Depletion of rafts in late endocytic membranes is controlled by NPC1-dependent recycling of cholesterol to the plasma membrane

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SUMMARY

In mammalian cells, cholesterol is thought to associate with sphingolipids to form lateral membrane domains termed rafts. Increasing evidence suggests that rafts regulate protein interactions, for example, during signalling, intracellular transport and host-pathogen interactions. Rafts are present in cholesterol-sphingolipid-enriched membranes, including early and recycling endosomes, but whether rafts are found in late endocytic organelles has not been analyzed. In this study, we analyzed the association of cholesterol and late endosomal proteins with low-density detergent-resistant membranes (DRMs) in normal cells and in cells with lysosomal cholesterol-sphingolipid accumulation. In normal cells, the majority of [^{3}H]cholesterol released from [^{3}H]cholesterol ester-LDL associated with detergent-soluble membranes, was rapidly transported to the plasma membrane and became increasingly insoluble with time. In Niemann-Pick C1 (NPC1) protein-deficient lipidosis cells, the association of LDL-cholesterol with DRMs was enhanced and its transport to the plasma membrane was inhibited. In addition, the NPC1 protein was normally recovered in detergent-soluble membranes and its association with DRMs was enhanced by lysosomal cholesterol loading. Moreover, lysosomal cholesterol deposition was kinetically paralleled by the sequestration of sphingolipids and formation of multilamellar bodies in late endocytic organelles. These results suggest that late endocytic organelles are normally raft-poor and that endocytosed LDL-cholesterol is efficiently recycled to the plasma membrane in an NPC1-dependent process. The cholesterol-sphingolipid accumulation characteristic to NPC disease, and potentially to other sphingolipidoses, causes an overcrowding of rafts forming lamellar bodies in the degradative compartments.

Key words: Cholesterol, Glycolipid, Detergent-resistant membranes, Late endosomes, Niemann-Pick disease

INTRODUCTION

Cholesterol is postulated to be a key constituent of membrane rafts, which are cholesterol-sphingolipid-rich lateral membrane domains that form platforms for regulated protein interactions and thus orchestrate, for example, cell signalling, intracellular membrane transport, cell adhesion and host-pathogen interactions (Simons and Ikonen, 1997; Brown and London, 2000). However, not much is known of how the association of cholesterol itself with rafts is regulated, nor how this is connected to the tightly controlled content and distribution of cholesterol in mammalian cells. When cultured in the presence of serum, cells acquire cholesterol by receptor-mediated internalization of low density lipoproteins (LDLs), degrade LDL in lysosomes and redistribute cholesterol to other organelles (Brown and Goldstein, 1986). The influx of LDL-cholesterol to the cell downregulates cholesterol synthesis and LDL receptors. However, these homeostatic responses fail if free cholesterol is not mobilized from the endocytic organelles. In the genetic disorder Niemann-Pick disease type C (NPC), massive amounts of unesterified cholesterol accumulate in lysosomes owing to mutations in the NPC1 gene (Carstea et al., 1997). The disease manifests as a progressive, eventually fatal neurovisceral storage disorder (Pentchev et al., 1995).

The NPC1 protein is a multispanning membrane protein that contains a sterol-sensing domain (SSD) in the putative membrane spans 4-8 (Davies and Ioannou, 2000). The protein localizes to late endosomal vesicles that contain Lamp-2, and has been found in other endocytic membranes that contain caveolin-1, and in the trans-Golgi network (Garver et al., 2000; Higgins et al., 1999; Neufeld et al., 1999; Patel et al., 1999). LDL-cholesterol loading increases the association of NPC1 with cholesterol-containing lysosomes, presumably thereby facilitating cholesterol eggress (Neufeld et al., 1999). The precise function of NPC1 remains unknown but it seems to act

in concert with membrane transport machinery (Hölttä-Vuori et al., 2000).

In addition to cholesterol, sphingolipids accumulate in NPC (Pentchev et al., 1995). Cholesterol accumulation is also observed in several other sphingolipidoses (Puri et al., 1999), and it has been postulated that lysosomal storage diseases are caused by the accumulation of rafts in late endosomes and lysosomes (Simons and Gruenberg, 2000). In normal cells, rafts are present in early and recycling endosomes (Gagescu et al., 2000; Hornick et al., 1997; Mukherjee et al., 1998). However, the lipid composition of late endosomes seems to differ from that of earlier endocytic compartments, being enriched in triglycerides, cholesterol esters and select phospholipids, including lysobisphosphatidic acid (Brotherus and Renkonen, 1977; Kobayashi et al., 1998; Wherrett and Huterer, 1972). Whether cholesterol-sphingolipid rafts are present in late endosomal or lysosomal membranes has not been analyzed. In this work, we studied the partitioning of LDL-cholesterol and NPC1 into rafts in normal cells and in cells with lysosomal cholesterol accumulation. In parallel, we analyzed the ultrastructure of late endocytic compartments and measured the availability of LDL-cholesterol to extracellular acceptors.

MATERIALS AND METHODS

[1α,2α(n)-^{3}H]Cholesteryl linoleate, [^{3}H]-CE (specific activity 47.0 Ci/mmol) was from Amersham Pharmacia. Filipin, progesterone and sphingomyelinase were from Sigma. Apolipoprotein A-I (apoA-I) was from the Swiss Red Cross Laboratory. PKF 058-035 was a generous gift from Novartis, and U18666A (3-β-[2-(Diethylamino)ethoxy]-androst-5-en-17-one) from Upjohn. Mouse monoclonal antibodies against human Lamp-2 were from Developmental Studies Hybridoma Bank, and rabbit polyclonal antibodies against cation-independent mannose-6-phosphate receptor (MPR) from Varpu Marjomäki (Dept of Biological and Environmental Science, University of Jyväskylä, Finland). Mouse IgM antibodies against 3′-LM1, globotriaocylceramide and GD3 have been described previously (Fredman et al., 1990). FITC-conjugated secondary antibodies were from Immunotech, and IgG-HRP conjugates from Bio-Rad. All other reagents were as described previously (Heino et al., 2000).

Generation of anti-NPC1 antibodies

A 5′ fragment of the human NPC1 cDNA encoding amino acids Cys25-Asp266 was amplified by PCR using the primers 5′TCGCGGATCCTGTGTTTGGTATGGAGAG and 5′TCGCGGATCCTCAGTCCAAGCCAAGGAT, subcloned into BamHI-site of the pGAT-4 expression vector (from Johan Peränen, Institute of Biotechnology, University of Helsinki, Finland) and expressed as a His_6-GST fusion protein in *Escherichia coli* JM109(DE3). The insoluble protein was purified from cell debris on preparative SDS-polyacrylamide gels and used for immunization of New Zealand White rabbits.

Cell culture and transfections

F92-99 control fibroblasts and 93.41 NPC fibroblasts obtained as described (Hölttä-Vuori et al., 2000) were cultured in Eagle's Minimum Essential Medium (MEM) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 IU/ml penicillin, 100 μg/ml streptomycin and 10 mM Hepes pH 7.4. The Chinese hamster ovary (CHO) CT60 cells, generously provided by T.-Y. Chang (Dartmouth College School of Medicine, Hanover, NH), and normal CHO cells were cultured in Dulbecco's MEM supplemented with 10% FBS, 2 mM L-glutamine, 100 IU/ml penicillin, 100 μg/ml streptomycin, and non-essential amino acids. Cells were transfected with NPC1/pCR3.1 (Carstea et al., 1997) or NPC1 P691S/pSV-SPORT-1 (Watari et al., 1999b) using LipofectAMINE PLUS transfection reagent (Life Technologies) according to the instructions of the manufacturer.

Immunoblotting

Proteins were separated on SDS-polyacrylamide gels, and transferred to Hybond-C extra membrane (Amersham Pharmacia) by western blotting. Unspecific antibody binding was blocked by incubating the filter in 5% nonfat milk, 0.1% Tween-20 in Tris-buffered saline; the filter was then incubated with the primary antibodies diluted in blocking solution, and the staining was visualized by HRP-conjugated secondary antibodies and enhanced chemiluminiscence (ECL Western blotting detection reagent, Amersham Pharmacia), and quantitated using the Millipore Bio-Image equipment.

LDL labeling

Human LDL (d=1.019-1.063) was isolated from the plasma of fasted healthy volunteers by sequential ultracentrifugation using KBr for density adjustments (Goldstein et al., 1983). [^{3}H]CE-LDL was prepared as described (Paananen et al., 1995), except that, instead of using a Bio-Gel A-5 m column, we used a Superose 6HR gel filtration column (Pharmacia) with 10 mM Tris/1 mM EDTA/150 mM NaCl (pH 7.4) as the eluent.

Radiolabeling of cells and cholesterol efflux

Before [^{3}H]CE-LDL uptake, CHO cells were sterol starved and labeled by growing for 72 hours in 5% lipoprotein-deficient serum (LPDS) supplemented with [^{14}C]cholesterol (50 nCi/ml). Cells were then labeled by a 30 minute pulse of [^{3}H]CE-LDL (10 μg/ml, 13×10^6cpm/ml) in serum-free medium containing 2 μg/ml of PKF 058-035 (acyl-coenzyme A: cholesterol acyltransferase inhibitor) and chased up to 6 hours in the same medium complemented with 50 μg/ml unlabeled LDL. Fibroblasts were starved and labeled with [^{14}C]cholesterol (50 nCi/ml) in 5% LPDS medium for 20 hours, followed by 18 hours incubation with 10 μg/ml [^{3}H]CE-LDL in the presence of 2 μg/ml PKF 058-035 and a further 2 hours in serum-free medium. The cells were scraped into ice-cold PBS, harvested by centrifugation and resuspended in 2% NaCl. [^{3}H]- and [^{14}C] radioactivity was determined from an aliquot of the suspension. Lipids were extracted and separated by thin layer chromatography as described (Heino et al., 2000). The cholesterol and cholesteryl ester bands were determined based on the co-migration of a cholesterol and a cholesterol ester standard, scraped, and [^{3}H]- and [^{14}C]-radioactivity measured by liquid-scintillation counting, and corrected for any losses based on the recovery of total [^{3}H]- and [^{14}C]-radioactivity.

Efflux to 100 mM methyl-β-cyclodextrin was carried out for 5 minutes on ice in serum-free medium. Efflux to ApoA-I (10 μg/ml) in the presence or absence of sphingomyelinase (100 mU/ml) was carried out for 8 hours in serum-free medium.

Detergent extractions

Detergent insolubility was analyzed either by flotation in Optiprep density gradient in the presence of 1% Triton X-100 as described previously (Harder et al., 1998, Heino et al., 2000). Alternatively, the detergent-insoluble material was pelleted. Cells were washed with PBS on ice and lysed in lysis buffer (150 mM NaCl, 10 mM Tris-HCl, pH 8.0, 1 mM EDTA, 1% Triton X-100 and protease inhibitors: 25 μg/ml of each chymostatin, leupeptin, antipain and pepstatin A) on ice. After incubating for 30 minutes on ice, detergent-insoluble material was pelleted (30 minutes at 15,000 ***g*** at 4°C). The pellet was resuspended in lysis buffer, and proteins precipitated from the supernatant and pellet either with TCA or using methanol/chloroform extraction.

Immunofluorescence and electron microscopy

Cells fixed with 4% paraformaldehyde were incubated in 10% FBS supplemented with 0.05% filipin for 30 minutes at 37°C, followed by primary antibodies (20 μg/ml) diluted in 5% FBS at 4°C overnight, and secondary antibodies diluted in 5% FBS. The coverslips were viewed with a Zeiss Axiophot photomicroscope with appropriate filters.

For transmission electron microscopy cells were fixed for 1 hour with 2.5% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4 at room temperature, followed by a post-fixation with 1% OsO_4. Where indicated, filipin was added to the fixative at 100 μg/ml and incubated for 3 hours on a shaking platform covered from light. After dehydration, the samples were embedded in LX-112 (Ladd Research Industries) and cut horizontally. The sections were viewed with JEOL JEM EX-1200 microscope operated at an acceleration voltage of 60 kV.

RESULTS

Detergent resistance and hydrolysis of [^{3}H]CE-LDL

To analyze the cellular hydrolysis and raft association of LDL-cholesterol, we first labeled LDL with [^{3}H]CE using cholesteryl ester transfer protein and purified it by gel filtration. The labeled LDL had a particle size similar to that of native LDL (Fig. 1a). 87% of the transferred label in the LDL was CE, the rest being free cholesterol. This is comparable with the esterified versus free cholesterol ratio in native LDL (Lund-Katz and Phillips, 1986). The raft association of radiolabeled LDL was analyzed by extraction with 1% Triton X-100 on ice and flotation in a Triton-Optiprep gradient. Rafts form detergent-resistant assemblies that can be recovered in the top gradient fractions (Brown and Rose, 1992). The vast majority of both [^{3}H]CE and free [^{3}H]cholesterol was found at the bottom of the gradient (Fig. 1b). The efficient solubilization of radiolabeled cholesterol was not due to low levels of lipid in the sample compared with detergent, as similar results were obtained when the analysis was carried out with radiolabeled LDL mixed with excess unlabeled fibroblast lysate (data not shown). This result indicates that cholesterol in LDL is present in an environment that does not promote the formation of detergent-resistant membranes (DRMs). Internalization of radiolabeled LDL into CHO cells led to the hydrolysis of [^{3}H]CE, with 93% of the esters hydrolyzed by 2 hours of chase (Fig. 1c). Cellular uptake of radiolabel from [^{3}H]CE-LDL was completely inhibited by incubation with 50-fold excess of unlabeled LDL (data not shown). The hydrolysis of LDL-cholesterol was not significantly or only marginally inhibited in the NPC1-deficient CHO cell line CT60, which exhibits massive lysosomal cholesterol deposits (Cruz et al., 2000) (Fig. 1c).

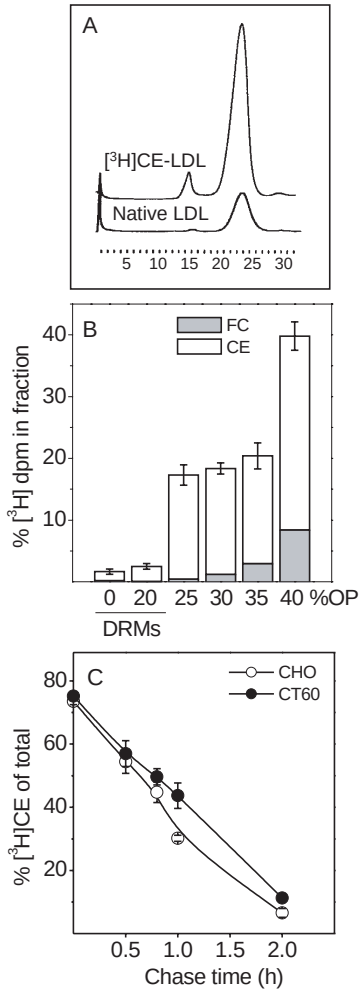

Fig. 1. Characteristics of [^{3}H]CE-LDL. Size-exclusion chromatography of LDL particles eluted at a flow rate of 0.5 ml/minute. The elution time (minutes) is indicated (A). Detergent resistance of [^{3}H]CE-LDL analyzed by Triton-Optiprep flotation gradient. The ratio of free and esterified cholesterol (FC, CE) in each fraction is shown (B). Cellular hydrolysis of [^{3}H]CE-LDL after 30 minutes of labeling and chasing for the indicated times (C). Results are mean±s.e.m. of 3 experiments.

Association of LDL-cholesterol and pre-existing cellular cholesterol with DRMs

We next explored the raft association of endocytosed LDL-cholesterol and compared it with that of pre-existing cellular [^{14}C]cholesterol. CHO cells were labeled with [^{14}C]cholesterol for 3 days and then pulse-labeled with [^{3}H]CE-LDL for 30 minutes. The detergent resistance of cholesterol was analyzed directly after the labeling and after increasing chase times. Immediately after labeling, the majority of the hydrolyzed [^{3}H]cholesterol was detergent soluble and only ~15% associated with DRMs whereas, in the same cells, ~50% of [^{14}C]cholesterol was recovered in DRMs (Fig. 2a). This suggests that the bulk of hydrolyzed cholesterol initially partitions into non-raft domains, and is in a membrane environment clearly different from that of the pre-labeled cholesterol. With increasing chase times, the insolubility of [^{3}H]cholesterol increased (~35% in DRMs at 6 hours of chase), and that of [^{14}C]cholesterol stayed about the same (Fig. 2a). We then compared these data with the detergent resistance of cholesterol in CT60 cells. We found that [^{3}H]cholesterol was more efficiently recovered in DRMs at each time point analyzed, the difference being most apparent immediately after the pulse (1.6 times more [^{3}H]cholesterol in DRMs in CT60 than in normal CHO cells, Fig. 2a,b). In addition, more of the [^{14}C]cholesterol was associated with DRMs in CT60 cells (~70%). The insolubility of [^{14}C]cholesterol stayed similar with increasing chase time (Fig. 2b).

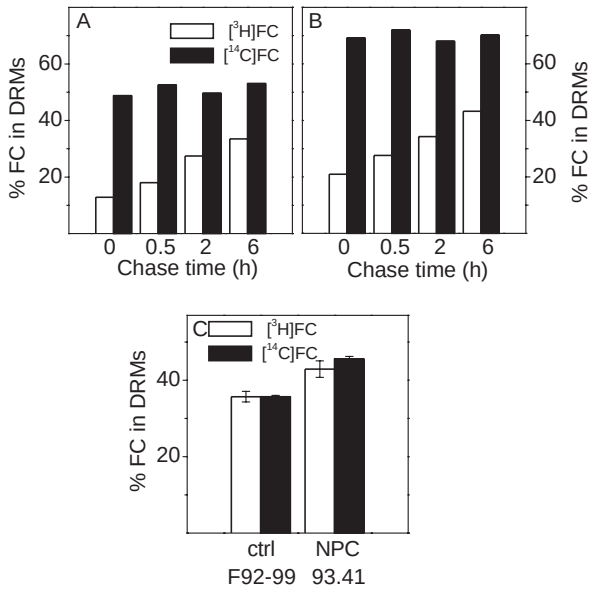

Fig. 2. Association of endocytosed [^{3}H]cholesterol and pre-existing [^{14}C]cholesterol with DRMs. CHO (A) and CT60 (B) cells were pre-labeled with [^{14}C]cholesterol and pulsed with [^{3}H]CE-LDL for 30 minutes, followed by chase for the indicated times. Detergent solubility was analyzed by Triton-Optiprep flotation gradient as in Fig. 1, and the percentage of radioactive free cholesterol (FC) in the two top fractions representing DRMs is shown. Representative results of 3 independent experiments. Control (F92-99) and NPC (93.41) fibroblasts pre-labeled with [^{14}C]cholesterol were incubated with [^{3}H]CE-LDL for 18 hours and chased for 2 hours, and the association of radioactive cholesterol with DRMs analyzed as for CHO cells. Results are mean±s.e.m. of 2-3 experiments (C).

To analyze the differences in the raft association of cholesterol between control and NPC patient cells, primary human fibroblasts were labeled with [^{14}C]cholesterol for 20 hours followed by 18 hours with [^{3}H]-CE-LDL, and fractionated as above. We found that [^{3}H]-CE became efficiently (>80%) hydrolyzed in both cell types and [^{3}H]cholesterol equilibrated with the [^{14}C]cholesterol label in the gradient. Significantly more of both radiolabels were found in DRMs in the patient cells, in accordance with the results from CT60 cells (45% DRMs in NPC cells versus 35% in control cells) (Fig. 2c).

U18666A causes rapid accumulation of multilamellar bodies in late endocytic organelles

Cholesterol transport blocks induced by the amphiphilic drugs progesterone or U18666A mimic the NPC cellular lesion as demonstrated by defective cholesterol esterification and by the formation of filipin-positive lysosomal cholesterol deposits (Butler et al., 1992; Liscum and Faust, 1989). We therefore used U18666A to acutely induce lysosomal cholesterol deposition in normal CHO cells. We found that already after 2-3 hours of incubation, U18666A started to induce the formation of bright, perinuclear filipin-positive cholesterol punctae (Fig. 3a,d). Electron microscopy revealed the appearance of a few multilamellar/vesicular elements in the cells (Fig. 3c). By 6 hours of drug treatment, the filipin-positive deposits were more numerous and had grown in size, obviously corresponding to the multiple lamellar bodies visualized by electron microscopy (Fig. 3g,i). The concentric lamellae accumulated free cholesterol as judged by the typical undulation of the membrane observed after filipin treatment (Fig. 3j). These multilamellar bodies correspond to late endosomes or lysosomes based on the accumulation of internalized BSA-gold after 2 hours of chase (data not shown), and closely resemble the characterized storage lysosomes in NPC patient fibroblasts (Blanchette-Mackie, 2000). Progesterone has previously been shown to result in the intracellular accumulation of GM2 ganglioside (Sato et al., 1998). To address the kinetics of glycosphingolipid redistribution in U18666A-treated cells, filipin-stained cover slips were double-labeled with anti-ganglioside 3′-LM1 antibodies. In non-treated cells, the antibodies visualized the plasma membrane and small punctate structures throughout the cytoplasm (Fig. 3b). After 3 hours of U18666A-treatment, the staining became more pronounced in the perinuclear area, where it co-localized with the internal filipin-positive structures (Fig. 3e). After 6 hours of treatment, the punctate intracellular staining was further accentuated and co-localized extensively with the filipin-positive organelles (Fig. 3h). Similar rapid redistribution of anti-3′LM1 staining was observed in human fibroblasts, and when using antibodies against two other glycosphingolipids, the ganglioside DG3 and the neutral glycosphingolipid globotriaocylceramide (data not shown).

Detergent-resistance of cholesterol in U18666A-treated cells was examined as in Fig. 2, by chasing CHO cells for 6 hours in the absence or presence of the drug. Both LDL-derived [^{3}H]cholesterol and the pre-existing [^{14}C]cholesterol were more avidly associated with DRMs in the U18666A-treated cells (Fig. 3k), suggesting that the multilamellar structures evident at 6 hours of chase contain DRMs.

Lysosomal cholesterol loading increases the association of NPC1 with DRMs

We next analyzed the raft association of NPC1 by using Triton-Optiprep gradient fractionation and western blotting of the precipitated proteins. The majority of the endogenous NPC1 of control fibroblasts was associated with detergent-soluble membranes (Fig. 4a), in accordance with a recent report on the raft association of murine liver NPC1 (Garver et al., 2000). We then studied whether loading fibroblasts with LDL in the presence of progesterone affects the raft association of NPC1. The cells were solubilized in 1% Triton X-100 on ice and the DRMs pelleted, while detergent-soluble material remained in the supernatant. When the cells were cultured in normal FBS containing medium, only 7% of NPC1 was recovered in the pellet (Fig. 4b). Prior to cholesterol loading, the cells were pre-incubated for 24 hours in LPDS medium. This treatment did not alter the detergent solubility of NPC1. However, when the cells were incubated for a further 24 hours in the presence of progesterone and LDL, the percentage of NPC1 remaining detergent insoluble increased about fourfold (Fig. 4b). The cholesterol-filled lysosomal membranes also contain the lysosomal membrane proteins (Lamps) and the mannose-6-phosphate receptor (MPR) (Kobayashi et al., 1999). Interestingly, Lamp-2 and MPR remained entirely detergent soluble in the cholesterol loaded cells (Fig. 4b). This indicates that the increase in raft association observed for NPC1 is not

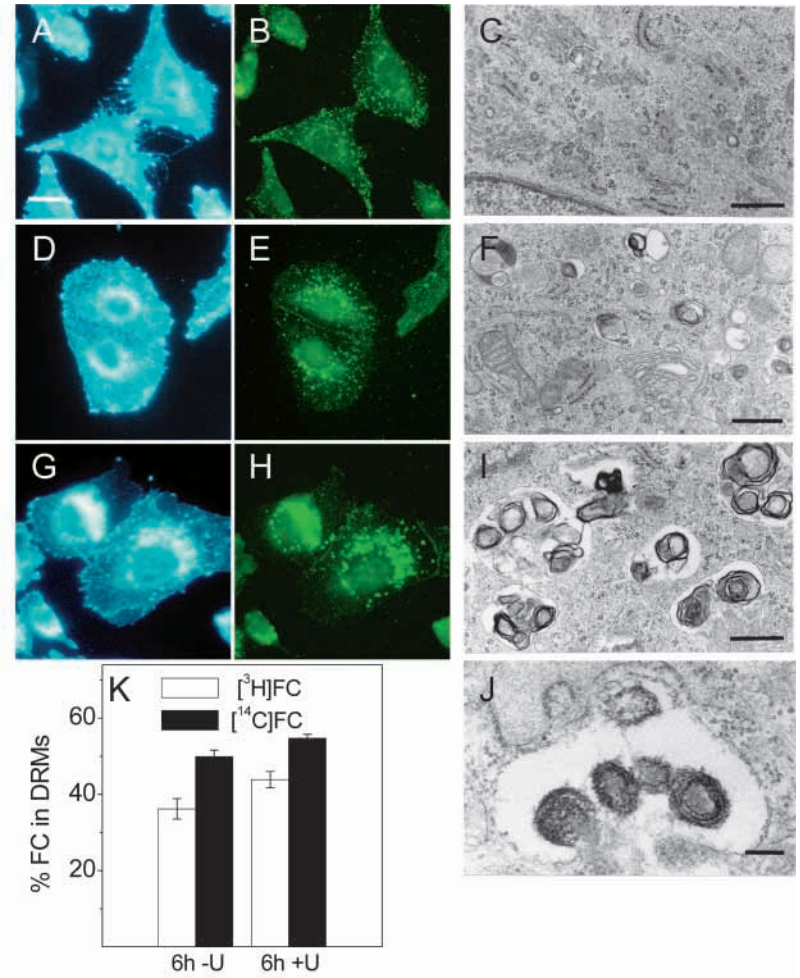

Fig. 3. Accumulation of cholesterol and glycolipid in late endocytic organelles of U18666A-treated cells. CHO cells were treated with 1 μg/ml U18666A for 0 hours (A,B,C), 3 hours (D,E,F) or 6 hours (G,H,I) and stained with filipin (A,D,G), anti-glycolipid 3′LM1 antibodies (B,E,H) or visualized by transmission electron microscopy (C,F,I,J). With increasing time, U18666A causes the accumulation of cholesterol and sphingolipid and the formation of lamellar bodies in late endocytic organelles. The lamellar membranes and the encircling membrane of the storage lysosomes are cholesterol rich, as indicated by the filipin induced deformations (J). Cells pre-labeled with [^{14}C]cholesterol were pulsed with [^{3}H]CE-LDL for 30 minutes and chased for 6 hours in the presence or absence of U18666A (U). The association of free cholesterol (FC) with DRMs is shown (mean±s.e.m. of 3 experiments) (K). Bars, 15 nm (A), 500nm (C, F, I), 160 nm (J).

a general characteristic of all proteins embedded in the cholesterol-filled late endocytic membranes.

Because lysosomal cholesterol deposition enhanced the raft association of NPC1, we analyzed the effect of introducing the NPC1 protein into NPC1-deficient lipidosis cells. Wild-type CHO cells or CT60 cells were transfected with the NPC1 cDNA and the raft association of NPC1 analyzed by flotation gradient at 3 days post-transfection. As with fibroblasts, NPC1 was found mostly in non-raft fractions of wild-type CHO cells (Fig. 4c). However, in CT60 cells, a slightly higher proportion of NPC1 associated with DRMs (Fig. 4c). In these cells, transient expression of the NPC1 protein is associated with gradual discharge of the lysosomal cholesterol accumulation (Watari, et al., 1999a). It was therefore possible that the small fraction of NPC1 recovered in rafts would represent a pool of the protein actively engaged in removing the deposited cholesterol. To address this, the cells were transfected with a construct encoding a nonfunctional NPC1 protein, NPC1 P691S. This protein harbors a mutation in the putative SSD and although targeted to the storage lysosomes, is unable to complement the NPC phenotype (Watari, et al., 1999b). We found that the P691S mutant behaved essentially similarly to the wild-type NPC1 protein in the gradient fractionation. The majority of the protein was detergent soluble in both cell types, but slightly more of it was recovered in DRMs in CT60 cells (<5% in DRMs in CHO cells versus ~13% in CT60 cells; Fig. 4c). Thus, the raft association of the inactive NPC1 was also enhanced in the cholesterol-loaded cells. The results indicate that an intact SSD is not a prerequisite for the association of NPC1 with rafts, and that the partitioning of NPC1 with rafts does not directly correlate with the activity of the protein in clearing lysosomal cholesterol stores. Rather, the raft association of NPC1 was most pronounced in conditions with non-physiological lysosomal cholesterol deposition.

Efflux of radiolabeled cholesterol pools to extracellular acceptors

To study how the increasing association of [^{3}H]cholesterol with DRMs is related to its availability to extracellular acceptors, cells were labeled and chased as in Fig. 2, and extracted with cyclodextrin. We used a 5 minute incubation with 100 mM methyl-β-cyclodextrin on ice, which extracted 17-20% of pre-labeled [^{14}C]cholesterol from wild-type CHO cells (Fig. 5a). In these cells, there was a clear increase in the cyclodextrin availability of free [^{3}H]cholesterol between 0.5-2 hours of chase, suggesting movement of hydrolyzed cholesterol from intracellular sites to the surface (Fig. 5a). At 2 and 6 hours of chase, the efflux was actually somewhat higher than that of [^{14}C]cholesterol, potentially contributed by the weaker raft association of [^{3}H]cholesterol (Fig. 5a). In CT60 cells, the extractability of [^{14}C]cholesterol was lower than in wild-type cells (13-15%). The difference increased with time, probably

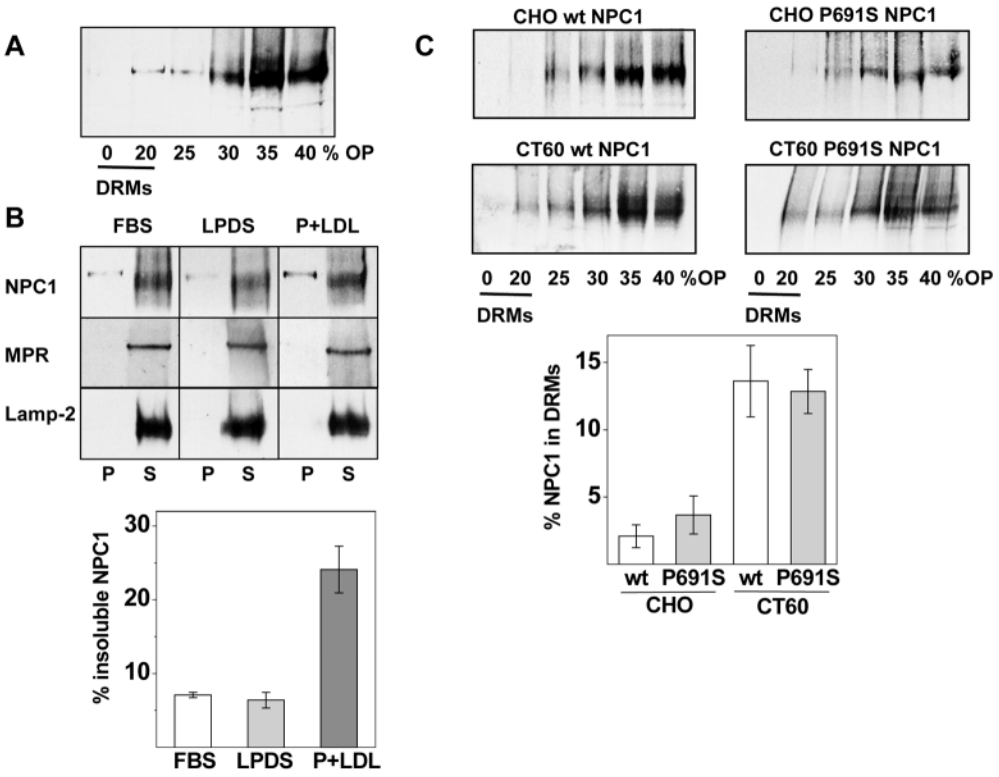

Fig. 4. Sterol loading results in increased raft association of wild-type and SSD mutant NPC1. (A) Endogenous NPC1 of control fibroblasts analyzed by Triton-Optiprep flotation gradient and western blotting. (B) Control fibroblasts grown in 10% FBS medium (FBS), in 5% LPDS medium for 48 hours (LPDS), or in LPDS medium for 24 hours, followed by loading with 50 μg/ml LDL and 10 μg/ml progesterone for 24 hours (P+LDL) were lysed in 1% Triton X-100 on ice and detergent-insoluble material pelleted. Proteins from soluble and insoluble fractions were analyzed by western blotting using anti-NPC1, anti-MPR or anti-Lamp-2 antibodies. Densitometric scanning of the intensity of the NPC1 band in insoluble fractions as a percentage of total NPC1 is presented (mean±s.e.m. of 3 experiments). (C) Wild-type CHO or CT60 cells were transfected with wt or P691S NPC1 cDNA, lysed in 1% Triton X-100 on ice, and fractionated in Triton-Optiprep gradient. Methanol/chloroform-extracted proteins were analyzed by western blotting using anti-NPC1 antibodies. Densitometric scanning of the intensity of the NPC1 band in DRMs as a percentage of total NPC1 is presented (mean±s.e.m. of 3 measurements). Note that the proportion of detergent-insoluble NPC1 is somewhat higher when analyzed by sedimentation (B) compared with flotation (C).

due to the presence of unlabeled LDL during the chase, which enhanced intracellular cholesterol sequestration in CT60 cells. Importantly, there was a clear inhibition in the availability of [^{3}H]cholesterol to cyclodextrin in CT60 cells both at 2 and 6 hours of chase, suggesting intracellular trapping of the hydrolyzed cholesterol (Fig. 5a).

Cholesterol efflux to the physiological acceptor apoA-I has been shown to increase when raft integrity is perturbed by sphingomyelinase treatment (Ito et al., 2000). We therefore analyzed [^{14}C]cholesterol efflux from wild-type CHO and CT60 cells to apoA-I in the absence or presence of sphingomyelinase. We found that cholesterol was more readily effluxed to apoA-I in wild-type than in CT60 cells (Fig. 5b). Sphingomyelinase treatment increased the removal of cholesterol to apoA-I in both cells, however, the effect was more pronounced in wild-type cells (Fig. 5b). This suggests that cholesterol efflux to apoA-I is defective in CT60 cells, and that, although raft cholesterol is normally freed for efflux by sphingomyelinase treatment, this process is disturbed in CT60 cells.

DISCUSSION

Our study emphasizes the role of cholesterol both as a structural component and a regulator of rafts, and demonstrates

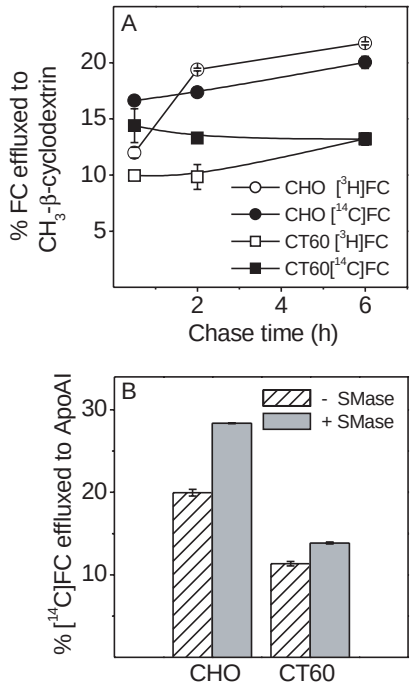

Fig. 5. Availability of cholesterol pools to extracellular acceptors. Cells pre-labeled with [^{14}C]cholesterol were pulsed with [^{3}H]CE-LDL for 30 minutes and chased for the indicated times, followed by 5 minutes cyclodextrin extraction on ice (A). Cells labeled with [^{14}C]cholesterol for 3 days were incubated with ApoA-I in the absence or presence of sphingomyelinase (SMase) for 8 hours. The percentage of cholesterol effluxed to ApoA-I is indicated (B). Results are mean±s.e.m. of 3 measurements.

the usefulness of using detergent solubility as a parameter in dissecting cholesterol pools. Importantly, we show that endocytosed LDL-cholesterol associates first with non-raft membranes, probably because of the low abundance of rafts in normal late endocytic membranes. This state seems to be maintained by efficient exclusion of cholesterol from these membranes via mechanisms involving NPC1. Analogously, the ER membrane harbors the SSD-containing proteins controlling cholesterol biosynthesis (Brown and Goldstein, 1999), and cholesterol synthesized de novo in the ER is preferentially associated with detergent-soluble membranes (Heino et al., 2000). Further, the combined results on cholesterol raft association and efflux to cyclodextrin suggest that the hydrolyzed cholesterol moves rapidly to the plasma membrane but equilibrates with the existing cholesterol pool only gradually.

The arrival of LDL-derived cholesterol to the plasma membrane was defective in cells that lack a functional NPC1 protein. However, the block in cholesterol recycling to the surface was not complete, as a significant proportion of [^{3}H]cholesterol was removed by cyclodextrin from the CT60 cells at the first time point analyzed (see Fig. 5a). Part of this label is likely to derive from the free [^{3}H]cholesterol present in the LDL particle that does not need to become hydrolyzed before mobilization. Part of it could also derive from early endocytic compartments participating in cholesterol ester hydrolysis. However, the precise identity of these organelles and their potential contribution to the degradation of LDL remain at present unknown. The importance of late endocytic organelles in cholesterol ester hydrolysis is supported by the high activity of acid lipase in lysosomes (Runquist and Havel, 1991), the observed time course of hydrolysis (Fig. 1c), and the inhibition of LDL degradation and cholesterol ester hydrolysis observed in cells overexpressing the dominant-negative mutant of Rab 7, Rab7N125I (Bucci et al., 2000; M. Hölttä-Vuori and E. Ikonen, unpublished). Notably, the difference in [^{3}H]cholesterol extractability to cyclodextrin between normal and CT60 cells became more pronounced with time. In particular, the rapid increase in [^{3}H]cholesterol efflux between 0.5-2 hours of chase taking place in normal cells was completely lacking in the mutant cells (see Fig. 5a). These findings suggest that NPC1 is involved in the transport of hydrolyzed LDL-cholesterol to the plasma membrane, in accordance with the conclusion reached by Cruz and coworkers (Cruz et al., 2000). They reported, however, that the initial movement of LDL-derived cholesterol to the plasma membrane did not require NPC1; it was only after reaching the plasma membrane and subsequent reinternalization that cholesterol trafficking back to the plasma membrane did involve NPC1. This difference may be due to the differences in the labeling and cyclodextrin extraction procedures applied.

We show that the excessive storage of cholesterol in lysosomes that is characteristic of the NPC disease and drug-treated cells, was accompanied by increased partitioning of endocytosed cholesterol and NPC1 in DRMs. This was paralleled by sequestration of glycosphingolipids and formation of multilamellar profiles in late endocytic organelles. The reason for the relatively slow incorporation of endocytosed cholesterol into DRMs in CT60 cells (see Fig. 2b) remains elusive, but it may reflect impaired membrane dynamics of the late endocytic organelles. Together, the data suggest that in the lipidosis cells, cholesterol-sphingolipid-containing rafts accumulate in late endocytic organelles forming multilamellar bodies, a known form of lipid storage in a variety of cells (Schmitz and Muller, 1991). The results support the idea that aberrant raft formation in lysosomes may be a general mechanism in lysosomal storage lipidoses where the deposition of one raft constituent could lead to the trapping of others (Simons and Gruenberg, 2000).

To further understand the molecular basis of the altered cellular responses in NPC and potentially other sphingolipidoses, it will be relevant to analyze how lysosomal raft accumulation is reflected in the lipid composition and raft-dependent functions of other cellular membranes. For instance, the observed inhibition of cholesterol efflux to apoA-I may be related to plasma membrane raft derangements aggravating the lipid deposition. It can be envisioned that some of the early disease manifestations result from subtle alterations in raft-dependent molecular interactions prior to the rapid accumulation of storage products. This may help to explain some of the defective signaling functions observed in embryonic neurons of the NPC mouse, where there is no overt cholesterol storage (Henderson et al., 2000).

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