Distinct Endosomal Compartments in Early Trafficking of Low Density Lipoprotein-derived Cholesterol*

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Shigeki Sugii, Patrick C. Reid, Nobutaka Ogami, Hong Du, and Ta-Yuan Chang††
From the ‡Department of Biochemistry, Dartmouth Medical School, Hanover, New Hampshire 03755 and the §Division of Human Genetics, Children's Hospital Medical Center, Cincinnati, Ohio 45229

We previously studied the early trafficking of low density lipoprotein (LDL)-derived cholesterol in mutant Chinese hamster ovary cells defective in Niemann-Pick type C1 (NPC1) using cyclodextrin (CD) to monitor the arrival of cholesterol from the cell interior to the plasma membrane (PM) (Cruz, J. C., Sugii, S., Yu, C., and Chang, T.-Y. (2000) J. Biol. Chem. 275, 4033–4032). We found that newly hydrolyzed cholesterol derived from LDL first appears in certain CD-accessible pool(s), which we assumed to be the PM, before accumulating in the late endosome/lysosome, where NPC1 resides. To determine the identity of the early CD-accessible pool(s), in this study, we performed additional experiments, including the use of revised CD incubation protocols. We found that prolonged incubation with CD (>30 min) caused cholesterol in internal membrane compartment(s) to redistribute to the PM, where it became accessible to CD. In contrast, a short incubation with CD (5–10 min) did not cause such an effect. We also show that one of the early compartments contains acid lipase (AL), the enzyme required for liberating cholesterol from cholesteryl ester in LDL. Biochemical and microscopic evidence indicates that most of the AL is present in endocytic compartment(s) distinct from the late endosome/lysosome. Our results suggest that cholesterol is liberated from LDL cholesteryl ester in the hydrolytic compartment containing AL and then moves to the NPC1-containing late endosome/lysosome before reaching the PM or the endoplasmic reticulum.

In mammalian cells, low density lipoprotein (LDL) binds to its receptor at the cell surface and is recruited into clathrin-coated endocytic vesicles. After endocytosis, LDL enters the endosomal/lysosomal system, where cholesteryl ester, a major lipid found in LDL, is hydrolyzed by the enzyme acid lipase (AL) (1). Mutations in AL cause cholesteryl ester to eventually accumulate in the lysosome (2, 3). After the hydrolytic action by AL, the transport of LDL-derived cholesterol from the endosome/lysosome to the plasma membrane (PM) or to the endoplasmic reticulum for re-esterification requires the protein named Niemann-Pick type C1 (NPC1). Mutations in NPC1 cause unesterified cholesterol and other lipids to accumulate in the late endosome and lysosome. Despite significant advances, the events that led to eventual accumulation of cholesterol in the late endosome/lysosome remain unclear. To delineate the early trafficking events of LDL-derived cholesterol, we previously performed pulse-chase experiments using [3H]cholesterol linoleate-labeled LDL ([3H]CL-LDL) in Chinese hamster ovary (CHO) mutant cells defective in the npc1 locus, CT43, along with their parental cells, 25RA (4). To monitor the arrival of [3H]cholesterol at the PM, we utilized a cyclodextrin (CD)-based intact cell assay. CD is a water-soluble molecule that has a high affinity for cholesterol and has been widely used to monitor the arrival of cholesterol at the PM from the cell interior (5–8). Our results show that [3H]cholesterol, newly released from the hydrolysis of [3H]CL-LDL, emerges in the early pool(s) in a manner unaffected by the npc1 mutation. Subsequently (within 2 h), in the parental cells, [3H]cholesterol is distributed to the PM and the endoplasmic reticulum. In CT43 cells, [3H]cholesterol accumulates in the characteristic aberrant endosome/lysosome (9). Cholesterol that is present in the early pool(s) is extractable by CD, whereas [3H]cholesterol that accumulates in the aberrant endosome/lysosome is resistant to extraction by CD. Based on this CD sensitivity test, the early pool(s) was assumed to be the PM (4). These results led us to hypothesize that, in NPC1 cells, cholesterol liberated from cholesteryl ester in LDL first moves to the PM independent of NPC1 and then moves back to the cell interior and accumulates in the aberrant late endosome/lysosome. Using a similar CD-based assay, other investigators independently reached the same conclusion (7).

The original CD-based assay used by us and by others involved continuous incubation of cells with CD for 30 min or longer. Thus, it is possible that prolonged incubation of cells with CD may cause redistribution of cellular cholesterol, so cholesterol originally residing in internal membranes moves to the PM and becomes extractable by CD. Recently, Haynes et al. (9) showed that, in CHO cells, depending on the incubation time used (ranging from 30 s to 20 min), CD is capable of extracting cellular cholesterol from two or three kinetically distinct pools; rearrangement of cholesterol between these pools could occur under various treatments. In this work, we further investigated the early trafficking events of LDL-derived cholesterol. To follow the fate of newly hydrolyzed cholesterol more precisely, we redesigned the procedures for the
pulsed-chase experiment and the CD treatment. We also performed biochemical and immunofluorescence experiments to define the hydrolytic compartment(s) involved in producing LDL-derived cholesterol. A model, revised from the one previously proposed by this laboratory (4), describing the early itinerary of LDL-derived cholesterol in the context of the endocytic pathway is presented.

EXPERIMENTAL PROCEDURES

Materials—Fetal bovine serum (FBS), protease inhibitor mixture, Nonidet P-40, 2-hydroxypropyl-β-cyclodextrin, monoclonal antibody against dinitrophenyl (DNP), paraformaldehyde, and human apoA-I were from Sigma. The monoclonal antibody against ABCA1 was from BD Biosciences. Monoclonal antibody against caveolin-1, and syntaxin-6 were from BD Biosciences. Monoclonal antibody against α-amanitin was from Roche Applied Science. The ProLong antifade kit, Alexa 488- or Alexa 568-conjugated goat anti-rabbit or anti-mouse IgG, Lysotracker Red (DND-99), 5-(2,4-dinitroanilino)-3-aminon-methylidipropylamine (DAMP), and Zeta rabbit IgG labeling kits were from Molecular Probes, Inc. Monoclonal antibodies against EEA1, cavin-1, and syntaxin-6 were from BD Biosciences. Monoclonal antibody against Na+/K-ATPase was from Upstate Biotechnology, Inc. Monoclonal antibody against hamster LAMP2 (lysosomal-associated membrane protein-2) was from the Developmental Studies Hybridoma Bank maintained by the University of Iowa. Rabbit polyclonal antibodies against α-amanitin were produced as described (10). Monoclonal antibody against caveolin-1 (V-ATPase) was a generous gift from Prof. Susanne Pfeffer (Stanford University) (12). Delipidated FBS was prepared as described (13). LDL (density of 1.019–1.063 g/ml) was prepared from fresh human plasma by sequential flotation as previously described (20). High density lipoprotein (HDL; density of 1.063–1.21 g/ml) was prepared by sequential flotation as previously described (14). High density lipoprotein (HDL; density of 1.063–1.21 g/ml) was prepared by the same flotation method and purified by heparin affinity chromatography.

Cell Lines and Cell Culture—25RA is a CHO cell line that is resistant to the cytotoxicity of 25-hydroxycholesterol (15) and that contains a gain-of-function mutation in SCAP (SREBP cleavage-activating protein) (16). CHO cells were maintained in medium A (Ham’s F-12 medium plus 15% FBS and 1% sodium bicarbonate) with 5% CO2. When medium B (Ham’s F-12 medium supplemented with 5% delipidated FBS plus 35 μl oleic acid and 10 μg/ml gentamycin) was used at lower temperatures (18 °C or lower), Ham’s F-12 medium (titrated to pH 7.4 without sodium bicarbonate) was used, and cells were placed in a water bath without CO2. A human fibroblast (c-f) cell line derived from an NPC patient (No. 93.22) was the generous gift of Dr. Peter Pentchev (National Institutes of Health). Hf cell lines isolated cultured for 2 days in medium B (to deplete stored cholesterol within previously described (4, 20). Prior to each experiment, the cells were treated to pH 7.4 without sodium bicarbonate) was used, and cells were titrated to pH 7.4 within 1 week before usage. An acyl-CoA cholesterol O-acyltransferase inhibitor (2 μM F12511) was included whenever cells were incubated at 37 °C. F12511 was previously shown to inhibit acyl-CoA cholesterol O-acyltransferase activity at the submicromolar level (21). Labeled cellular lipids were quantitated as described (17). The3H-labeled lipids were calculated as [3H]cholesterol counts divided by the sum of [3H]Cl and [3H]cholesterol counts. For cholesterol efflux experiments, cells were incubated with 4% 2-hydroxypropyl-β-cyclodextrin (CD) in medium B in the presence of the acyl-CoA cholesterol O-acyltransferase inhibitor at 37 °C for the indicated times. The labeled lipids were extracted and analyzed as described (4, 17). The percent cholesterol efflux was calculated as [3H]cholesterol counts in the medium divided by the sum of [3H]Cl counts in the cell and [3H]cholesterol counts in the cell and in the medium.

Isolation of the PM—To isolate the PM from the cells, we used the 30% Percoll gradient procedure essentially as described (20). All procedures were performed at 4 °C. Briefly, after the pulse-chase experiment, cells in two 150-mm dishes were collected. The cells were scraped in cold 30% sucrose, 1 mm EDTA, and 20 mm Tricine (pH 7.8) and broken with 15 strokes using a stainless steel tissue grinder (Dura-Grind, Wheaton). The post-nuclear supernatant was loaded onto a 30% Percoll gradient. After centrifugation at 84,000 × g for 30 min, fractions were collected from the top. The PM fractions usually corresponded to fractions 9 and 10, as evidenced by a visible white membrane band; this band showed high enrichment in Na+/K-ATPase and caveolin-1 protein (20). In addition, we performed bioradiolysis of PM proteins in intact cells at 4 °C for 10 min using sulfosuccinimidyl-6-biotinamidohexa-2,4-dienoic acid (Pierce), which showed the highest enrichment in fractions 9 and 10 for caveolin-1 and the highly enriched lysosomal proteins (data not shown). The [3H]-labeled lipids were extracted using chloroform/methanol and analyzed by TLC as previously described (20).

11% Percoll Gradient Analyses—All procedures were performed at 4 °C. The fractionation method was performed as described previously (4, 22). Briefly, after the pulse-chase experiment, cells from one 150-mm dish were scraped into homogenization buffer (0.25% sucrose, 1 mm EDTA, and 20 mm Tricine (pH 7.4)) and homogenized with 15 strokes using the same steel tissue grinder described above. To minimize breakage of membrane vesicles, 250 mm sucrose was included in the buffer. To increase recovery, the pellet was resuspended in buffer and homogenized a second time. The combined post-nuclear supernatant from cells was loaded onto a 30% Percoll gradient and centrifuged at 200,000 × g for 30 min. The supernatant (200,000 × g) was loaded onto a Beckman Model Ti-70.1 rotor. 10 fractions were collected from the top. >80% of the PM marker (Na+/K-ATPase) was concentrated in fractions 1 and 2, whereas >80% of the late endosomal/lysosomal markers (LAMP1/LAMP2) were concentrated in fractions 9 and 10 as previously described (4). The [3H]-labeled lipids were extracted using chloroform/methanol and analyzed by TLC as previously described (20).

Optiprep Gradient Analyses—The procedure was based on a previously described method (23) with modifications. Cells grown in one 150-mm dish were homogenized at 4 °C as described above. The post-nuclear supernatant (1 ml) was placed onto 9 ml of a linear 5–20% Optiprep gradient prepared in homogenization buffer at 4 °C. Gradi- ent centrifugation was performed at 27,000 rpm for 20 h at 4 °C using a Beckman SW 41 rotor. 20 fractions (0.5 ml each) were carefully collected from the top. Immunoblot analyses were performed using antibodies against individual organelle markers as indicated. The [3H]-labeled lipids were extracted using chloroform/methanol and analyzed by TLC as previously described (20).

Immunoblot and Spectrofluorometric Analyses of Percoll Fractions—For immunoblot analysis, each Percoll fraction was ultracentrifuged either at 100,000 × g for 90 min or at 150,000 × g for 30 min to remove the Percoll particles. Afterward, the samples (located on top of the Percoll particles) were carefully collected using Pasteur pipettes. Proteins present in these fractions were concentrated by chloroform/meth- alcohol precipitation (24). The concentrated protein pellet was resuspended in 100 mm Tris (pH 8.0), 0.2% NaCl, 1% Nonidet P-40, 1 mm EDTA, and 1× protease inhibitor mixture), separated on SDS-polyacrylamide gel, and immunoblotted with polyclonal anti-AL antibodies (1:1000). To quantify the LysoTracker signal (a late endosomal/lysosomal mark-
er), we used a highly sensitive fluorometer to measure the fluorescence intensities present in various Percoll fractions. The method is briefly described as follows. Cells were incubated with 100 nM LysoTracker Red for 2 h at 37 °C and then fractionated on a Percoll gradient at 4 °C. The Percoll fractions were ultracentrifuged at 150,000 g for 30 min to remove the Percoll particles. Each fraction was then quantitated for its fluorescence at Ex/em 577 nm/Em590 nm using a PC1 photon counting spectrophotometer from ISS Inc. (Champaign, IL). For detection of the green fluorescent protein (GFP) signal in GFP-transfected or NPC1-GFP-expressing cells, a modified method was needed (because Percoll particles exhibited autofluorescent signals that strongly interfered with the GFP signal). Each Percoll fraction was solubilized with the non-fluorescent detergent Thesit (Roche Applied Science) at 0.2%, and the solubilized material was ultracentrifuged at 150,000 g for 10 min. Values are the averages of duplicate dishes; results are representative of two independent experiments. Error bars indicate S.E.

RESULTS

Early Trafficking of LDL-derived Cholesterol Probed with a Long Versus Short Incubation with CD—We grew CT43 and 25RA cells in cholesteryl-free medium for 2 days and pulse-
labeled them with \[^{3}H\]CL-LDL for 5 h at 18 °C. At this temperature, LDL was internalized, but accumulated in pre-lysosomal compartments without significant hydrolysis of CL. When the temperature was increased to 37 °C, CL in LDL was rapidly hydrolyzed to free cholesterol and transported to desmosomal compartments without significant hydrolysis of CL. For labeling, we pulsed cells with \[^{3}H\]CL-LDL. The PM isolated by this method contained minimal contamination from internal membranes. Using this procedure, we monitored the \[^{3}H\]cholesterol content in the PM after various chase times. The results show that the \[^{3}H\]cholesterol content in the PM increased significantly with time, reaching a maximum at ~75 min in 25RA cells, whereas such an increase was hardly observed in the CT43 cells (Fig. 2C).

**Percoll Gradient Analyses of Various Membrane Fractions Containing LDL-derived \[^{3}H\]Cholesterol**—To detect the presence of \[^{3}H\]cholesterol in various membrane fractions, we performed Percoll gradient centrifugation using cell homogenates prepared from labeled cells. The Percoll fractions consisted of 10 fractions of increasing density, with light fractions (fractions 1–4) enriched in the PM and early endosome and with heavy fractions (fractions 9 and 10) enriched in the late endosome and lysosome (4, 22). For labeling, we pulsed cells with \[^{3}H\]CL-LDL for 5 h at 18 °C. As shown in Fig. 3A, when the chase time was 0 min, \[^{3}H\]CL was predominantly present in the lighter fractions (fractions 1–4). At the 30-min chase time, a significant decrease in \[^{3}H\]CL occurred in both cell types; concomitantly, a significant increase in the \[^{3}H\]cholesterol counts occurred in both cell types (Fig. 3B). The cholesterol counts were distributed in the lighter fractions (fractions 1–4), medium density fractions (fractions 5–8), and heavy fractions (fractions 9 and 10). Importantly, the \[^{3}H\]cholesterol distribution patterns in 25RA and CT43 cells were similar at the 30-min chase time point. In contrast, when the cells were chased for a longer time period (for 1 h) in the absence of CD, a significant difference in the \[^{3}H\]cholesterol distribution was seen between 25RA and CT43 cells (Fig. 3C, solid bars): in 25RA cells, \[^{3}H\]cholesterol was distributed in the lighter fractions (fractions 1–4), medium density fractions (fractions 5–8), and heavy fractions (fractions 9 and 10).
FIG. 3. Early trafficking of LDL-derived transport analyzed by Percoll gradient centrifugation. A and B, 25RA and CT43 cells were pulse-labeled with [3H]CL-LDL for 5 h at 18°C and chased for either 0 min (no chase) or 30 min. The cells were subjected to Percoll gradient analysis. [3H]CL (A) and [3H]cholesterol (B) in each Percoll fraction were analyzed according to the procedures described under “Experimental Procedures.” Within each cell type, to normalize variation in total 3H counts recovered from different samples, the values reported were normalized so that the sum of counts in cellular cholesterol and CL was the same for different samples. C, after the pulse, 25RA and CT43 cells were chased at 37°C in the presence or absence of CD for 1 h. [3H]Cholesterol in each Percoll fraction and in the medium was counted. For each cell type, the values reported were normalized so that the sum of the counts in cellular cholesterol and cholesterol in the medium was the same for different samples. D, after the pulse, 25RA and CT43 cells were chased for 1 h and then chased for an additional 1 h with or without CD. The counts were analyzed as described for C. Results are representative of two independent experiments.
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5–8), and heavy fractions (fractions 9 and 10), whereas in CT43 cells, [3H]cholesterol accumulated mainly in the heavy fractions (fractions 9 and 10). If CD was included during the 1-h chase period, [3H]cholesterol present in the heavy fractions (fractions 9 and 10) and light fractions (fractions 1–4) significantly decreased, resulting in a blurring of the difference in the [3H]cholesterol distribution between 25RA and CT43 cells (Fig. 3C, hatched bars). In a separate experiment, the chase time was increased to 2 h. We found that, in the absence of CD, [3H]cholesterol continued to accumulate in the heavy fractions (fractions 9 and 10) in CT43 cells (Fig. 3D, solid bars); if CD was included during the last hour of the 2-h chase time, the cholesterol that accumulated in the heavy fractions of CT43 cells was resistant to extraction by CD (Fig. 3D, compare hatched and solid bars).

Identification of the AL Compartment(s) in Percoll Fractions—The results shown in Fig. 3 suggest that [3H]cholesterol newly liberated from [3H]CL-LDL may be present in multiple membrane fractions (Percoll fractions 1–8) before it is sequestered in the late endosome/lysosome (Percoll fractions 9 and 10). Because more than one membrane compartment may be present in any of the Percoll fractions, the identities of these early fractions could not be positively determined at present. On the other hand, hydrolysis of cholesteryl ester in LDL requires the action of the enzyme AL. Therefore, the compartment(s) that contains AL is involved during the early trafficking of [3H]cholesterol liberated from [3H]CL-LDL. In the literature, the localization of AL has been assumed to be in lysosomes (1); however, direct evidence is lacking. We thus focused our effort to identify the compartment(s) containing AL. We used the specific antibodies against AL (10) to perform immunoblot analyses on various Percoll fractions. These antibodies identified a single 41-kDa protein band. The results show that, in both 25RA and CT43 cells, all of the AL-positive signals were distributed in the buoyant fractions (fractions 1–3); no detectable signal could be found in either the heavy or medium density fractions. Representative results are shown in Fig. 4A. A control experiment showed that LysoTracker, a marker for the late endosome and lysosome, was predominantly found in the heavy fractions (fractions 9 and 10) (Fig. 4B). In another experiment, the NPC1-GFP fusion protein expressed in CT43 cells was also predominantly found in the heavy fractions (Fig. 4C); a control experiment showed that GFP alone expressed in CT43 cells was predominantly localized in the buoyant fractions (Fig. 4C).

Monitoring the Early Fate of [3H]Cholesterol Using Optiprep Gradients—The results shown in Figs. 3 and 4 demonstrate that, on a 11% Percoll gradient, the AL-containing membranes were located in light fractions (fractions 1–3). However, because the PM fractions were also enriched in these fractions, one could not determine whether [3H]cholesterol newly liberated from [3H]CL-LDL was present in the AL compartment(s) or in the PM. To clarify this issue, we used another subcellular fractionation method that separates the PM from the endosomal compartments using the Optiprep gradient procedure first developed by Sheff et al. (23). As shown in Fig. 5A, immunoblot analyses demonstrated that both AL and the early endosomal marker EEA1 were predominantly located in early fractions (fractions 3–5), whereas the PM marker Na+/K+-ATPase was broadly enriched in heavier fractions (fractions 11–19). The late endosomal marker Rab9 was located mainly in fractions 7 and 8; the trans-Golgi network (TGN) marker syntaxin-6 was located mainly in fractions 9 and 10. Next, we pulse-labeled 25RA and CT43 cells with [3H]CL-LDL as described above, chased the cells at 37 °C for various times as indicated (0 min to 2 h), and analyzed [3H]cholesterol present in cell homogenates after Optiprep gradient fractionation. At zero time, as expected, little [3H]cholesterol was present in various fractions in both cell types (Fig. 5B). A control experiment showed that, at zero time, the unhydrolyzed [3H]CL was located as a broad peak in fractions 1–7 in both cell types (data not shown). When cells were chased for 30 min, the [3H]cholesterol fractions emerged and were seen as a broad peak (fractions 4–11) that centered at fraction 7 in both cell homogenates (Fig. 5C). When cells were chased for 2 h, [3H]cholesterol fractions continued to accumulate as a broad peak (fractions 5–12) that centered at fraction 9 in CT43 cells. In contrast, in 25RA cells, a significant

FIG. 4. Distribution of AL and NPC1 on Percoll gradients. A, immunoblot analysis of AL. Each Percoll fraction from 25RA cells grown in medium B at 37 °C was analyzed by SDS-PAGE and immunoblotted for AL as described under “Experimental Procedures.” A single band at ~41 kDa was detected in the light fractions. The results shown are representative of two independent experiments. The same results were obtained when CT43 cells were used for analysis. B, distribution of LysoTracker. C, distribution of GFP or NPC1-GFP in Percoll fractions from CT43 cells transfected with GFP alone or with NPC1-GFP. In B and C, the fluorescent signal in each Percoll fraction was quantitated using the fluorometer as described under “Experimental Procedures.”
Fig. 5. Monitoring the early fate of [3H]cholesterol using Optiprep gradients. A, AL and various subcellular organelle markers were subjected to immunoblot analyses. EEA1 (for the early endosome), Na⁺/K⁺-ATPase (for the PM), Rab9 (for the late endosome), and syntaxin-6 (for the TGN) were immunoblotted as described under “Experimental Procedures.” The results shown are the blots made using CT43 cells grown in medium B for 48 h. The same results were obtained using 25RA cells. Under the cell homogenization conditions described, the PM fractions of the 25RA and CT43 cells were consistently found as a broad peak, ranging from fractions 11 to 19 (results of three independent experiments). B–D, 25RA and CT43 cells were pulse-labeled with [3H]CL-LDL for 5 h at 14 °C and chased at 37 °C for 0 min (B), 30 min (C), or 2 h (D); the post-nuclear cell homogenates were fractionated on an Optiprep gradient and analyzed as described under “Experimental Procedures.” Results are representative of two independent experiments.

portion of [3H]cholesterol was redistributed to various regions across the entire gradient, including the heavier fractions where the PM was located (Fig. 5D). These results, along with the results shown in Figs. 2–4, show that [3H]cholesterol newly liberated from [3H]CL-LDL was absent from the PM, but was present in various endocytic compartment(s), including those containing AL.

Identification of the AL Compartment(s) by Fluorescence Microscopy of Intact Cells—The results shown in Figs. 4 and 5 suggest that AL resides mainly in the membrane compartment(s) with buoyant density. For a different approach to identify the AL-containing compartment(s), we performed fluorescence microscopy with intact cells. CT43 cells were transiently transfected with the NPC1-GFP construct and viewed under a fluorescence microscope. The AL signal (red) was identified using the anti-AL antibodies as the primary antigen in indirect immunofluorescence. The NPC1-GFP signal (green) was identified by the intrinsic fluorescence from GFP. The results show that little NPC1-GFP signal colocalized with the AL signal (Fig. 6A). In a separate experiment, the two signals were viewed under a confocal laser scanning microscope. The results clearly show that these signals did not colocalize with each other (Fig. 6B).

Previously, Neufeld et al. (31) reported that the NPC1 protein resides mainly in a compartment that contains LAMP2, a marker for the late endosome and lysosome; the NPC1-containing compartment(s) does not contain CI-MPR. The CI-MPR protein shuttles between the TGN and various endocytic compartments (32). Consistent with the finding of Neufeld et al., we found that NPC1-GFP significantly overlapped with the LAMP2 protein and LysoTracker (Fig. 7A, upper and middle panels), but did not significantly overlap with the CI-MPR signal except in the TGN region (lower panels). As expected, the AL signal did not colocalize with the LysoTracker signal or the LAMP2 signal (Fig. 7B, upper and middle panels). We also found that the AL signal (green) did not significantly colocalize with any of the following organelle markers: EEA1 (an early endosomal marker), syntaxin-6 (a TGN marker), and caveolin-1 (a caveola marker) (Fig. 7B, lower panels). In addition, we found that the AL signal did not colocalize with the flotillin-1 signal (another caveola marker) or the GM130 signal (a Golgi marker) (data not shown).
AL is deficient (data not shown). To further test whether AL resides mainly in acidic compartment(s), we used the compound DAMP. As first shown by Anderson et al. (26), DAMP readily diffuses into the cells and is concentrated inside various acidic organelles; its presence can be detected by immunostaining using antibodies against the DNP group. This method is capable of detecting weakly acidic compartments (27). As shown in Fig. 9B, in HepG2 cells, a significant portion (>50%) of the AL-positive signals colocalized with the DAMP-positive signals. Similar results were obtained in Hf and THP-1 cells (data not shown). These results strengthen the interpretation that AL resides mainly in an acidic environment.

AL is one of the numerous acid hydrolases that contain mannose 6-phosphate residues. Based on this motif, the MPR protein directs the hydrolases from the TGN to various endocytic compartments (32). We therefore tested the localization of
CI-MPR against that of AL in Hf cells. The results show that, on average, >60% of the AL signal colocalized with the CI-MPR signal. Typical results are shown in Fig. 9C (upper and middle panels). To demonstrate the specificity of the signals elicited by the anti-AL and anti-CI-MPR antibodies, we performed the colocalization experiments in Hf cells from mucolipidosis II (I-cell disease) patients. In cells with I-cell disease, lysosomal enzymes including AL are released and secreted into the extracellular milieu due to lack of the covalently modified mannose 6-phosphate residues (32). Our results show that, there was very little AL signal found inside the I-cell Hf cells; also, the CI-MPR signal had a distribution pattern resembling that of a TGN marker (Fig. 9C, lower panels).

**DISCUSSION**

The results presented here suggest that, under the conditions used, the following scenario occurs. Upon warming up at 37 °C, [3H]cholesterol is abundantly released from [3H]CL-LDL within a 30–60-min chase time. It is first present in certain early compartment(s) in a manner independent of NPC1. [3H]Cholesterol in the early compartment(s) is resistant to a short incubation with CD (5–10 min), but is sensitive to a longer incubation with CD (30 min or longer). After the 45–60-min chase time, in CT43 cells, [3H]cholesterol enters and accumulates in the late compartment(s) and becomes resistant to both short and long CD incubations. During the chase period, continuous inclusion of CD induces the [3H]cholesterol efflux from the early compartment(s) and prevents its entry into the late compartment(s). Once entering the late compartment(s), [3H]cholesterol in CT43 cells becomes resistant to CD even after a prolonged incubation time (60 min). The late compartment(s) probably consists mainly of the aberrant late endosome/lysosome characteristic of all the NPC1 cells. The early compartment(s) appears mainly in buoyant density fractions on Percoll gradients, whereas the late compartment(s) is concentrated in heavy density fractions. In contrast to what we previously believed, the early compartment(s) contains little PM for the following reasons. During the first 30 min of the chase period, [3H]cholesterol was resistant to a short incubation with CD (Fig. 1E). Also, within the first 75 min, the [3H]cholesterol content steadily increased in the isolated PM fraction of 25RA cells; its increase was hardly seen in that of CT43 cells (Fig. 2C). In addition, when HDL or apoA-I was used, significant efflux of [3H]cholesterol from [3H]CL-LDL occurred only in 25RA cells, but not in CT43 cells (Fig. 2, A and B).

Our results are consistent with early studies describing the function of NPC1, i.e. transport of LDL-derived cholesterol to the PM requires NPC1 (6, 35). While this manuscript was under review, Wojtanik and Liscum (36) reported that LDL-derived cholesterol moves directly to the NPC1-containing compartment(s) without passing through the PM first. Building on the same model, our work demonstrates that the free cholesterol liberated from cholesteryl ester of LDL is present in early intracellular compartment(s) before it is transported to the NPC1-containing compartment. The early compartment(s) may consist of various internal membrane vesicles/organelles. One of the early compartments contains AL. The AL compart-
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To explain the heterogeneity observed in the degree of colocalization between the AL compartment and LysoTracker signals in Hf cells, we speculate that the AL compartment may fuse with the late endosome; the fusion may occur at different rates in different cell types. Before fusion occurs, the two compartments would be found in close proximity. In Hf cells, the fusion event may occur at a considerably slower rate than in other cell types, thus accounting for some degree of apparent overlap between the AL signal and the late endosomal/lysosomal signal seen in Hf cells. Other possibilities cannot be ruled out at present. For example, vesicular transport may account for the transit of cholesterol from the AL compartment to the late endosome; the cell type-specific difference described here may be attributed to the difference in the vesicular trafficking rate. It is also possible that, in Hf cells (and in other cell types yet to be examined), a certain portion of AL is physically present in the late endosome/lysosome, in addition to its presence in the non-lysosomal compartment described in our current work. The results presented in Fig. 3 suggest that, in addition to the AL compartment, LDL-derived cholesterol may traverse to other endocytic compartment(s) before it finally appears in the late endosome/lysosome. Further biochemical investigation at the cellular level is required to elucidate the exact relationship of these compartments.
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Shigeki Sugii, Patrick C. Reid, Nobutaka Ohgami, Hong Du and Ta-Yuan Chang

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