and the statistical difference from the control transfected with control RNA was examined by the Student's *t*-test (*P<0.001).

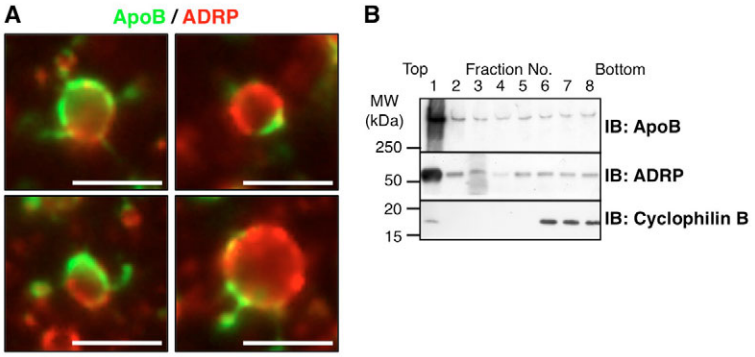

Fig. 6. ApoB in the ApoB-crescent tightly adheres to the LD surface through non-ionic interactions. (A) Gallery of isolated LDs labeled for ApoB (green; goat polyclonal) and ADRP (red). LDs were isolated from Huh7 cells treated with 0.4 mM DHA for 12 hours. ApoB and ADRP were observed in complementary areas of LDs. Bar, 5 µm. (B) LDs isolated from DHA-treated cells were incubated with 0.1 M sodium carbonate for 30 minutes at 4°C, and fractionated by the second round of density-gradient centrifugation. Using western blotting, most ApoB and ADRP were recovered from the top floating LD fraction, whereas cyclophilin B was shifted to the bottom soluble fraction.

stalled the LD formation process at an intermediate stage. In hepatocytes, a similar situation might also occur during the putative repeated fission and fusion cycles of LD and ER (Gibbons et al., 2000; Murphy, 2001). Lipids stored in LDs are preferentially used for VLDL maturation, and LD-ER fusion has been hypothesized to mobilize lipids for this process (Gibbons et al., 2000). Therefore, it is relevant for this function that lipidated ApoB binds to the ER membrane underneath the fused LD. The fusion might be only transient in the normal cell, but the LD might be trapped and form the ApoB-crescent due to anomalous binding of lipidated ApoB to the ER membrane (Fig. 7).

Our previous results have suggested that ApoB in the ApoB-crescent is destined for proteasomal and autophagic degradation (Ohsaki et al., 2006b). Combined with our present results, the ApoB-crescent is likely to be related to the degradation mechanism that copes with lipidated ApoB (Liao et al., 1998; Fisher et al., 2001; Olofsson and Boren, 2005). For this degradation function, and especially for proteasomal degradation, ApoB in the ER must be transported to the cytoplasm. Lipidated ApoB, however, might be too large to pass through protein channels such as the translocon, and special transport mechanisms might be required. One possible mechanism is the 'hatching' of lipid-ester globules together with the ER membrane, as has been recently hypothesized (Ploegh, 2007) (Fig. 7). This could make lipidated ApoB that binds to the luminal surface of the ER membrane accessible to the cytoplasmic proteasome; the whole hatched globule might be also processed by autophagy. Another intriguing possibility is that lipidated ApoB is first de-lipidated at the LD surface that faces the ER lumen and then retrotranlocated to the cytoplasmic side. It is physiologically relevant that lipids that are liberated from lipidated ApoB are recovered to the LD because they can be reused in subsequent rounds of VLDL synthesis. Whatever the mechanism, the degradation mechanism for lipidated ApoB might be intimately linked to the process of LD biogenesis and/or recycling, and it is worthy of further investigation.

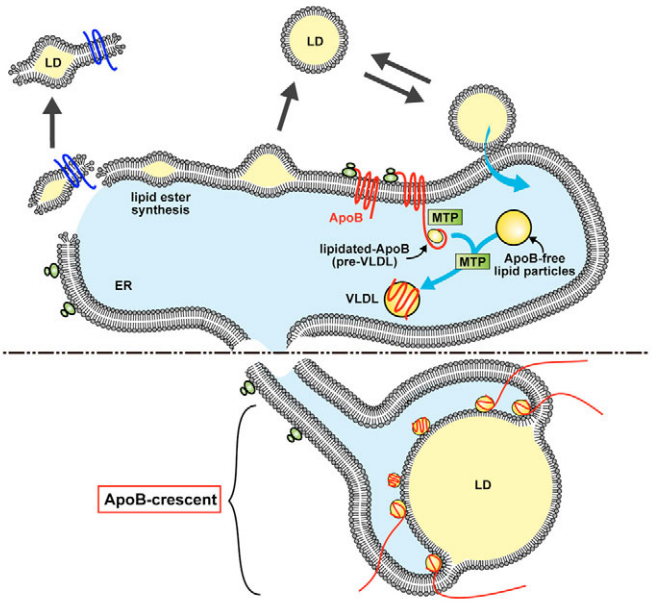

Fig. 7. A putative mechanism for ApoB-crescent formation. (Top) Under normal conditions, lipid esters synthesized in the ER membrane continuously leave the ER membrane by forming LDs. LDs might leave the ER membrane by hatching, budding, or other mechanisms. In hepatocytes, LDs might recycle to the ER to supply lipids for VLDL formation. ApoB is co-translationally lipidated and acquires additional lipids to become mature VLDL. (Bottom) Abnormalities in lipidated ApoB might cause it to bind tightly to the ER membrane and arrest the LD departure from the ER. This results in the accumulation of lipid esters in the ER membrane and induces formation of the ApoB-crescent structure.

Disorders in VLDL synthesis and secretion could lead to LD accumulation in hepatocytes. Recent studies have shown that production of the hepatitis C virus (HCV) requires both ApoB and MTP (Huang et al., 2007), and that the virus is likely to assemble in the vicinity of LDs (Miyanari et al., 2007). These results suggest that HCV takes advantage of the LD and its role in ApoB processing for its reproduction. It will be interesting to determine whether factors known to induce steatosis, including HCV infection and excessive intake of alcohol, influence the ApoB degradation process and the mechanisms of LD biogenesis.

Materials and Methods

Cells and transfection

Huh7, HepG2, COS-7, and HeLa cells were obtained from the Japanese Collection of Research Bioresources Cell Bank and cultured in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% FCS and antibiotics, at 37°C in a humidified atmosphere containing 5% CO_2. When appropriate, cells were incubated in medium containing 10 µM ALLN (Sigma), 25 µM lactacystin (Sigma), or 10 µM MG132 (Sigma) to inhibit proteasomal functions. DHA (Sigma) complexed with fatty-acid-free BSA (Wako) at a molar ratio of 6:1 was also applied at a final fatty acid concentration of 400 µM. Cells were transfected with cDNA or small interfering RNA (siRNA) using Lipofectamin 2000 (Invitrogen) according to the manufacturer's instruction.

Antibodies and reagents

Mouse monoclonal anti-human ApoB-100 (clone 6H12 from INTRACEL; clones 5F8, 4C11, and 2E3 from MONOSAN), goat polyclonal anti-human ApoB-100 (Rockland), mouse anti-ADRP (Progen), mouse anti-PDI (Affinity Bioreagents), and goat anti-Grp78 (Santa Cruz Biotech) antibodies were obtained from the respective suppliers. Secondary antibodies conjugated to fluorochromes (Jackson ImmunoResearch Laboratory) and colloidal gold (BioCel) were also purchased. The

MTP inhibitors BAY13-9952 and WM1159 were kindly provided by Bayer Healthcare and Wakunaga Pharmaceutical Co., Ltd, respectively. Streptolysin O was obtained from Sucharit Bhakdi (Johannes Gutenberg-University Mainz).

Subcellular fractionation and western blotting

Cells were disrupted by nitrogen cavitation and subjected to sucrose-density-gradient ultracentrifugation as previously described (Ohsaki et al., 2006a). In a single experiment, the top-floating LD fraction was treated with 0.1 M sodium carbonate (pH 11) for 30 minutes at 4°C and subjected to a second round of ultracentrifugation. Fractions were precipitated with 10% TCA, dissolved in sample buffer and analyzed by western blotting. The western blot signal was detected by chemiluminescence.

Immunofluorescence microscopy and data analysis

Cells were fixed with 3% formaldehyde ±0.015% glutaraldehyde in 0.1 M phosphate buffer for 15 minutes and permeabilized either with 0.01% digitonin in PBS for 30 minutes or with 0.1% Triton X-100 in PBS for 5 minutes before blocking and incubating with antibodies. LDs and nuclei were stained with BODIPY493/503 (Invitrogen) and DAPI, respectively. For isolated LDs, samples were adhered to coverslips that had been pre-treated with poly-L-lysine and glutaraldehyde, fixed with the aldehyde mixture, and incubated with antibodies after blocking. Images were captured using an LSM 5 PASCAL/Axioscope 2 laser-scanning microscope or Apotome/Axiovert 200M microscope (Carl Zeiss), using an Apochromat 63× lens with a 1.40 numerical aperture. Specimens were moved in a pre-determined manner, and pictures were taken randomly along the path of movement. The color, brightness and contrast of the images were adjusted using Adobe Photoshop 7.0. Cell numbers and colocalization of ApoB and other markers were quantified using ImageJ.

Electron microscopy

For conventional EM, cells cultured on coverslips were fixed with 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer and post-fixed in a mixture of 1% osmium tetroxide and 0.1% potassium ferrocyanide in the same buffer (White et al., 1979). Isolated LDs adhering to coverslips were fixed with a mixture of 2.5% glutaraldehyde and 1% osmium tetroxide. For glucose-6-phosphatase histochemistry, cells fixed with 0.5% glutaraldehyde in 0.1 M sodium cacodylate buffer were processed using the lead-nitrate method (Griffiths et al., 1983). For pre-embedding immunoEM, cells were fixed and permeabilized as for immunofluorescence microscopy and labeled sequentially by a primary antibody and a fluoro-nanogold-conjugated secondary antibody (Nanoprobes). The samples were treated with GoldEnhance (Nanoprobes) to visualize nanogold particles. Labeling by filipin was performed as previously described (Thyberg, 2002). After dehydration through an ethanol series, samples were embedded in Quetol812 resin. Ultrathin sections were observed using a JEOL 1200CX electron microscope at 100 kV.

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