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Loss of cation-independent mannose 6-phosphate receptor expression promotes the accumulation of lysobisphosphatidic acid in multilamellar bodies

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SUMMARY

A number of recent studies have highlighted the importance of lipid domains within endocytic organelles in the sorting and movement of integral membrane proteins. In particular, considerable attention has become focussed upon the role of the unusual phospholipid lysobisphosphatidic acid (LBPA). This lipid appears to be directly involved in the trafficking of cholesterol and glycosphingolipids, and accumulates in a number of lysosomal storage disorders. Antibody-mediated disruption of LBPA function also leads to mis-sorting of cation-independent mannose 6-phosphate receptors. We now report that the converse is also true, and that spontaneous loss of cation-independent mannose 6-phosphate receptors from a rat fibroblast cell line led to the formation of aberrant late endocytic structures enriched in LBPA. Accumulation of LBPA was directly dependent upon the loss of the receptors, and could be reversed by expression of bovine cation-independent mannose 6-phosphate receptors in the mutant cell line.

Ultrastructural analysis indicated that the abnormal organelles were electron-dense, had a multi-lamellar structure, accumulated endocytosed probes, and were distinct from dense-core lysosomes present within the same cells. The late endocytic structures present at steady state within any particular cell likely reflect the balance of membrane traffic through the endocytic pathway of that cell, and the rate of maturation of individual endocytic organelles. Moreover, there is considerable evidence which suggests that cargo receptors also play a direct mechanistic role in membrane trafficking events. Therefore, loss of such a protein may disturb the overall equilibrium of the pathway, and hence cause the accumulation of aberrant organelles. We propose that this mechanism underlies the phenotype of the mutant cell line, and that the formation of inclusion bodies in many lysosomal storage diseases is also due to an imbalance in membrane trafficking within the endocytic pathway.

Key words: Massose 6-phosphate receptor, Endosome, Lysosome, LBPA

INTRODUCTION

In mammalian cells dense core lysosomes (DCL) are of fundamental importance to the maintenance of cellular homeostasis. They are required not only for the processing of endocytosed nutrients such as lipoprotein particles, but also for cellular responses to external stimuli including the down-regulation of ligand activated growth-factor receptors, and autophagy (reviewed by Kornfeld and Mellman, 1989). Loss of lysosomal function can have severe consequences, and there are over 30 distinct diseases directly attributable to lysosomal dysfunction (Gieselmann, 1995). Although lysosomal storage diseases (LSD) generally arise from defects in a single acid hydrolase, they are often characterised by complex pathologies, which in many cases remain poorly understood.

In order to understand the intracellular consequences of LSD a knowledge of DCL biogenesis is essential. Ultrastructural analysis of fibroblasts has identified lysosomes as spherical electron-dense organelles approximately 300-500 nM in diameter in which endocytosed tracers accumulate. Thus traditional models of the endocytic pathway have regarded DCLs as the terminal destination, and primary site of degradation, for endocytosed macromolecules (Kornfeld and Mellman, 1989). However, a number of recent studies have suggested that the situation may be more complex, and that the late endocytic pathway is much more dynamic than the traditional model suggests (reviewed by Luzio et al., 2000). In particular one revised model suggests that late endosomes and DCLs undergo multiple cycles of fusion and condensation, and highlights the importance of the late endosome-lysosome hybrid organelle (ELH) in this process (Bright et al., 1997; Futter et al., 1996; Mullock et al., 1998).

The majority of lysosomal hydrolases are delivered to the endocytic pathway via a mannose 6-phosphate (M6P) recognition signal (Dahms et al., 1989). This signal is created through the sequential actions of two enzymes, UDP-GlcNAc: lysosomal enzyme GlcNAc-phosphotransferase, and GlcNAc-1-phosphodiester α-N-acetylglucosaminidase during passage of the newly synthesized hydrolase to and through the Golgi (Baranski et al., 1991; Kornfeld and Mellman, 1989). Subsequently the mannose 6-phosphorylated proteins bind to specific receptors (M6PRs) in the trans Golgi network (TGN) and are thus transported to the endocytic pathway. Following delivery, the acidic endosomal milieu causes dissociation of hydrolase and receptor, with the latter recycling to the TGN to mediate further rounds of transport (Dahms et al., 1989). Failure to form the M6P signal due to defective phosphotransferase activity is the underlying defect in the LCDs mucolipidosis types II and III (Lang et al., 1985), and a similar phenotype to mucolipidosis type II (I-cell disease) is obtained in mice lacking M6PRs (Dittmer et al., 1998).

Trafficking of the cation-independent M6PR (ciM6PR) within the endocytic pathway can be perturbed by antibodies against lysobisphosphatidic acid (LBPA; Kobayashi et al., 1999). This lipid is mainly restricted to internal membranes within late endocytic organelles, and has an unusual stereoconfiguration which renders it resistant to most phospholipases, and particularly prone to forming non-bilayer structures (Kobayashi et al., 1998a). LBPA-rich membranes are likely to be involved in membrane protein sorting from late endosomes, as evidenced by the perturbation of CD63 trafficking in cells treated with drugs that disrupt the function of this lipid (Kobayashi et al., 2000). They also appear to be the primary sites of glycosphingolipid breakdown (Schuette et al., 1999), and play a major role in cholesterol metabolism (Kobayashi et al., 1999).

In the present study we show that the spontaneous loss of ciM6PR from a transfected rat fibroblast line resulted in the accumulation of abnormal late endocytic structures enriched in cholesterol and LBPA. This effect could be reversed by expression of the bovine receptor. Quantitative electron microscopic analysis of the mutant cell line revealed an abnormal accumulation of multi-lamellar bodies (MLB) and concomitant decrease in multi-vesicular late endosomes and DCLs. These results suggest a central role of lipid trafficking and/or metabolism in controlling the biogenesis of lysosomes. We propose that a common defect of membrane trafficking through the late endocytic pathway may underlie the pathologies of many LSDs.

MATERIALS AND METHODS

Cell lines

Fischer Rat Embryonic Fibroblasts (Rat1; Topp, 1981) were maintained in RPMI 1640 medium supplemented with 10% foetal calf serum, 1% non-essential amino acids, 10 mM Na pyruvate, 2 mM glutamine, 50 i.u./ml penicillin, and 50 μg/ml streptomycin, in a humidified 95% air/5% CO_2 atmosphere at 37°C. Cells were transfected by electroporation with vectors conferring resistance to hygromycin, and clonal cell lines derived essentially as described previously (Reaves and Banting, 1994). Clones were maintained in medium supplemented with 200 μg/ml hygromycin (Roche, Lewes, UK). For the recovery experiments shown in Fig. 8, clone 2 cells were transfected with pSREP93-ciM6PR using Fugene 6 (Roche) according to the manufacturer's instructions. After 24 hours the media was changed and fresh media containing 5 μg/ml puromycin added. Cells were processed for immunofluorescence microscopy after a further 10 days in culture in the continued presence of puromycin.

Antibodies and cDNA

Cathepsin D was isolated from rat liver by pepstatin A affinity chromatography as described by Stewart and co-workers (Stewart et al., 1994). Polyclonal antisera were raised against the purified protein in New Zealand White rabbits by established techniques (Harlow and Lane, 1988). Polyclonal antisera towards rat ciM6PR (Reaves et al., 1996) and TGN38 (Luzio et al., 1990), and monoclonal GM-10 (anti-rat lgp120; Reaves et al., 1996) have been described elsewhere. Rabbit anti-(γ-adaptin; Seaman et al., 1996) was a gift from Dr M. Robinson (University of Cambridge), rabbit anti-EEA1 from Dr M. Clague (University of Liverpool), and mouse monoclonal 6C4 against LBPA (Kobayashi et al., 1998b) from Dr T. Kobayashi (Saitama, Japan). FITC-labelled goat anti-(mouse IgG), Texas Red-labelled donkey anti-(rabbit IgG) and peroxidase-labelled donkey anti-(rabbit IgG) were all obtained from Amersham (Little Chalfont, UK). Goat anti-mouse IgG conjugated to 10 nm colloidal gold was purchased from Sigma (Poole, UK).

The cloning and mutagenesis of cDNA encoding rat Vps34p will be described elsewhere (P. E. Row et al., unpublished). Wild-type and mutant constructs were sub-cloned into the mammalian expression vector ΔpMEP4 (Girotti and Banting, 1996). Bovine ciM6PR cDNA (Lobel et al., 1989) was kindly provided by Dr S. Kornfeld (St Louis, USA). The entire coding sequence was sub-cloned into pSREP93, a derivative of pMEP4 (Invitrogen, Groningen, Holland) in which the metallothionein promotor was replaced with the SRα promotor (Takebe et al., 1988), and the *hph* gene replaced by the *pac* gene conferring resistance to puromycin (de la Luna and Ortin, 1992).

Biosynthetic radiolabelling

Rat1 cell lines (>80% confluent) growing on 60 mm tissue culture dishes were washed twice with PBS, then incubated in methionine and cysteine free DMEM (Sigma) containing 2.2 g/l Na bicarbonate, 5% v/v dialysed new-born calf serum, and inhibitors if required for 1 hour at 37°C in a gassed incubator. After washing twice with PBS, 1.2 ml of methionine and cysteine free medium containing 100 μCi Trans^{35}S-Label (ICN Biomedicals Ltd, Thame, UK) was added, and the cells incubated at 37°C for 30 minutes. They were then transferred to ice, washed three times with ice-cold PBS/5 mg/ml BSA and lysed as described previously (Davidson, 1995). Labelled ciM6PR was immunoprecipitated, and analysed by SDS-PAGE and phosphorimaging.

Starvation induced autophagy

Starvation-induced autophagy was assayed by a modification of the method of Petiot and colleagues (Petiot et al., 2000). Rat1 cell lines (>80% confluent) growing on 60 mm tissue culture dishes were washed twice with PBS, then incubated in methionine free α-MEM (Sigma) containing 2.2 g/l Na bicarbonate and 5% v/v dialysed new-born calf serum for 2 hours at 37°C in a gassed incubator. After washing twice with PBS, 1.5 ml of methionine free medium containing 50 μCi Trans^{35}S-Label (ICN Biomedicals Ltd, Thame, UK) and 10% v/v dialyzed serum was added and the cells incubated for a further 16 hours. The labelled cells were then washed with PBS and autophagy induced by incubation at 37°C in 2 ml Hanks' balanced salt solution (Sigma) containing 0.1 mM methionine, 0.1% BSA and inhibitors as required. After 1 hour the medium was replaced and the incubation continued for a further 4 hours. The final chase medium was retained and the cells recovered from the dishes by scraping into PBS. All fractions were treated with ice-cold TCA (10% w/v final concentration) and separated into soluble and precipitated material by centrifugation. The various fractions were then analysed by liquid

scintillation counting. Results are expressed as the sum of the TCA soluble material in the cells and final medium as a percentage of the total recovered radioactivity.

Microscopy

Indirect immunofluorescence microscopy was performed as described previously (Reaves et al., 1996) using Rat1 cell lines grown on glass coverslips. Prior to staining of unesterified cholesterol using filipin (Sigma), cells were incubated in the presence or absence of 3 μg/ml U18666A (Affiniti research products, Exeter, UK) for 6 hours at 37°C. They were then fixed with 2% paraformaldehyde in PBS for 20 minutes at room temperature, washed 3 times in PBS and permeabilized and blocked with 0.05 mg/ml filipin in 0.2% BSA/PBS for 20 minutes. All subsequent incubations, including antibody solutions, contained filipin and 0.2% BSA in PBS.

Prior to preparation for electron microscopic analysis BSA-5 nm gold was internalised for 4 hours followed by a 20 hour chase as described previously (Bright et al., 1997). Quantitation of the number, size and LBPA labelling density of BSA-gold positive late endocytic structures was performed as in our previous studies (Bright et al., 1997; Mullock et al., 1998). Late endosomes were defined as structures containing the BSA-gold which were electron-lucent, and MLBs as labelled structures which had an electron-dense luminal content and multilamellar intraluminal membranes. DCL were defined as previously (Bright et al., 1997). To generate the data shown in Fig. 6C-E derived from samples embedded in epoxy resin, 34 cell profiles containing a total of 69 BSA-gold loaded organelles, and 70 cell profiles containing a total of 81 BSA-gold loaded organelles, were scored from clones 1 and 2, respectively. To generate the data shown in Fig. 7C derived from ultra-thin frozen sections labelled with anti-LBPA, 96 cell profiles containing a total of 191 BSA-gold loaded organelles, and 90 cell profiles containing a total of 107 BSA-gold loaded organelles, were scored from clones 1 and 2, respectively. The distribution of gold, and the size of gold-labelled organelles was not affected by the sample preparation method.

RESULTS

Clone 2 cells do not express ciM6PR

Previously, we demonstrated a role for phosphatidylinositol 3-kinase activity in trafficking within the endocytic pathway (Reaves et al., 1996). During studies designed to extend this work we isolated a hygromycin resistant cell line (clone 2) deficient in ciM6PR. Western blotting of clone 2 cells showed that in contrast to the parental cell line, or clones 1, 3 or 4, it did not express any detectable ciM6PR staining at steady state (Fig. 1). The result shown utilised an antibody directed towards the cytoplasmic tail of the receptor, but an identical pattern was observed when a luminal epitope was used (data not shown). All lines expressed equivalent amounts of the control protein γ-adaptin (Fig. 1). A number of mouse cell lines which apparently lack ciM6PR have been identified. For example $P388D_1$ cells do not synthesize any gene product, whereas MOPC 315 cells synthesize an apparently normal protein which is rapidly degraded (Goldberg et al., 1983). To distinguish between these two alternatives for clone 2 cells, we biosynthetically radiolabelled the cells with [^{35}S]methionine for 30 minutes, and immunoprecipitated labelled ciM6PR from the clarified cell lysate. As shown in Fig. 2, in contrast to the parental cells or clones 1, 3, and 4, radiolabelled ciM6PR was undetectable in clone 2 immunoprecipitates, consistent with a failure to synthesize this protein. No increase in signal was observed in clone 2 cells pre-treated with either the proteosome

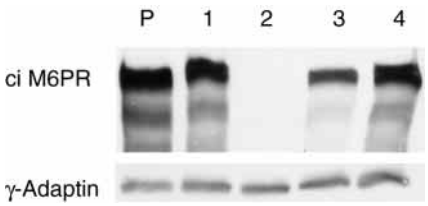


Fig. 1. Western blotting of Rat1 cell lines. Equal numbers of parental Rat1 fibroblasts (P), and clones 1-4, were harvested and processed for immunoblotting as described in Materials and Methods. Prior to antibody treatment, blots were stained with Ponceau S (Sigma), and divided into regions containing the 300 kDa ciM6PR and the 100 kDa γ-adaptin subunit of AP-1. Each region was then processed with the appropriate primary and secondary antibody solutions.

inhibitor lactacystin, or the lysosomal hydrolase inhibitor E64d (data not shown). No correlation between the expression of the protein encoded by the cDNA with which the various clones had been transfected (a kinase-dead rat Vps34p,) and the expression of ciM6PR could be determined (data not shown). Thus it appeared that loss of ciM6PR expression from clone 2 was unrelated to the expression of the cDNA with which it had been transfected, and was a consequence of the transfection and/or selection procedure per se.

Clone 2 cells contain functional lysosomal hydrolases

In addition to the ciM6PR, mammalian cells also possess a second M6PR, the 46 kDa cation-dependent receptor (cdM6PR; Dahms et al., 1989). Although neither receptor can completely compensate for the loss of the other, intracellular concentrations of lysosomal hydrolases are typically between 40% and 80% of control levels in mouse fibroblasts lacking either the ciM6PR or cdM6PR (Ludwig et al., 1994; Pohlmann et al., 1995). Consistent with the hypothesis that lysosomal hydrolases would still be targeted efficiently via the cdM6PR in clone 2 cells, cathepsin D could readily be detected by indirect immunofluorescence (Fig. 3A and B). Typically cathepsin D staining was slightly less intense in clone 2 cells than in the other lines tested (compare A and B), which correlates with the modest increase in secretion of newly synthesized procathepsin D from this cell line which we observed (data not shown). However, no difference in the abilities of the various clones to degrade endocytosed proteins was seen (data not shown).

The appearance of the TGN marker protein TGN38 (Luzio et al., 1990), was essentially identical in all of the clones

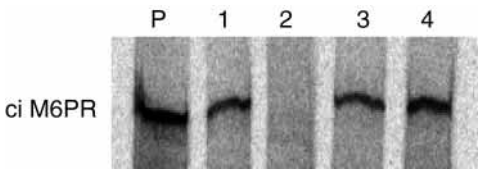


Fig. 2. Biosynthetic radiolabelling of ciM6PR. Parental Rat1 fibroblasts (P), and clones 1-4, were radiolabelled for 30 minutes with [^{35}S]methionine as described in Materials and Methods. Labelled ciM6PR was recovered from clarified cell lysates by immunoprecipitation, and detected by SDS-PAGE and phosphorimaging.

examined (Fig. 3C and D), as was the distribution of the early endosomal marker protein EEA1 (Mu et al., 1995; Fig. 3E and F). In contrast, an abnormally intense staining pattern for the lysosomal membrane protein lgp120 was seen in the mutant cell line (compare I and J), although analysis of the steady state levels of lgp120 in the various cell lines did not reveal any significant differences (data not shown). Consistent with the other results described above, ciM6PR staining was undetectable in clone 2 cells (G and H). In all microscopic studies, clone 1 was a representative example of ciM6PR expressing Rat1 cells.

In addition to degrading exogenous proteins, DCLs are also required for turnover of intracellular organelles and proteins by macroautophagy. Recent studies have implicated mammalian Vps34p in the control of this process (Petiot et al., 2000). Thus although we did not believe that the defect in ciM6PR expression by clone 2 was directly due to expression of the mutant Vps34p encoded by the cDNA with which it was transfected, we thought it important to examine autophagy in this cell line. As shown in Fig. 4A, degradation of radiolabelled proteins in response to amino acid deprivation occurred to an essentially identical extent in clone 1 and clone 2 cells during the 4 hour chase. Importantly, and consistent with the reported involvement of Vps34p (Petiot et al., 2000), starvation-induced autophagy was significantly inhibited by treatment with wortmannin and 3-methyladenine, in addition to the lysosmotrophic drug chloroquine (Fig. 4B). As predicted, no significant difference in the inhibitor sensitivities of induced protein turnover in clones 1 and 2 was observed (Fig. 4B, compare open and solid bars).

Clone 2 cells accumulate LBPA and unesterified cholesterol

Recent studies have identified the unusual acidic phospholipid LBPA (also known as bis(monoacylglycero)phosphate) as a marker of internal membranes in late

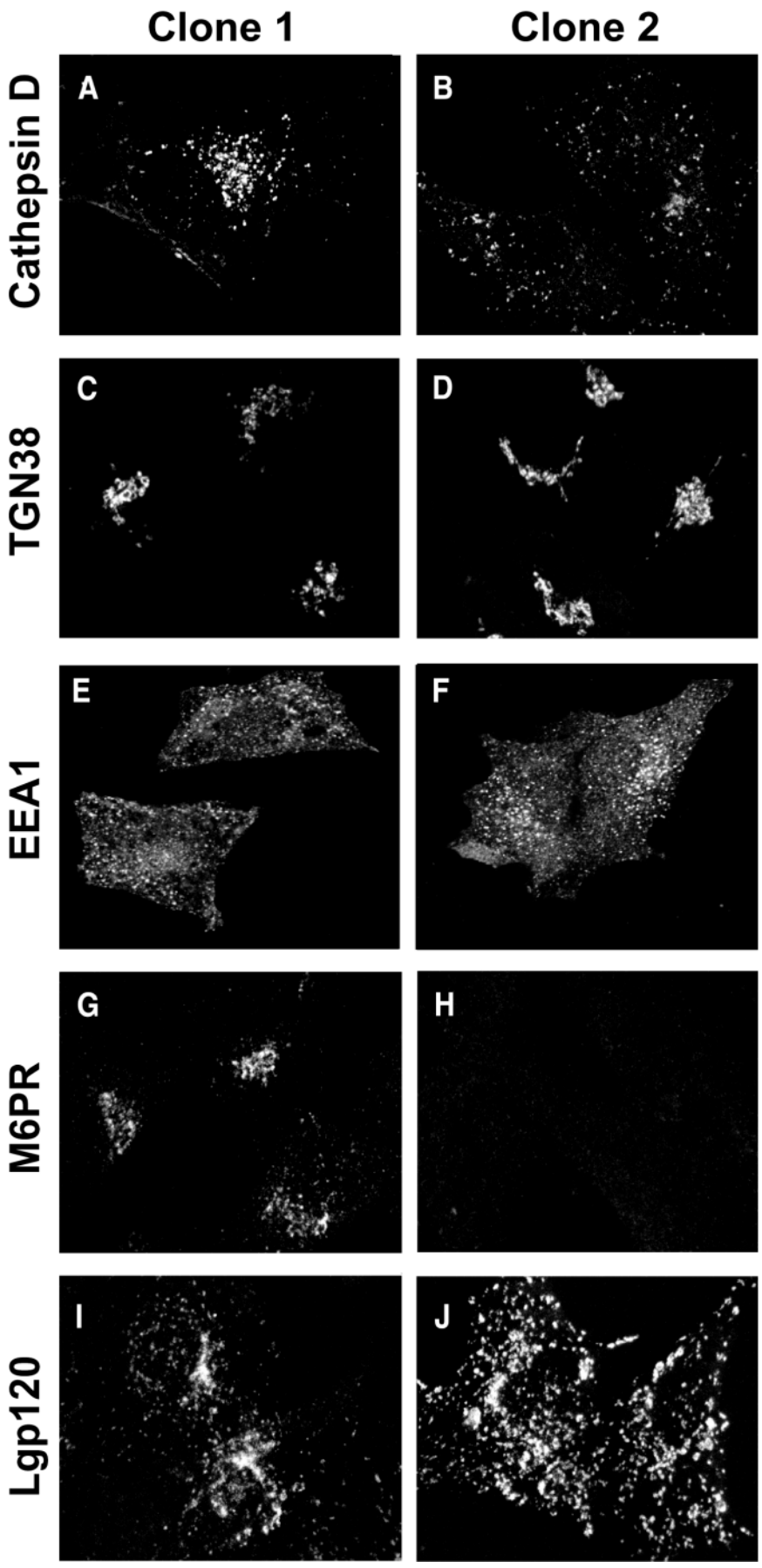


Fig. 3. Immunofluorescence microscopy of transfected Rat1 fibroblasts. Clone 1 (a representative of Rat1 cell lines expressing ciM6PR) and clone 2 cells were fixed with methanol and processed for indirect immunofluorescence as described in Materials and Methods. The panels show the localizations of rat cathepsin D, TGN38 (Luzio et al., 1990), EEA1 (Mu et al., 1995), ciM6PR (using an antibody to the cytosolic tail; Reaves et al., 1996), and lgp120 (Grimaldi et al., 1987; Reaves et al., 1996).

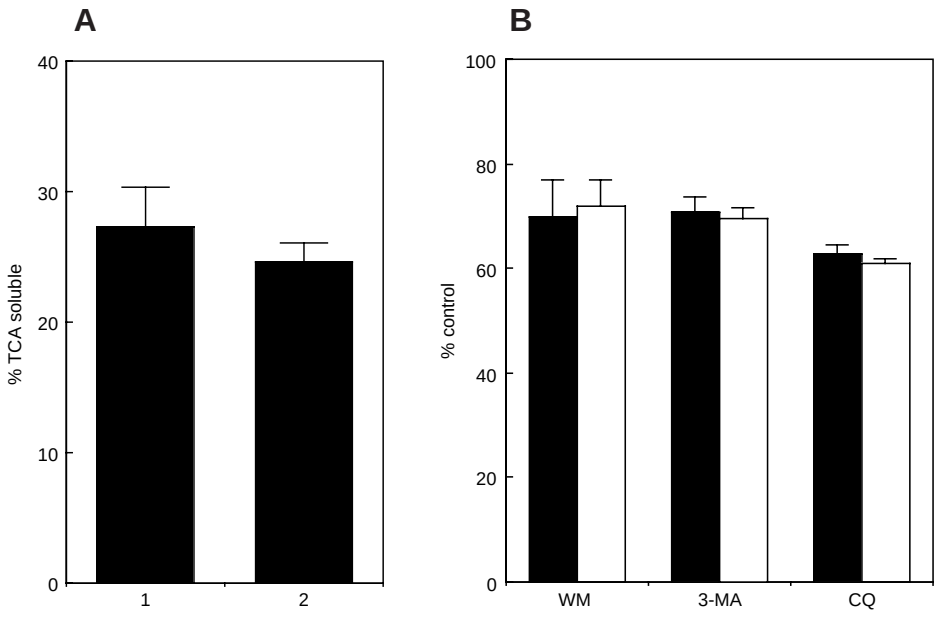


Fig. 4. Analysis of starvation-induced autophagy in clones 1 and 2. Clone 1 or 2 cells (>80% confluent) were labelled for 16 hours with [^{35}S]methionine as described in Materials and Methods. (A) Labelled cells were starved of amino-acids without additions and autophagy assessed. Results represent the mean ± s.d. of 11 separate determinations. (B) Labelled clone 1 cells (closed bars) or clone 2 cells (open bars) were starved of amino-acids in the presence of 100 nM wortmannin (WM), 10 mM 3-methyladenine (3-MA), or 200 μM chloroquine (CQ), and autophagy assessed. Results are expressed relative to cells starved in the absence of any inhibitors, and represent the mean ± s.d of 3 separate determinations.

endosomal/prelysosomal compartments (Kobayashi et al., 1998b), and shown that this lipid accumulates in some LSDs (Kobayashi et al., 1999). Given the unusual staining pattern of lgp120 in clone 2 cells (Fig. 3J), we thought it important to examine LBPA distribution in these cells. As shown in Fig. 5A, in clone 1 cells LBPA staining was confined to a restricted number of widely distributed punctate structures together with a diffuse peri-nuclear localization. In contrast, LBPA staining in clone 2 cells was considerably more intense, and focussed in the peri-nuclear region (C). A link between LBPA accumulation and the trafficking of un-esterified cholesterol has recently been demonstrated (Kobayashi et al., 1999). Consistent with this observation, staining with the fluorescent antibiotic filipin was considerably brighter in clone 2 cells as compared to the other clones (compare B and D), and in contrast to the control cells, showed extensive overlap with the LBPA staining.

Recent studies have also shown that LBPA and cholesterol accumulation can be induced by treatment of cells with the hydrophobic amine U18666A (Kobayashi et al., 1999). As predicted, treatment of clone 1 cells with U18666A caused an increase in anti-LBPA and filipin staining, and a redistribution of LBPA into the cholesterol-rich structures (Fig. 5E and F). Under these conditions the drug-treated clone 1 cells could not be distinguished from untreated clone 2 cells (compare C and D with E and F). In contrast, no additional effect on LBPA and cholesterol staining in clone 2 cells was observed following treatment with U18666A (G and H).

LBPA accumulates in MLBs in clone 2 cells

To further characterise the structures in which LBPA accumulated we conducted electron microscopic and morphometric analyses of the various late endocytic structures in Rat1 fibroblasts. Consistent with our previous analyses of NRK fibroblasts (Bright et al., 1997), in control cells greater

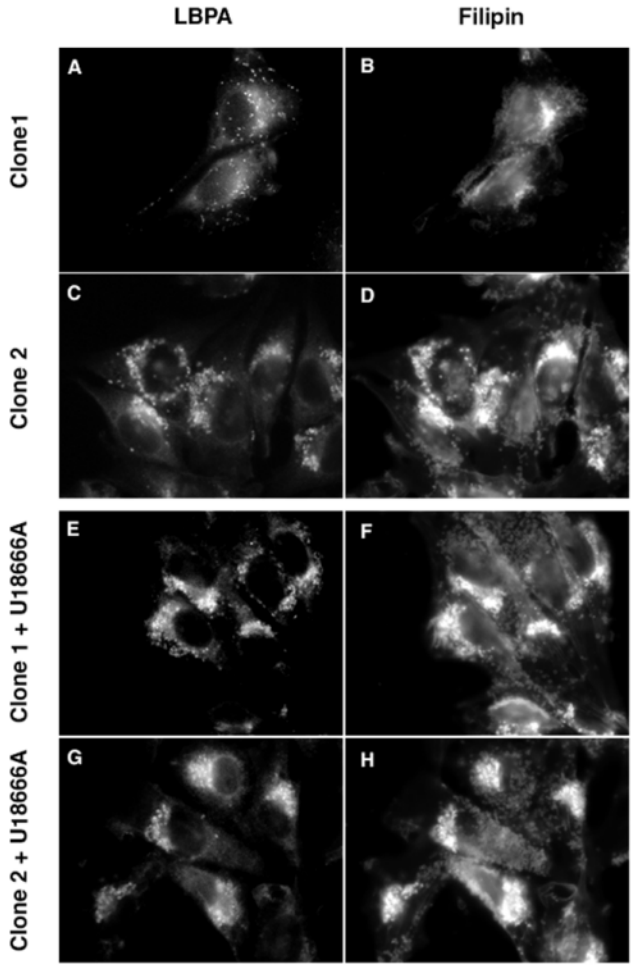
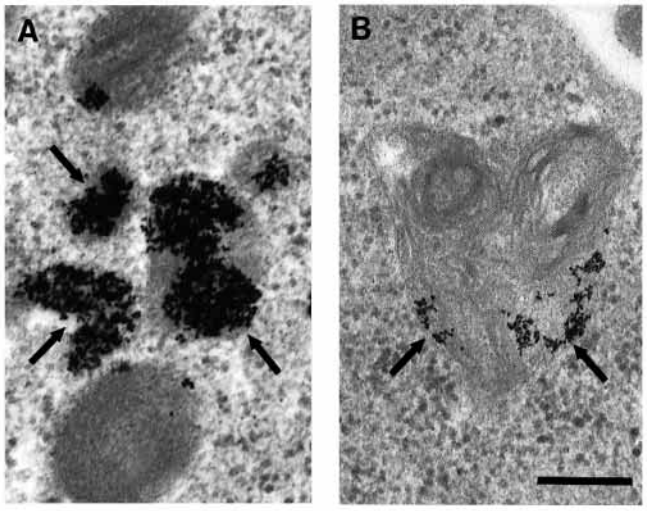
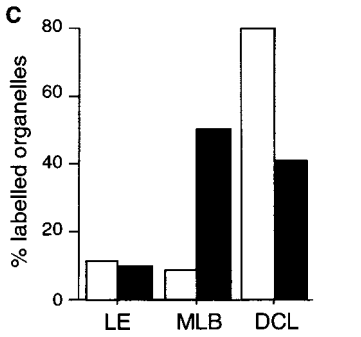
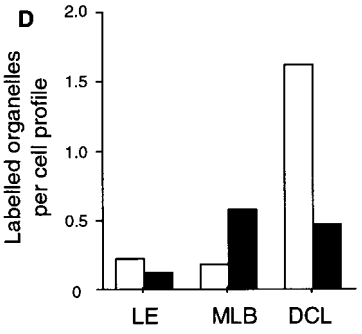


Fig. 5. LBPA and cholesterol localization in clones 1 and 2. (A,B,E,F) Clone 1 (a representative of Rat1 cell lines expressing ciM6PR) and (C,D,G,H) clone 2 cells were incubated in the presence or absence of 3 μg/ml U18666A, fixed with 2% paraformaldehyde in PBS, and processed for indirect immunofluorescence as described in Materials and Methods. LBPA was visualised using monoclonal 6C4 (Kobayashi et al., 1998b), and unesterified cholesterol with filipin.







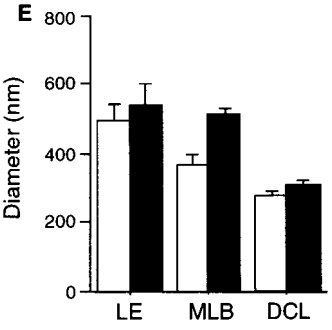
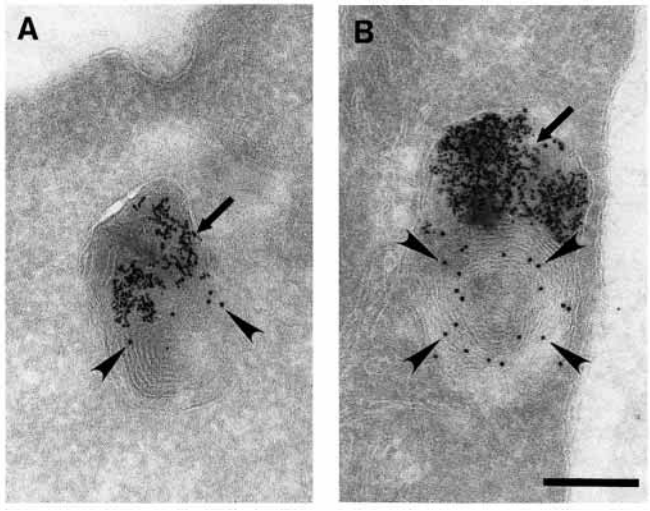


Fig. 6. Electron microscopy and morphometry. Clone 1 and clone 2 Rat1 fibroblasts were allowed to endocytose BSA-5 nm gold (A,B, arrows) and processed for electron microscopy as described in Materials and Methods.
(A,B) Representative thin-sections showing electron dense lysosomes and late endocytic structures in clone 1 (A) and clone 2 (B) cells. Bar, 200 nm.
(C-E) Quantitation from sections of clone 1 cells (open bars) or clone 2 cells (filled bars) of the proportion of late endosomes (LE), multilamellar bodies (MLB), and dense core lysosomes (DCL) labelled with BSA-gold (C), of the number of BSA-gold labelled structures per cell profile (D), and of the diameter of the labelled structures (E).



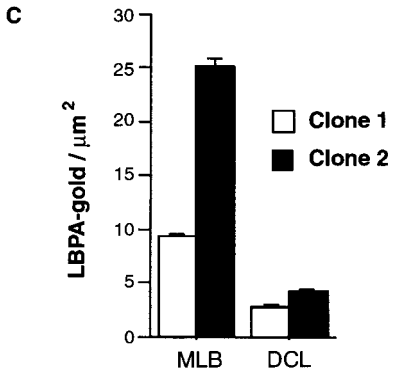


Fig. 7. Immunogold labelling of late endocytic structures. Clone 1 and clone 2 Rat1 fibroblasts were allowed to endocytose BSA-5 nm gold (A,B, arrows) and processed for immunoelectron microscopy as described in Materials and Methods. (A,B) Representative frozen thin sections immunolabelled with the late endosome/lysosome phospholipid marker LBPA (10 nm gold, arrowheads) from clone 1 (A) and clone 2 (B) cells. Bar, 200 nm. (C) Quantitation of the density of LBPA labelling of BSA-gold loaded late endocytic structures in clone 1 (open bars) or clone 2 (filled bars) cells.

than 80% of the organelles labelled by BSA-gold following a 4 hour pulse and 20 hour chase were DCLs (Fig. 6A and C). In contrast, the most abundant BSA-gold labelled structures in clone 2 cells were identified as MLBs (B and C). Quantitative analysis revealed that the average number of labelled organelles per cell profile was reduced by almost 50% in the mutant cell line (2.03 vs 1.16), but that the number of MLBs was increased almost 3-fold (D). Moreover, the mean diameter of these organelles was significantly greater in clone 2 cells as compared to the other cell lines (510±25 nm vs 370±30 nm; E). Together this resulted in the total cellular volume occupied by late endocytic organelles in clone 2 cells becoming at least 1.5 times greater than that of the other cell lines. Quantitative immuno-staining confirmed that the internal membranes of the MLBs contained the bulk of the cell associated LBPA, and showed that the aberrant structures present in clone 2 cells contained approximately 2.5-fold more LBPA than the equivalent structures in the control cells (Fig. 7).

LBPA accumulation can be reversed by re-expressing ciM6PRs

The data presented above suggests that the loss of ciM6PR expression is associated with a defect in DCL biogenesis, which leads to LBPA and cholesterol accumulation. To confirm

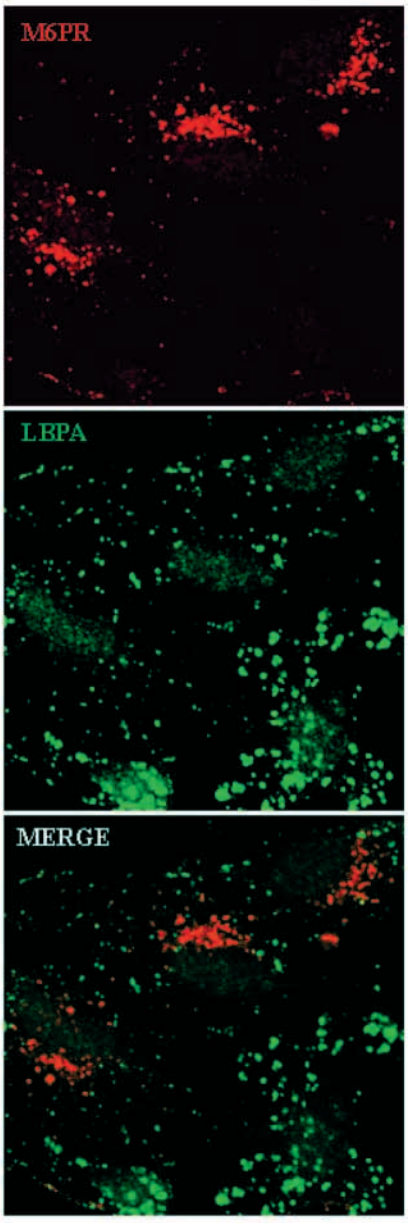


Fig. 8. Expression of bovine ciM6PR in clone 2 cells. Clone 2 cells were transfected with pSREP93-bovine ciM6PR as described in Materials and Methods. After 10 days selection with puromycin the cells were fixed in 2% paraformaldehyde and stained for ciM6PR using the polyclonal antiserum (which recognises an epitope conserved between the rat and bovine enzymes; top panel) and LBPA (centre panel). The merged image is shown in the lower panel.

that this is a direct effect we investigated whether it could be reversed by expressing the bovine ciM6PR. The data shown in Fig. 8 shows clearly that this is the case. In a mixed population of clone 2 cells transfected with cDNA encoding the bovine receptor, only those cells showing normal ciM6PR distributions showed a normal LBPA staining pattern (compare the LBPA staining in the upper 3 cells which are ciM6PR positive with that in the lower 3 cells which are negative). This eliminates the possibility that the ciM6PR loss was a consequence of the LBPA accumulation, or that they were unconnected events.

DISCUSSION

The results described above demonstrate that loss of ciM6PR expression in Rat1 fibroblasts causes the accumulation of abnormal MLBs. Loss of the receptor occurred spontaneously, and at present we are uncertain of the precise mechanism responsible. Since clone 2 cells were selected following transfection with a dominant negative form of rat Vps34p, our initial assumption was that loss of ciM6PR was directly attributable to expression of the mutant protein. However, closer examination provided abundant evidence that this was not the case. Loss of ciM6PR synthesis was not observed in numerous other clones from the same, or a subsequent, transfection, many of which expressed equal or greater basal levels of mutant Vps34p. Similarly, an equivalent phenotype to that expressed by clone 2 could not be obtained in any other clone by heavy metal ion mediated induction of the metallothionein promotor controlling expression of the mutant cDNA. Recent reports which utilised the alternative approach of anti-sense oligonucleotide treatment to disrupt Vps34p function indicated that loss of this enzymatic activity resulted in an inhibition of starvation-induced autophagy (Petiot et al., 2000), and perturbation of EEA1 localization (Siddhanta et al., 1998). Neither of these effects was manifest in uninduced clone 2 cells, which strongly suggests that the low levels of mutant Vps34p expressed in the absence of heavy metal ion mediated induction are insufficient to negate the endogenous wild-type protein expressed by the cells.

In addition to its role in lysosomal enzyme delivery, the 300kDa cation-independent M6PR (ciM6PR) also functions as an endocytic receptor for other ligands including insulin-like growth factor II, retanoic acid, and urokinase-type plasminogen activator receptor (reviewed by Braulke, 1999). The gene encoding the ciM6PR is imprinted, and in rodents, only the maternal allele is expressed in peripheral tissues (Mills et al., 1998). Thus loss of expression in clone 2 cells may simply have arisen through disruption of the single functional copy of the gene through direct integration of the transfected plasmid. Alternatively, since expression is controlled through differential methylation of elements within the maternal and paternal alleles, the transfection and/or selection procedure may have perturbed this process, leading to inappropriate silencing of the active gene in clone 2 cells. This explanation appears more consistent with the observation that ciM6PR expression is lost or greatly reduced in many human and mouse tissue culture cell lines (for example Goldberg et al., 1983; Wenk et al., 1991) and has been identified as a suppressor gene in several human tumours (Devi et al., 1999). Nevertheless, whatever the mechanism by which expression was lost, we considered the resultant morphological changes in the late endosomal organelles to be of sufficient interest to warrant further investigation. In particular we were intrigued by the possibility that the unusual structures we observed might be related to some of the inclusion bodies detected in LSDs.

LSD inclusion bodies contain material that would normally be digested or exported from lysosomes (Gieselmann, 1995). For example, the sphingolipidoses such as Gaucher's, Faber's, and Fabry's diseases all result in accumulation of ceramide derivatives, whilst Pompe's disease patients accumulate glycogen. Inclusion bodies accumulate endocytosed probes, indicating that they are derived from the late endocytic pathway, and in the most severe cases may represent up to 50% of the total cell volume (Karageorgos et al., 1997). However, their precise relationship to the remainder of the endocytic compartments remains unclear. Recently, analysis of cells

derived from several distinct sphingolipidoses revealed that in addition to the accumulation of products whose metabolism is impaired by the specific gene defect, there is also a common disorder in lipid handling characterised by the accumulation of un-esterified cholesterol and LBPA (Chen et al., 1999; Ridgway, 2000).

A similar defect in cholesterol and LBPA metabolism to that observed in sphingolipidoses was seen in clone 2 cells suggesting that a common mechanism might be involved. One trivial possibility is that the ciM6PR is exclusively required for the delivery of a key hydrolase to Rat1 endosomes, and that loss of the receptor caused aberrant secretion of this enzyme, and a consequent failure to degrade a key substrate. At present we cannot exclude this possibility, but consider it unlikely to be the correct explanation. Although the cdM6PR and ciM6PR show different affinities for individual lysosomal hydrolases, mouse fibroblasts lacking only one of the two receptors typically contain at least 50% of the wild-type levels of each enzyme (Pohlmann et al., 1995). The dominant feature in determining the relative affinities of the two receptors appears to be related to the number of phosphorylated oligosaccharides possessed by an individual enzyme, and the number of phosphate esters added to specific sugar chains, and to date no hydrolase whose trafficking is entirely dependent on the ciM6PR has been identified. Moreover, at least one key component of sphingolipid metabolism, prosaposin, can be retrieved from the extracellular medium by a non-M6P dependent route (Hiesberger et al., 1998).

An alternative explanation for the defect in clone 2 cells, which we favour, is that it is directly due to a perturbation of the balance of membrane flux within the endocytic pathway, resulting from the reduction of membrane recycling from late endosomes to the TGN. At present there is considerable debate concerning the organization and function of the late endosomal pathway. Traditional ultrastructural analysis following labelling with endocytosed probes typically reveals an heterogeneous population of vesicular structures clustered in the peri-nuclear region of the cell. Based upon the kinetics of labelling by internalised probes, the degree of electron density, the appearance of internal membranes, and the presence or absence of particular marker proteins, various organelles such as multi-vesicular bodies (MVBs), prelysosomal compartments (PLCs), MLBs, ELHs, and DCLs have been defined. However, in many cases there is significant variation between the structures seen in different cell types, and it remains difficult to extrapolate data between distinct tissues. Part of the apparent inconsistency can be reconciled by the realisation that the entire endocytic pathway is much more dynamic than has previously been recognised (Sönnichsen et al., 2000). Thus the variations between tissues at steady state may simply reflect differences in the net flux of membrane and cargo into and out of individual endocytic compartments, and the consequent accumulation of distinct intermediates in each cell type.

Studies on clathrin adaptor complex AP-1 binding to the TGN have indicated that it is directly influenced by the levels of M6PRs (Le Borgne and Hoflack, 1997), suggesting that the receptors are directly involved in nucleating transport vesicle formation. Although the precise mechanism by which ciM6PRs are retrieved from late endosomes is currently uncertain, it is likely that a coat complex is involved, and that recruitment and subsequent vesicle formation is at least partially dependent upon the presence of the cargo (Rohn et al., 2000). Thus loss of the ciM6PR might reduce the rate of recycling of material from the late endocytic pathway to the TGN. In this regard it should be noted that studies of the Niemann-Pick type C protein NPC1, which is implicated in the retrieval of cholesterol from late endocytic compartments, have revealed a close association with ciM6PRs. NPC1 also appears to cycle between endosomes and the TGN (Mukherjee and Maxfield, 1999), and cells expressing mutant NPC1 accumulate both cholesterol and ciM6PR in LBPA rich membranes (Kobayashi et al., 1999). This observation is consistent with the hypothesis that the trafficking of NPC1 and ciM6PR from late endosomal compartments are related events, even though they may not share the same transport vesicles, and that the loss of ciM6PRs might directly inhibit NPC1 transport, which would lead to the lipid accumulation which we observed.

Our results clearly indicate that trafficking of endocytosed material to DCLs is inhibited in clone 2 cells. However, at present we are uncertain as to the precise nature of the MLBs that accumulate in clone 2 cells, and hence the stage at which the block is occurring. Two distinct possibilities exist, the first of which is that it is fusion between endosomes and lysosomes which is inhibited. Consistent with this hypothesis is the observation of structures showing a distinct resemblance to those seen in clone 2 cells in human Hep2 cells during a study in which late endosome-lysosome fusion was prevented by ablation of the DCLs with HRP (Futter et al., 1996). Under the inhibitory conditions used by Futter and colleagues the pre-lysosomal organelles observed in Hep2 cells became swollen and accumulated internal membranes. This suggests that if fusion is inefficient, or occurs at a slower rate than the accumulation of LBPA, MLBs can arise from the maturation of MVBs. Such a hypothesis is consistent with the morphology of the PLCs seen in NRK cells (Griffiths et al., 1988). Moreover, our recent studies have highlighted the importance of the internal milieu of endosomes and lysosomes in the fusion event (Pryor et al., 2000), which might explain how accumulation of material within a pre-lysosomal compartment could prevent its subsequent fusion.

However, it should be noted that the MLBs in clone 2 were electron dense, consistent with their containing active lysosomal enzymes. This appears to be in contrast with the arrested structures detected in Hep2 cells, which were unable to degrade EGF-EGF receptor complexes (Futter et al., 1996). Although newly synthesized hydrolases delivered from the early endocytic pathway or TGN may become active in late endosomes, our previous studies have suggested that the acquisition of electron density is primarily a consequence of ELH formation (Bright et al., 1997). This suggests an alternative hypothesis in which the MLBs which accumulate in clone 2 cells are abnormal ELHs. In this regard it is interesting to note that DCLs morphologically similar to the structures observed in rat liver, appear to be present at particularly low levels, or absent, in many tissue culture cell lines, and that in such cells structures resembling MLBs are regarded as lysosomes (for example Kleijmeer et al., 1997). The acquisition of a dense-core is probably related to the balance between the generation of degraded material within ‘lysosomes,’ and the export of this material into the cytoplasm.

Our current model suggests that re-condensed ELHs can undergo multiple rounds of fusion (Luzio et al., 2000), which might lead to a gradual accumulation of electron dense material. Thus accumulation of material within ELHs in clone 2 cells might lower the efficiency of re-condensation, and consequently reduce the rate of maturation into DCLs. However, whatever the precise mechanism involved in the accumulation of the aberrant MLBs in clone 2 cells, our results clearly indicate a link in membrane trafficking events and storage disorders, and highlight the importance of trafficking within the late endocytic pathway in the development of the pathologies of LSDs.

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