Late endosomal membranes rich in lysobisphosphatidic acid regulate cholesterol transport

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The fate of free cholesterol released after endocytosis of low-density lipoproteins remains obscure. Here we report that late endosomes have a pivotal role in intracellular cholesterol transport. We find that in the genetic disease Niemann–Pick type C (NPC), and in drug-treated cells that mimic NPC, cholesterol accumulates in late endosomes and sorting of the lysosomal enzyme receptor is impaired. Our results show that the characteristic network of lysobisphosphatidic acid-rich membranes contained within multivesicular late endosomes regulates cholesterol transport, presumably by acting as a collection and distribution device. The results also suggest that similar endosomal defects accompany the anti-phospholipid syndrome and NPC.

Most animal cells acquire cholesterol by receptor-mediated endocytosis of low-density lipoproteins (LDL), which are then transported first to early endosomes and then towards late endosomes/lysosomes for degradation[1]. Although this pathway is well established, the fate of free cholesterol remains obscure. In animal cells, late endosomes function not only as an obligatory station for LDL and other endocytosed ligands destined to be degraded, but also as a major protein-sorting compartment[2]. Lysosomes, in contrast, are generally considered as the end-point of the endocytic pathway, and delivery of endocytosed ligands to lysosomes may be mediated by direct fusion of late endosomes and lysosomes, leading to the formation of a hybrid organelle[3]. One of the characteristic features of late endosomes is a complex system of internal membranes within the lumen[4]. We previously showed that this membrane network contains high amounts of the unique, poorly degradable phospholipid lysobisphosphatidic acid (LBPA), and thus forms a specialized membrane domain within endosomes[5]. Here, we report investigations into the role of LBPA-rich membrane domains in intracellular cholesterol transport.

Results

Cholesterol accumulates in late endosomes in Niemann–Pick type C fibroblasts. Electron-dense multivesicular and/or multilamellar structures are often observed in cells affected by lysosomal storage diseases. In the human autosomal recessive Niemann–Pick type C disease (NPC), an abundance of these structures is accompanied by intracellular accumulation of unesterified cholesterol[6]. The precise function of the protein encoded by the *NPC* gene is not known, although it is presumably involved in the regulation of cholesterol transport[7–10]. Accumulated cholesterol is conveniently detected by light microscopy after cell fixation using filipin as a fluorescent marker[11] (Fig. 1A). In skin fibroblasts from NPC patients, accumulated cholesterol is present within vesicles containing both the small GTPase Rab7, a late endosomal marker[12], and LBPA (Fig. 1Ae–h). This is in contrast to control fibroblasts (Fig. 1Aa,b) or fibroblasts from patients with Tay–Sachs disease (Fig. 1Ac,d), an autosomal recessive lipidosis resulting in ganglioside GM_2 accumulation. Immunogold labelling of cryosections from NPC cells showed that late endosomes in these cells (Fig. 1B) revealed the same characteristic distribution of LBPA within their internal membranes as did normal BHK cells[5]. Although LBPA is increased in the liver and spleen of NPC patients[13], we found similar amounts of LBPA in NPC and control fibroblasts (not shown). This agrees well with recent observations[14], and supports the view that lipid metabolism varies between tissues in NPC patients[13]. Altogether, our observations demonstrate that NPC is not only a lysosomal, but also an endosomal, storage disorder.

NPC fibroblasts have a late endosome sorting/trafficking defect. This finding has important implications, as late endosomes, unlike lysosomes, are not the end-point of the endocytic pathway. One of the main functions of late endosomes is the sorting of the multifunctional receptor (IGF2/MPR) for ligands bearing mannose 6-phosphate, which include lysosomal enzymes and insulin-like growth factor 2 (ref. 15). IGF2/MPR delivers newly synthesized lysosomal enzymes from the *trans*-Golgi network (TGN) to late endosomes, and then recycles back to the TGN for reutilization. We found that, at steady state, IGF2/MPR localized predominantly to the TGN in control fibroblasts or Tay–Sachs fibroblasts (Fig. 2). In NPC fibroblasts, however, the receptor was largely redistributed to abundant vesicles identified as late endosomes using CD63 as a marker[16] (Fig. 2). Immunogold labelling of cryosections showed that IGF2/MPR then co-localized with LBPA within internal membranes of large multivesicular endosomes (not shown). Some IGF2/MPR remained in the Golgi region of NPC cells (Fig. 2), perhaps corresponding, at least in part, to newly synthesized molecules. These experiments indicate that cholesterol accumulation in the NPC disease is accompanied by an endosomal sorting/trafficking defect, in agreement with studies suggesting that NPC fibroblasts are deficient in vesicle-mediated clearance of endocytosed [^{14}C]sucrose[17].

Cholesterol accumulates in late endosomes after U18666A treatment. The accumulation of LDL-derived cholesterol characteristic of NPC cells can be mimicked after treatment of healthy cells over a short time with hydrophobic amines such as U18666A (ref. 18). When BHK cells were treated with U18666A, cholesterol accumulated rapidly (within 2–8 h) in late endosomes that contained both LBPA and Rab7 (Fig. 3), much as in NPC fibroblasts. However, no

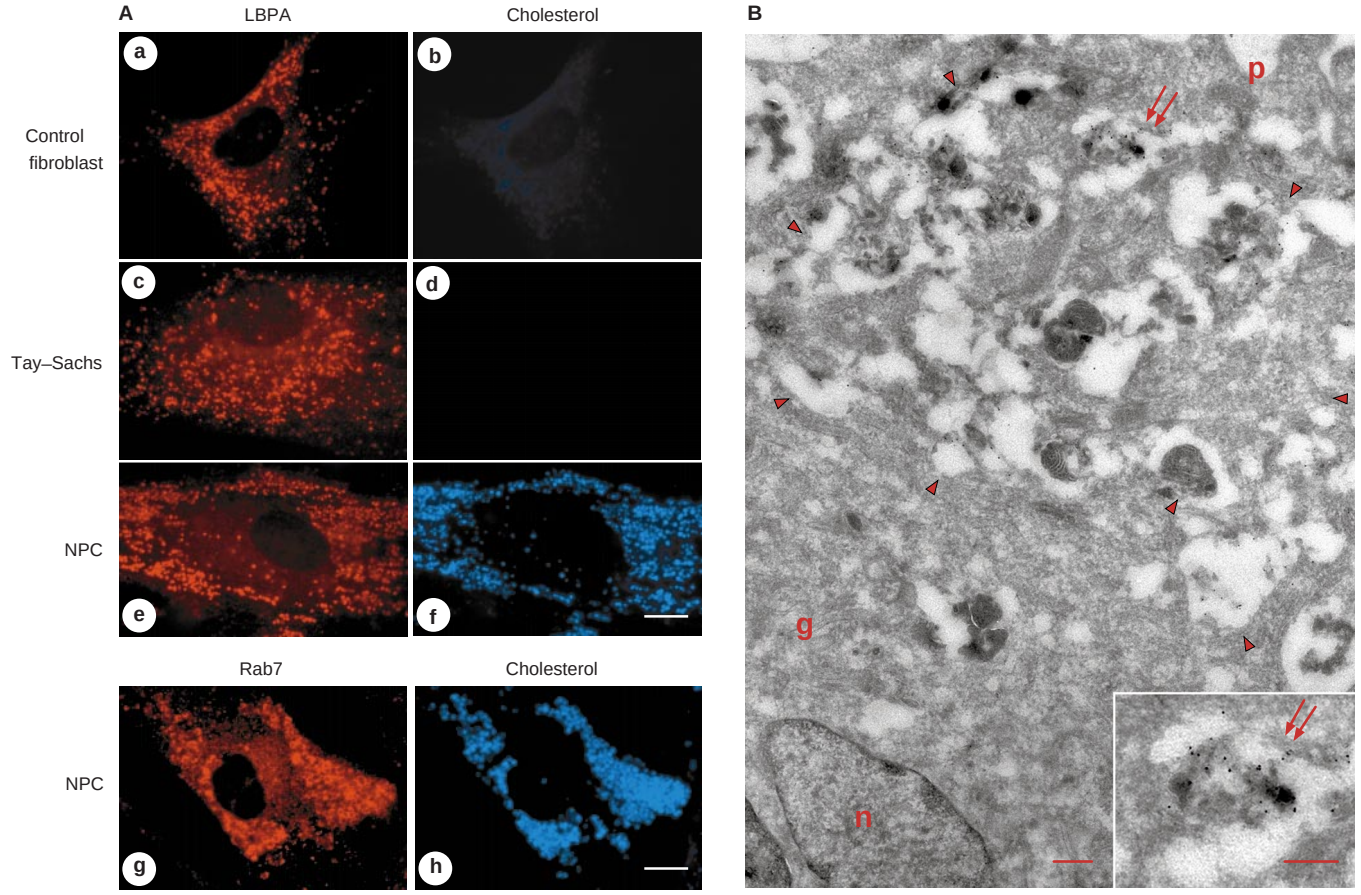

Figure 1 **Cholesterol accumulates in late endosomes of NPC fibroblasts. A,a–f**, Normal human skin fibroblasts, Tay–Sachs fibroblasts and NPC fibroblasts were fixed with paraformaldehyde and then double-labelled with a monoclonal anti-LBPA antibody (red fluorescence) to show LBPA and with filipin (blue fluorescence) to reveal intracellular cholesterol. **g**,**h**, NPC fibroblasts were also double-labelled with anti-Rab7 antibodies (red) and filipin. All vesicles containing cholesterol were also LBPA- or Rab7-positive after the short exposure times required to resolve individual cholesterol-positive vesicles unambiguously, but some LBPA- and Rab7-positive vesicles at first appeared to lack cholesterol. However, after longer exposure times of the same micrographs, these structures were clearly labelled with filipin (not shown), as was the plasma membrane in both NPC and control cells as expected (not shown). **B**, Low-magnification view of an ultrathin frozen section of an NPC fibroblast. Endosomes accumulate in large areas roughly delineated by arrowheads, which extend from the nucleus (n) and the Golgi region (g) to the plasma membrane (p), and which are labelled with anti-LBPA antibodies followed by rabbit antibodies against mouse IgG and 10-nm protein–A gold. Labelling is shown more clearly in the inset, which shows a higher-magnification view of the area indicated by red double arrows. Scale bars = 10 μm (**A**), and 200 nm (**B**).

significant change in total amounts of cellular cholesterol was detected after U18666A treatment (not shown), as observed by others[18]. This indicates that cholesterol accumulates and becomes concentrated within late endosomes, but that this pool remains small when compared with the large (diluted) pool of cholesterol present in other membranes. The ability of hydrophobic amines to bind negatively charged phospholipids may be responsible for their ability to block cellular cholesterol transport (reviewed in ref. 19). As late endosome membranes are rich in negatively charged lipids, in particular LBPA[5], we decided to test more directly the effects of cation accumulation within endosomes. In these experiments, we made use of BHK cells expressing ZnT2, a recently discovered Zn^{2+} transporter[20]. ZnT2 protects a zinc-sensitive BHK cell line from toxic concentrations of Zn^{2+} in the medium (0.1 mM), by loading the cation into an endocytic compartment, where it reaches concentrations ≥ 10 mM (ref. 20).

Cholesterol accumulates in late endosomes of cells expressing ZnT2 and treated with Zn^{2+}. Incubation of Znt2-BHK cells with 0.1 mM Zn^{2+} caused the appearance of numerous late endosomes containing both Rab7 and LBPA (Fig. 4A), as well as GFP–ZnT2 itself (not shown), as expected[20]. These late endosomes exhibited a highly electron-dense and compact multivesicular/multilamellar appearance (Fig. 4B).) Like NPC cells (Fig. 1B) and control cells[5], they contained LBPA within their internal membranes (Fig. 4B). As shown in Fig. 4A, Zn^{2+} addition caused a dramatic accumulation of unesterified cholesterol within late endosomes of ZnT2-BHK cells, as we predicted. However, as observed after U18666A treatment, total amounts of cellular cholesterol were not significantly affected by Zn^{2+} (not shown). Intense immunofluorescent labelling of the vesicle periphery presumably reflects the partial collapse of these delicate structures during sample preparation, as this was not observed after LBPA immunogold labelling on cryosections. It seems reasonable to suppose that the cation, which has accumulated within endosomes to high concentrations, is chelated by the headgroups of LBPA and other phospholipids, and that these interactions are responsible, at least in part, for the observed inhibition of cholesterol transport by Zn^{2+}.

Late endosome sorting/trafficking defect in cells treated with U18666A, or in cells expressing ZnT2 and treated with Zn^{2+}. Because we had observed that IGF2/MPR distribution is selectively affected in NPC cells, we tested whether U18666A treatment or Zn^{2+} accumulation in ZnT2 cells also affected late endosome sorting functions. As shown in Fig. 5, both conditions resulted in IGF2/MPR redistribution from the TGN to late endosomes. Similarly, the NPC1 protein itself is relocalized after cholesterol accumulation to cholesterol-laden endosomes/lysosomes[17]. In contrast, the distribu-

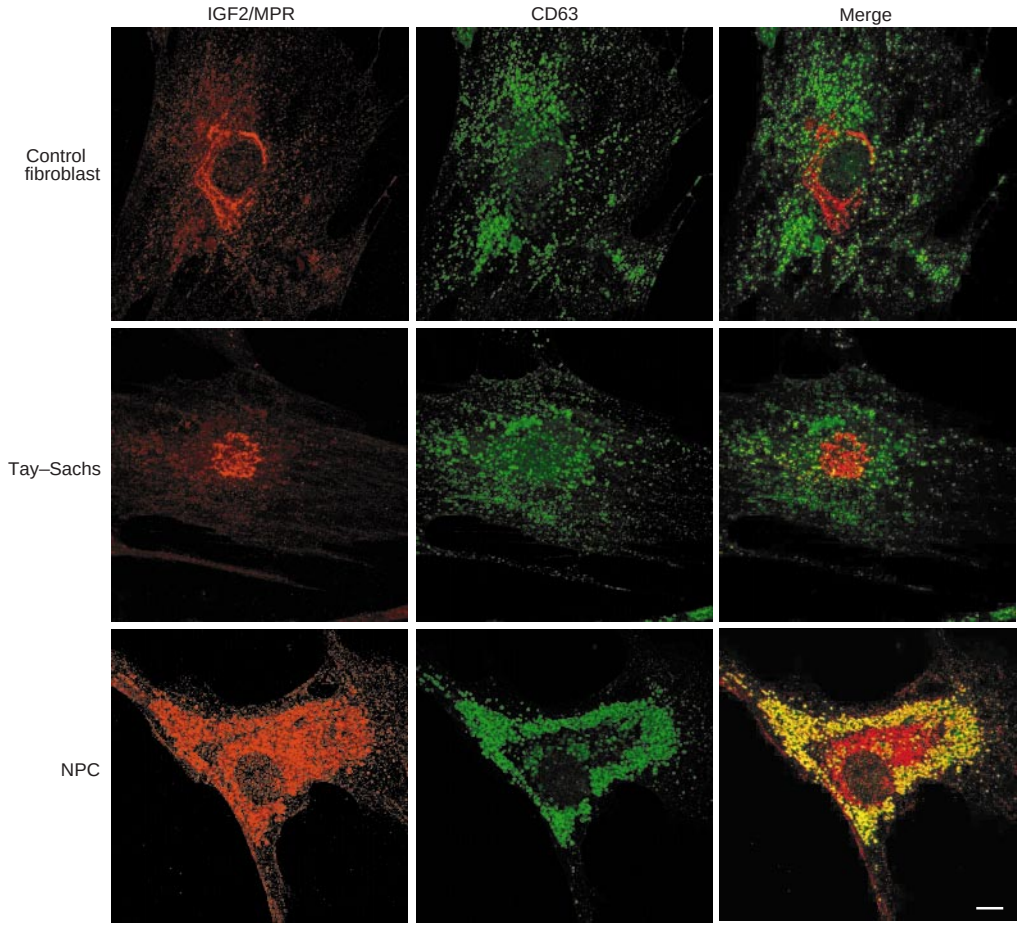

Figure 2 **IGF2/MPR is redistributed to late endosomes in NPC fibroblasts.** Normal human skin, Tay–Sachs fibroblasts and NPC fibroblasts were fixed and double-labelled with antibodies against IGF2/MPR (red fluorescence) and CD63 (green fluorescence). We used an anti-CD63 antibody not only because this antibody reacts with the human protein, but also because sample preparation is compatible with IGF2/MPR double labelling. In the merged images, yellow indicates co-localization of IGF2/MPR and CD63. Scale bar = 10 μm.

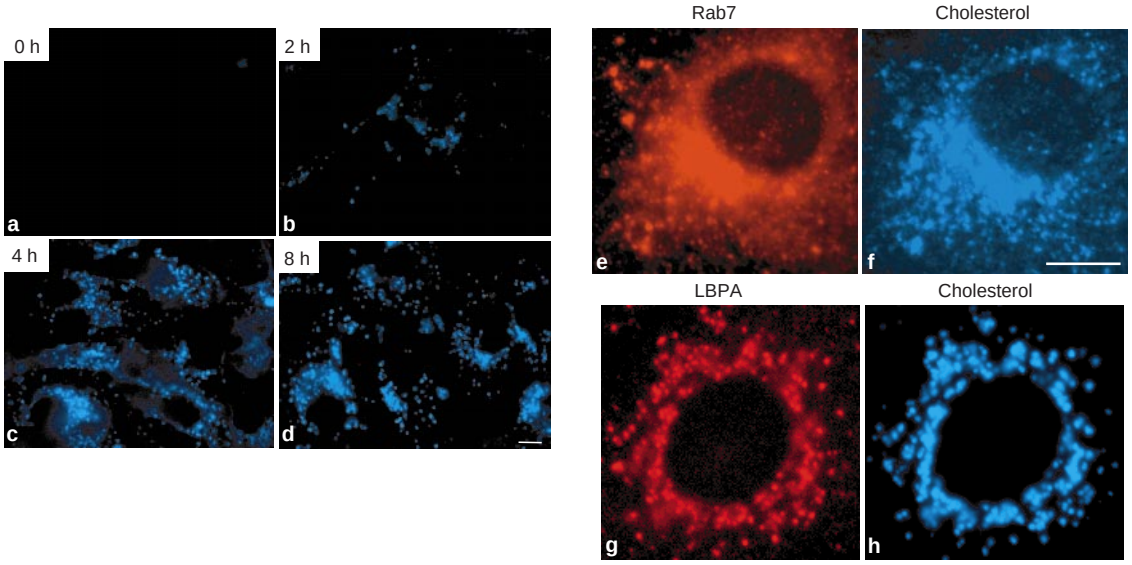

Figure 3 **U18666A causes cholesterol accumulation in late endosomes.** Cells were incubated with 3 μg ml^{-1} U18666A for 0, 2, 4 or 8 h. Cells were then fixed and labelled with filipin (**a–d**), or double labelled with filipin and antibodies against Rab7 (2 h) (**e**,**f**) or LBPA (8 h) (**g**,**h**). Scale bars = 10 μm.

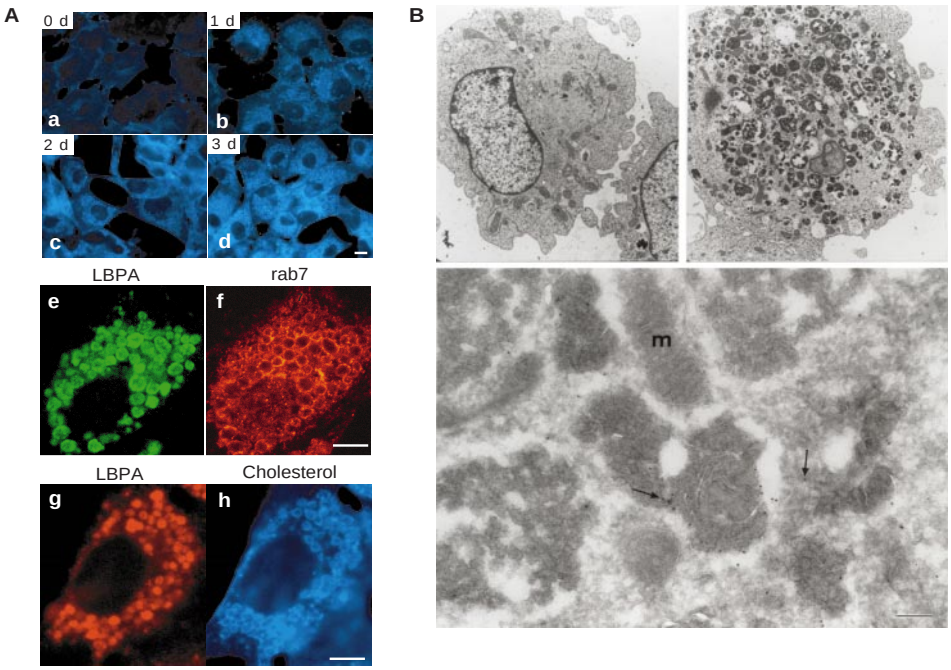

Figure 4 **Zn^{2+} causes cholesterol accumulation in late endosomes of cells expressing ZnT2. A**, Cells expressing ZnT2 were incubated with 0.1 mM Zn^{2+} for 0, 1, 2 or 3 days, fixed, and then labelled with filipin (**a–d**), or double labelled with antibodies against LBPA and Rab7 at day 2 (**e**,**f**), and against LBPA and filipin at day 3 (**g**,**h**). Differences in the appearance of late endosomes containing LBPA, Rab7 and cholesterol between human NPC fibroblasts (Fig. 1A), BHK cells treated with U18666A (Fig. 3) or BHK cells expressing ZnT2 and treated with Zn^{2+} may reflect, at least in part, differences in the preservation of the fragile endosomal ultrastructure after preparation of the samples for light microscopy. **B**, The upper panels show thin-section electron micrographs of control cells (left) and ZnT2-BHK cells treated for 3 days with Zn^{2+} (right), which causes a massive accumulation of huge electron-dense multivesicular structures throughout the cells. The bottom panel shows an ultrathin frozen section of these structures labelled with anti-LBPA antibodies (as in Fig. 1B). Labelling efficiency is lower than in NPC cells, perhaps because of reduced accessibility after Zn^{2+} accumulation; (m) mitochondrion. Scale bars = 10 μm (**A**) and 200 nm (**B**).

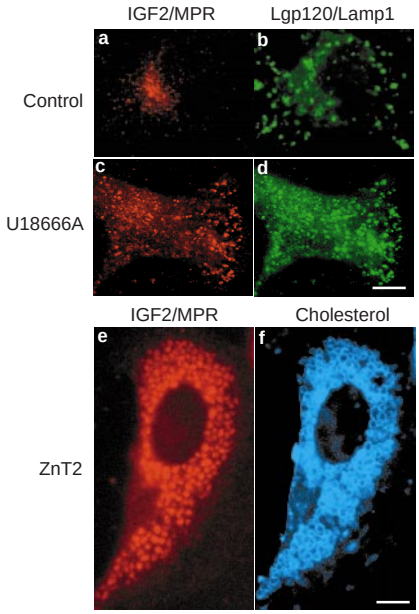

Figure 5 **IGF2/MPR is redistributed to late endosomes after treatment with U18666A or Zn^{2+}.** BHK cells were incubated with 3 μg ml^{-1} U18666A for 0 (control, **a**,**b**) or 48 h (**c**,**d**), fixed, and double labelled with antibodies against IGF2/MPR and Lgp120/Lamp1 (refs 5,21). We used an anti-Lgp120/Lamp1 antibody, because this antibody reacts with the BHK protein and because sample preparation is compatible with IGF2/MPR double-labelling. BHK cells expressing ZnT2 were incubated with 0.1 mM Zn^{2+} for 3 days, fixed, and double labelled with filipin and antibodies against IGF2/MPR (**e**,**f**). Scale bars = 10 μm.

tion of the protein TGN38, which also cycles between TGN and endosomes, was not affected by Zn^{2+} treatment (not shown), indicating that intralumenal accumulation of cations did not cause general perturbations of endosomal membranes, and suggesting that TGN38 may recycle from early, and not late, endosomes.

After U18666A addition, cholesterol accumulation was rapid (8 h, see Fig. 3), as expected from the known action of the drug[18]. In contrast, IGF2/MPR redistribution occurred more slowly (24–48 h, see Fig. 5). Similarly, after Zn^{2+} addition cholesterol was affected before IGF2/MPR (Fig. 4,5), although Zn^{2+} accumulation within endosomes required longer incubation. These observations suggest that the IGF2/MPR-sorting defect is a consequence, rather than a cause, of impaired cholesterol transport, and demonstrate that late endosomes have a critical role in intracellular cholesterol movement.

Cholesterol accumulates in late endosomes of cells treated with a monoclonal antibody against LBPA. In previous studies we showed that a well-characterized monoclonal antibody against LBPA, when internalized by fluid-phase endocytosis into living cells, selectively accumulated in late endosomes upon binding to its antigen[5]. This treatment caused late endosomes to become electron-dense and compact, and IGF2/MPR to redistribute from TGN to late endosomes. These striking similarities prompted us to test the effect of our antibody on cholesterol transport. As shown in Fig. 6A, treatment with the anti-LBPA antibody resulted in massive accumulation of cholesterol, much as in NPC cells or cells treated with U18666A or Zn^{2+}. However, as with our observations after Zn^{2+} or U18666A treatment, total amounts of cellular cholesterol were not significantly affected by internalization of anti-LBPA antibodies (not shown). Cholesterol then co-localized with the endocytosed antibody within late endosomes. In contrast, the distribution of cholesterol (not shown) or IGF2/MPR[5] was not affected after

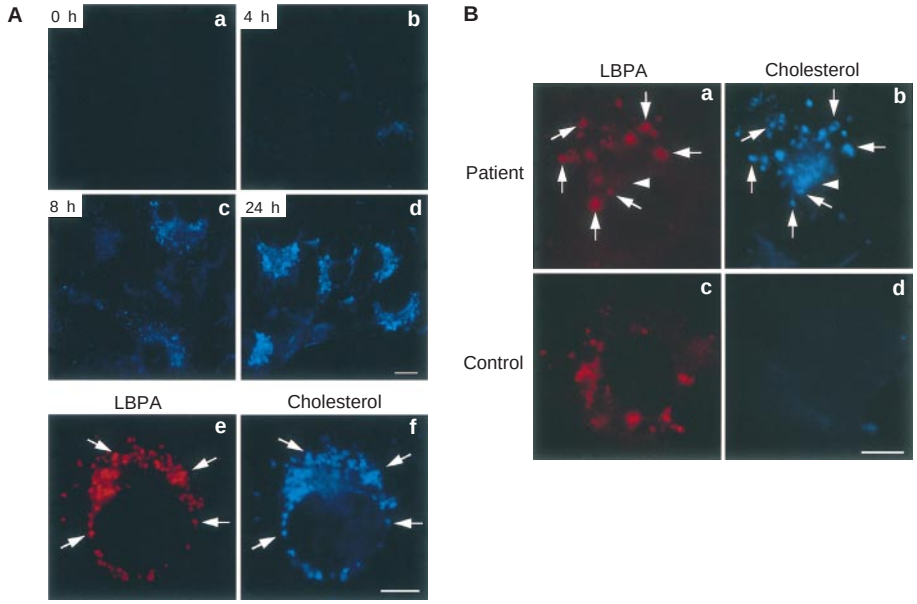

Figure 6 **Antibodies against LBPA cause cholesterol accumulation in late endosomes. A**, Cells were incubated with the monoclonal antibody against LBPA (50 μg ml^{-1}) for 0, 4, 8 or 24 h, fixed, and labelled with filipin (**a–d**), or double labelled with anti-mouse antibodies (to visualize the endocytosed anti-LBPA antibody) and filipin (arrows point at examples of double-labelled structures) (**e**,**f**). **B**, Typical examples of cells incubated with sera from patients with the anti-phospholipid syndrome (**a**,**b**) or with control sera (**c**,**d**), and then double labelled, after fixation, with antibody against LBPA and with filipin. Lower LBPA labelling intensity when compared with **A** is likely to reflect competition for LBPA molecules between the anti-LBPA monoclonal antibody used for detection and the internalized human antibodies, as both recognize LBPA[5]. Arrows point at double-labelled structures, and arrowheads at structures that do not seem to contain high levels of LBPA. Scale bars = 10 μm.

endocytosis of control antibodies, including a monoclonal antibody against Lgp120/Lamp1, which is a major constituent of the limiting membranes of the same compartment[5,21]. Much as after U18666A treatment, cholesterol accumulated rapidly after antibody addition (~ 4–8 h, see Fig. 6A), whereas ≥ 24-h incubations were necessary to cause IGF2/MPR redistribution[5], supporting the notion that IGF2/MPR trafficking was affected by cholesterol accumulation. More important, these data show that selective perturbation of LBPA-rich membrane domains is involved in the regulation of intracellular cholesterol transport.

Cholesterol accumulates in late endosomes of cells treated with sera from patients with anti-phospholipid syndrome. We had previously reported that antibodies from patients suffering from the anti-phospholipid (aPL) syndrome[22,23] specifically recognized LBPA *in vivo* and *in vitro*, and altered IGF2/MPR sorting when endocytosed by living cells, much like the monoclonal anti-LBPA antibody[5]. As shown in Fig. 6B, treatment with sera from patients, but not with sera from healthy individuals, caused significant accumulation of cholesterol within late endosomes containing LBPA, which was both reminiscent of Niemann–Pick type C, and similar to that observed with the monoclonal anti-LBPA antibody. We previously speculated that antibodies associated with the aPL syndrome affect IGF2/MPR sorting by altering LBPA-rich membrane dynamics[5]. In the light of our new observations, it is attractive to propose that such alterations result from cholesterol accumulation. Our observations also suggest that endosome functions may be affected in a similar manner in the genetic NPC disease and in the aPL syndrome, despite the fact that the onset of these diseases is entirely different.

Discussion

Internal, LBPA-rich membranes account for the vast majority of total late endosomal membranes, and form an extensive lumenal network[5]. Thus, newly internalized cholesterol, which is first hydrolysed to unesterified cholesterol, is very likely to partition preferentially within these internal membranes when released into the lumen. Then, free cholesterol is rapidly redistributed intracellularly, as it does not accumulate within late endosomes under normal steady-state conditions. However, intralumenal accumulation of cations, which is predicted to affect the structures of internal membranes, causes cholesterol accumulation. More important, cholesterol accumulation in late endosomes is caused by highly specific perturbations of internal membranes with a monoclonal antibody against LBPA, or with human antibodies which recognize LBPA. Thus, cholesterol transport depends not only on its intrinsic properties (diffusion, flip-flop), but also on the physicochemical and dynamic properties of LBPA-rich internal membranes. Accumulation of cholesterol within LBPA-rich internal membranes is predicted to alter membrane properties even further, and to contribute to the observed inhibition of IGF2/MPR-mediated sorting and trafficking. Consistent with this notion, IGF2/MPR accumulates within internal membranes rich in LBPA in cells treated with anti-LBPA antibodies[5]. Cholesterol accumulation in late endosomes, and the resulting sorting/trafficking defect, may also alter transfer from late endosomes to lysosomes, as recent studies suggest that this step occurs via the formation of a hybrid organelle[3]. Although the role of the NPC protein is not known, an attractive hypothesis is that it functions as a sensor of the endosomal cholesterol content[7–10].

In conclusion, we propose that the network of LBPA-containing internal membranes within late endosomes functions, in effect, as a collecting and distribution device, thereby controlling intracellular cholesterol transport. In addition, our observations support the notion that LBPA-rich membrane domains are affected in both NPC and the aPL syndrome, resulting in cholesterol accumulation within late endosomes and similar endosomal sorting/trafficking defects. □

Methods

Baby hamster kidney (BHK-21) cells, BHK cells expressing ZnT2, and their parental zinc-sensitive line 3286-8-8 (ref. 20) were grown and maintained as described[24]. Cultured skin fibroblasts from patients with NPC[25] or Tay–Sachs disease[26] and from healthy subjects were established and maintained as

described. Diagnosis was confirmed by the typical clinical manifestations and by lysosomal enzyme assays and filipin staining[11]. Briefly, double labelling with filipin and antibodies was performed on cells fixed with 3% paraformaldehyde[11]. Cells were incubated with the first antibodies in PBS containing 10% FCS and 50 μg ml^{-1} filipin, washed, and then incubated with the second antibodies in PBS-FCS only. Human antisera from patients with antiphospholipid syndrome were a gift from P. de Moerloose (University Hospital of Geneva). The monoclonal antibodies against LBPA[5] and Lgp120/Lamp1 (ref. 21) have been described, as well as the antibody against Rab7 (ref. 5). Polyclonal antibodies against IGF2/MPR and TGN38 were gifts from B. Hoflack (Institut Pasteur, Lille) and G. Banting (University of Bristol), respectively. The monoclonal antibody against CD63 was from Chemicon. U18666A was from Biomol, and filipin from Sigma. Immunofluorescence microscopy[5], and electron microscopy[27] were performed as described.

RECEIVED 26 MARCH 1999; REVISED 26 APRIL 1999; ACCEPTED 4 MAY 1999; PUBLISHED 15 MAY 1999.

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ACKNOWLEDGEMENTS

We thank R. Gagescu, G. van der Goot, F. Perez, and M. Rojo for critical reading of the manuscript. This work was supported by the Swiss National Science Foundation (J.G.), the NHMRC of Australia (R.G.P.) and by the International Human Frontier Science Program (J.G., R.G.P., T.K).

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