INTRODUCTION

The endocytic pathway is composed of dynamic membrane processes. Via this pathway, cells take up various kinds of ligands such as nutrients, hormones and growth factors and deliver them to their correct destinations (Goldstein et al., 1985). Some of the molecules involved in the early endocytic pathways have been identified and, in certain cases, the molecular mechanisms of the involvement of the identified molecules have been well described (Gruenberg and Maxfield, 1995; Mellman, 1996; Mukherjee et al., 1997; Simonsen et al., 1998). However, the understanding of the late endocytic pathways has just begun to emerge (Bright et al., 1997; Futter et al., 1996; Mullock et al., 1998). A detailed description of how the late endocytic transport proceeds awaits further dissection of this pathway, and many of the constituents of the machinery of the late endocytic processes have yet to be discovered (Griffiths, 1996; Mullock et al., 1998; Storrie and Desjardins, 1996).

The isolation and characterization of mutant cells defective in membrane transport should be a powerful approach to identify both membrane intermediate structures and factors involved in various transport steps (Pryer et al., 1992). Since the endocytic pathway is at present best characterized both morphologically and biochemically in mammalian cells, mammalian mutant cells in this pathway should be especially

SUMMARY

Chinese hamster ovary cell mutants defective in the post-uptake degradation of low-density lipoprotein (LDL) in lysosomes were selected from mutagenized cells by novel three-step screening. First, in the presence of LDL, clones sensitive to an inhibitor of the rate-limiting enzyme of the cholesterol biosynthetic pathway, 3-hydroxy-3-methylglutaryl-CoA reductase, were isolated. Second, from the selected clones, those lacking in the degradation of a constituent of a fluorescent LDL were qualitatively screened by microscopy. Third, the clones were further screened by previously established quantitative analytical flow cytometry that detects the early-phase disintegration of LDL by lysosomal acid hydrolases. One of the isolated mutant clones, LEX1 (Lysosome-Endosome X 1), was a recessive mutant, and exhibited a specific disorder in the late endocytic pathway. LEX1 cells showed an unusual perinuclear aggregate of vesicles, heterogeneous positive for lysosomal glycoprotein-B/cathepsin D and rab7, yet negative for the cation-independent mannose 6-phosphate receptor. The aggregate was formed around the microtubule organizing center, and was disrupted by nocodazole treatment. Internalized octadecyl rhodamine B-labeled LDL (R18-LDL) was accumulated in the perinuclear rab7-positive vesicles. In a Percoll density gradient, neither internalized R18-LDL nor internalized horseradish peroxidase was efficiently chased into heavy lysosomal fractions positive for β-hexosaminidase. LEX1 cells showed differences in the activity and subcellular distribution of lysosomal enzymes. These characteristics of LEX1 cells are consistent with the ideas that the perinuclear vesicle aggregate is an arrested intermediate of direct fusion or divergence between lysosomes and rab7-positive, cation-independent mannose 6-phosphate receptor-negative late endosomes, and that equilibrium between the lysosomes and the late endosomes is shifted towards the late endosomes in LEX1 cells. Such fusion or divergence between the late endosomes and the lysosomes would determine an appropriate equilibrium between them, and might thereby play an important role for proper lysosomal digestive functions. LEX1 mutant cells would be helpful for the dissection of the as yet unrevealed details of the late endocytic membrane dynamics and for the identification of factors involved in the process arrested by the mutation.

Key words: Lysosome, Endosome, Mutant, Microtubule, rab7
useful in dissecting the endocytic traffic. Several mammalian mutant cells defective in the internalization step of endocytosis have been isolated and characterized (Krieger et al., 1985). In certain cases, molecules whose functions are related to the mutant phenotypes were identified using these mutants (Guo et al., 1994; Podos et al., 1994). In addition, some mammalian mutants showing defects in the later stages of the endocytic pathway and/or lysosomal biogenesis were reported (Colbaugh et al., 1989). A mutant selection method concerning specifically the early endocytic pathway has also been described: Cain et al. (1991) have established mutant Chinese hamster ovary (CHO) cells defective in the recycling pathway by isolating temperature-sensitive mutants which showed both the retention of a pulse of fluorescent transferrin, and the accumulation of a pinocytic marker, FITC-dextran, at a restrictive temperature. Analysis of such a mutant has suggested the link between the recycling pathway and the lysosomal biogenesis pathway (Wilson et al., 1993). In an analogous manner, by devising new strategies, the establishment of more mammalian mutant cells with specific defects in the late endocytic pathway should be of special importance to understand the details of the late endocytic processes.

In the present report, we describe a novel systematic three-step screening procedure for the isolation of mutant cells defective in intracellular endocytic processing. This method selects cells that endocytose low-density lipoprotein (LDL) but that fail to disintegrate endocytosed LDL in lysosomes. By this method, we established a recessive mutant, LEX1 (Lysosome-Endosome X 1), with a specific disorder in the late endocytic pathway. LEX1 cells had an unusual perinuclear aggregate of vesicles, heterogeneously positive for lysosomal glycoprotein-B (Igp-B)/cathepsin D and rab7, yet negative for the cation-independent mannose 6-phosphate receptor (CI-MPR). From the characteristics of LEX1 cells, we propose that the perinuclear vesicle aggregate is an arrested intermediate of direct fusion or divergence between lysosomes and rab7-positive, CI-MPR-negative late endosomes. Such fusion or divergence between the late endosomes and the lysosomes would determine an appropriate equilibrium between late endosomes and lysosomes, and might thereby play an important role for proper lysosomal digestive functions. LEX1 mutant cells would be helpful for the dissection of the as yet unrevealed details of the late endocytic digestive processes and for the identification of factors involved in the process arrested by the mutation.

MATERIALS AND METHODS

Materials

Sodium pravastatin was a generous gift from Dr Y. Tsujita (Sankyo, Tokyo/Japan). Human LDL was prepared by centrifugation (Goldstein et al., 1983). Lipoprotein deficient serum (LPDS) was prepared from fetal calf serum by centrifugation as described (Goldstein et al., 1983), except that thrombin incubation was omitted. Mouse monoclonal antihamster Igp-B was prepared by ammonium sulfate precipitation and Protein A affinity chromatography from culture supernatants of hybridoma 1B3 (Uthayakumar and Granger, 1995). The hybridoma was originally produced by Dr Sandra Schmid (The Scripps Research Institute), and was kindly provided to us by Dr Bruce Granger (Montana State University). Anti-CI-MPR antibody (Brown and Farquhar, 1987), anti-rab7 antibody produced as described previously (Chavrier et al., 1990), and anti-cathepsin D antibody were generous gifts from Dr William Brown (Cornell University), Dr Takashi Ueno (Juntendo University), and Dr Kazumi Ishidoh (Juntendo University), respectively. Affinity-purified rabbit anti-human LDL IgG was from Biomedlical Technologies Inc (Stoughton, MA/USA). Mouse monoclonal anti-human LDL IgG was from Progen Biotechnik Gmbh (Heidelberg/Germany). Horseradish peroxidase (HRP) (type II) was from Sigma-Aldrich (Tokyo/Japan).

Cell culture

CHO-K1 cells and derivatives were maintained as described previously (Ohashi et al., 1992). For optical microscopy, cells were plated on glass coverslips. For electron microscopy and immunoelectron microscopy, cells were plated on plastic coverslips (Celldisk LF1, Sumitomo Bakelite Co., Ltd, Tokyo/Japan).

Fluorescent LDLs

RET-LDL was prepared by the reconstitution method of Krieger et al. (1978) as described previously (Ohashi et al., 1992). (DiO, SRh)LDL, LDL double-labeled with 3,3’-diodotiacetyloxycarbocyanine (DiO) and N-(Texas Red sulfonyl)-1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine (SRh-PE), was similarly prepared by reconstituting 18 nmol DiO and 18 nmol SRh-PE (both in benzene) together with 6 mg unlabeled cholesteroy linoleate (in heptane) into heptane-extracted LDL (1.9 mg protein). DiO-LDL was prepared omitting SRh-PE. Octadecyl rhodamine B (R18)-labeled LDL (R18-LDL) was prepared as described previously (Ohashi et al., 1992). Specific uptake of these LDLs via the LDL receptor was confirmed as described previously (Ohashi et al., 1992).

Flow cytometry

Flow cytometry was done as described previously (Ohashi et al., 1992). The procedure eliminates the cell-surface-bound LDL. The lysosomal disintegration of RET-LDL was quantified and expressed as two parameters, r and N(1-r), as described previously (Ohashi et al., 1992). Uptake of DiO-LDL was monitored by 530 nm fluorescence measurement.

Isolation of LEX1 cells

Mutagenesis

CHO-K1 cells (subcloned) were plated (2x10^5/75 cm^2 bottle), cultured for two days, and then mutagenized with 400 µg/ml ethyl methanesulfonate in culture medium for 16 hours at 37°C. After washing three times with culture medium, the cells were further incubated in culture medium for one day and were stored frozen.

First screening

Variant cells defective in LDL-derived cholesterol metabolism were isolated as cells sensitive to pravastatin, an inhibitor of the rate-limiting enzyme of the cholesterol biosynthetic pathway, 3-hydroxy-3-methylglutaryl-CoA reductase from the mutated CHO cells. Cholesterol is indispensable for cell growth, and is mainly supplied by the biosynthetic pathway and by endocytosis of LDL (Goldstein et al., 1985). In the presence of pravastatin, cells depend solely upon LDL-derived cholesterol. In this situation, cells defective in the LDL metabolism should not grow even in the presence of LDL. Such defective cells were selected by negative selection using 5-bromodeoxyuridine (BrDU) (Kao and Puck, 1974). Specifically, the frozen mutagenized cells were thawed, cultured at 37°C for two days and then plated at 5x10^5 cells/100 mm dish. These cells were incubated in a selection medium (thymidine-deficient Ham’s F12 medium containing 1 mg/ml sodium pravastatin, 10 µg/ml LDL, 0.25 mM mevalonic acid and 5% LPDS) at 37°C for two days during which cells sensitive to pravastatin cease growth. Then 10^-5 M (final) BrDU was added to the selection medium and the incubation was further continued for one day. The cells were then washed extensively and
were, in 10 ml Hanks–Hepes (Hanks’ balanced salt solution, 5 mM Hepes, pH 7.3) per dish, illuminated with intense fluorescent light to kill cells which had incorporated BrdU (Kao and Puck, 1974). The surviving cells were allowed to form colonies for seven days in thymidine-deficient F12 medium containing 10% fetal calf serum, dialyzed against 0.9% NaCl. Four colonies were isolated from five to 10 formed colonies per dish using cloning rings.

Second screening

The variant cells isolated by the first screening should contain cells lacking the LDL receptor activity and those with defects in the post-lysosomal metabolism of LDL-derived cholesterol, in addition to those defective in the post-uptake lysosomal degradation of LDL. To eliminate the former two variants, cells which maintain the ability to endocytose but fail to degrade LDL were qualitatively selected from the pravastatin-sensitive cells by microscopy using (DiO, SRh)LDL. The cells were allowed to endocytose (DiO, SRh)LDL (10 μg/ml) in 5% LPDS/F12 for 10 minutes, then chased for 90 minutes, at 37°C. They were then washed on ice, fixed for two hours with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) on ice, ice, fixed, and mounted, and observed under a Zeiss IM-35 fluorescent microscope. Conventional fluorescent and rhodamine filter sets were used to observe DiO and SRh, respectively. Cells that showed colocalized particulate staining of DiO and SRh, indicating substantial uptake of LDL but slower degradation of SRh-PE than wild-type cells, were selected and stored.

Third screening

The selected cells were quantitatively analyzed for their ability to endocytose and to degrade LDL by flow cytometry using RET-LDL in a pulse (10 minutes)-chase (20 minutes) experiment as described previously (Ohashi et al., 1992). Variant cells that showed a similar level of endocytosis to that of wild-type cells, but showed less RET-LDL disintegration, were selected. One clone showing stable expresion to that of wild-type cells, but showed less RET-LDL disintegration, were selected. One clone showing stable endocytosis and to degrade LDL by flow cytometry using RET-LDL was allowed to endocytose (DiO, SRh)LDL (10 μg/ml) in 5% LPDS/F12 for 10 minutes, then chased for 90 minutes, at 37°C. They were then washed, blocked with 0.1% gelatin in PBS. The samples were mounted in SlowFade Light (Molecular Probes) and were observed under a Carl Zeiss LSM 310 or 510 laser scanning microscope equipped with a PlanApo ×60 objective with an optical section set at <0.8 μm.

Immunofluorescence

For immunolocalization of hamster IgG-B, CI-MPR, human LDL, and cathepsin D, cells were fixed with 3% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for 20 minutes and were then permeabilized with 50 μg/ml digitonin in PBS for 10 minutes. Alternatively, when rab7 staining was included, cells were permeabilized with 50 μg/ml digitonin in 80 mM PIPES-K (pH 6.8), 5 mM EGTA, 1 mM MgCl2 for 5 minutes, fixed with 3% paraformaldehyde in PBS for 15 minutes, and treated with 50 μg/ml digitonin in PBS for 5 minutes followed by 50 μM NH4Cl/PBS for 10 minutes. These treated cells were washed, blocked with 0.1% gelatin in PBS for 20 minutes, and processed for indirect immunofluorescence using corresponding primary and secondary antibodies in 0.1% gelatin in PBS. The samples were mounted in SlowFade Light (Molecular Probes) and were observed under a Carl Zeiss LSM 310 or 510 laser scanning microscope equipped with a PlanApo ×60 objective with an optical section set at <0.8 μm.

Dominance test

Introduction of selectable dominant markers for cell-cell fusion

Variants of wild-type and LEX1 cells resistant to hygromycin B and blastcidin S (Funakoshi, Tokyo/Japan) were prepared by transfecting the cells with pSv2ph (Santerre et al., 1984) and pSV2bx (Izumi et al., 1991), respectively, using LipofectAMINE (Life Technologies, Inc., Rockville, MD/USA) according to the manufacturer’s instructions. The cells were screened for stably transfected cells with an appropriate selection medium (250 μg/ml hygromycin B or 5 μg/ml blastcidin S in growth medium).

Cell-cell fusion

Two kinds of cells to be fused were plated together (each at 2.5-3×10⁵ cells) in a 35 mm dish, and were cultured for 24 hours. After thoroughly removing the growth medium, the cells were treated with 1 ml of F12 medium containing 45% (w/w) polyethylene glycol and incubated at room temperature for one minute with gentle swirling (Sato et al., 1982). After washing four times with F12 medium, the cells were cultured at 37°C for 8 hours in growth medium and replated in a 100 mm dish in selection medium for hybrids (medium containing both hygromycin B and blastcidin S), and were cultured until negative controls (fusions between cells with an identical selectable marker) died away. The resultant hybridomas were analyzed for IgP-B localization by immunofluorescence and for RET-LDL degradation by flow cytometry.

Semi-quantification of colocalization in double fluorescence microscopy

A parameter, Col(A/B), indicating localization of fluorescence A within structures positive for fluorescence B in a cell, was defined as the percentage of fluorescence A included in region B, the region of structures positive for fluorescence B. Region B was created, using the Photoshop program, from fluorescence B image data by selecting areas showing fluorescence above background level within a cell profile. The background fluorescence level was unintentionally chosen, as the value limiting a group of pixels exhibiting the lowest continuous fluorescence in the Photoshop program. The calculation was done within each cell profile on the cell by cell basis using the NIH Image program.

Electron microscopy

For conventional electron microscopy, cells were fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer for one hour. The cells were washed in the same buffer for five minutes three times, and were post-fixed in 1% OsO4 in 0.1 M cacodylate buffer for 1 hour. After washing in distilled water, the cells were incubated with 50% ethanol for 10 minutes, and were block stained with 2% uranyl acetate in 70% ethanol for two hours. The cells were washed and dehydrated with a series of ethanol, and were embedded in epoxy resin. Ultra-thin sections were double stained with uranyl acetate and lead citrate, and were observed under a Hitachi H7000 electron microscope (Hitachi, Tokyo/Japan).

Immunoelectron microscopy

The pre-embedding silver enhancement immunogold method was performed as described by Mandai et al. (1997) with a slight modification. Cells were fixed in 4% paraformaldehyde and 0.1% glutaraldehyde in 0.1 M cacodylate buffer for 15 minutes. The cells were washed in the buffer for five minutes three times, and were incubated in Na-phosphate buffer (pH 7.4, PB) containing 0.25% saponin and 5% bovine serum albumin (BSA) for 30 minutes, and then for another 30 minutes with blocking solution (PB containing 0.005% saponin and 10% BSA, 10% normal goat serum, and 0.1% cold water fish skin gelatin) (Hartmann et al., 1997). The cells were then reacted with rabbit anti-rab7 antisera or mouse monoclonal antibody against IgG-B in the blocking solution overnight. Then, the cells were washed in PB containing 0.005% saponin for 10 minutes six times, and were incubated with goat anti-rabbit IgG or anti-mouse IgG conjugated to colloidal gold (1.4 nm in diameter, Nanogold, Nanoprobes, Stony Brook, NY) in the blocking solution for 2 hours. The cells were washed with PB for 10 minutes six times, and were fixated with 1% glutaraldehyde in PB for 10 minutes. After washing, the gold labeling was intensified using a silver enhancement kit (HQ silver, Nanoprobes) for 7.5 minutes at room temperature in the dark. After washing in distilled water, the cells were post-fixed with 0.5% OsO4 for 90 minutes at 4°C, washed in distilled water, incubated with 50% ethanol for 10 minutes, and block stained with 2% uranyl acetate in 70% ethanol for two hours. The cells were further dehydrated with a graded series of ethanol, and were embedded in epoxy resin. Ultra-thin sections were double stained with uranyl acetate and lead citrate.
Other methods
LDL uptake experiments were performed as described previously (Ohashi et al., 1992). Post-nuclear supernatants of cells were prepared and subjected to 20% isosmotic Percoll gradient fractionation as described previously (Ohashi and Ohnishi, 1991). Lysoosomal enzymes were assayed as described by Merton and Sly (1983). α-Mannosidase was assayed at pH 5.4 as described by Pool et al. (1983). HRP was assayed as described elsewhere (Ohashi and Ohnishi, 1991). The amount of R18 in Percoll gradients was determined by fluorometry as described elsewhere (Ohashi et al., 1992).

RESULTS

Disintegration of endocytosed RET-LDL was impaired in LEX1 cells
LEX1 mutant cells were established through three-step screening procedures as described in Materials and Methods. The endocytic uptake and the time-course of the lysosomal disintegration of LDL in LEX1 cells were quantitatively examined by flow cytometry using RET-LDL. LEX1 cells exhibited an about 50% higher uptake of RET-LDL than wild-type cells during a 10 minute pulse uptake period (Fig. 1A). In contrast, markedly less disintegration of RET-LDL was observed in LEX1 cells over a 30 minute chase period (Fig. 1B,C).

Spatial distribution of late endosomes and lysosomes was altered in LEX1 cells
To investigate morphology of the endocytic pathway in LEX1 cells, the distributions of various markers of the endocytic compartments were examined by immunofluorescence microscopy. Dramatic changes in the distribution of a late endosome/lysosome marker, lgp-B (Chen et al., 1985; Lewis et al., 1985), a late endosome marker, rab7 (Chavrier et al., 1990), and a lysosomal enzyme, cathepsin D, were observed. In wild-type cells, the majority of lgp-B, rab7 and cathepsin D were observed in a scattered particulate pattern (Fig. 2A,C, and data not shown). In LEX1 cells, these marker proteins were observed as aggregated dots at a perinuclear position (Figs 2B,D, 3B). This perinuclear nature was microtubule dependent, since both the lgp-B- and rab7-positive perinuclear structures were dispersed by nocodazole treatment and became indistinguishable from those observed in wild-type cells treated with nocodazole in the same manner (Fig. 2E,F, and data not shown). By staining with an acidotropic dye, Acridine Orange (Allison and Young, 1969), these perinuclear vesicles were also implicated to be acidic; in wild-type cells, Acridine Orange stained dots scattered in the cytoplasm; however, it stained perinuclearly aggregated dots in LEX1 cells (data not shown).

The distributions of other membrane markers such as the transferrin receptor (TfR) for early endosomes (Yamashiro et al., 1984), CI-MPR for late endosomes and other compartments involved in transport between the trans-Golgi network and endosomes (Griffiths et al., 1988), or BODIPY FL C5-ceramide for the trans-Golgi network (Pagano et al., 1991) did not show any recognizable difference between LEX1 and wild-type cells (data not shown).

The perinuclear aggregate in LEX1 cells was heterogeneously stained for lgp-B and rab7. The perinuclear dots positive for rab7 were also positive for lgp-B (Fig. 3A). However, some lgp-B-positive perinuclear dots were negative for rab7 (Fig. 3A). On the other hand, cathepsin D was mostly colocalized with lgp-B (Fig. 3B).

Although the CI-MPR-positive compartments also showed a perinuclear distribution, the CI-MPR staining hardly colocalized with the aggregated lgp-B in LEX1 cells (Fig. 3C). Rather, we noticed that the lgp-B-positive aggregate tended to avoid close contact with the CI-MPR-positive compartments. In addition, no codistribution of the perinuclear cathepsin D with TfR was observed in LEX1 cells (data not shown).

In electron micrographs, unusual aggregation of vesicles of heterogeneous appearance was observed around the microtubule organizing center (MTOC) in LEX1 cells (Fig. 4A,B). These vesicles were of heterogeneous size, some being of normal size as observed in wild-type cells, while others looking larger (Fig. 4A,B). They also showed heterogeneous contents with some containing filamentous material, others containing particulate material, and still others both filamentous and particulate materials (Fig. 4B). Multivesicular bodies were occasionally found in the aggregate (Fig. 4B).

![Fig. 1.](attachment:image) Disintegration of endocytosed RET-LDL was impaired in LEX1 cells, (A) RET-LDL uptake during a 10 minute pulse. Wild-type cells (WT) and LEX1 cells (LEX1) endocytosed 10 μg/ml RET-LDL for 10 minutes at 37°C. The cells were then subjected to dual-parameter flow cytometry. Five thousand cells were counted for each determination. The value N(1−r), indicating the mean amount of intact RET-LDL within a cell, was calculated from dual-parameter histograms. The value of LEX1 cells is expressed as percentage of that of wild-type cells. The mean ± s.d. (n=4) is shown.

(B,C) Time course of RET-LDL disintegration. Wild-type cells (WT) and LEX1 cells (LEX1) were incubated with 10 μg/ml RET-LDL for 10 minutes at 37°C. After washing the cells, chase incubations were performed at 37°C for various times as indicated. The cells were then subjected to dual-parameter flow cytometry. Five thousand cells were counted for each determination. Values r, indicating the extent of disintegration of RET-LDL within a cell (B), and N(1−r), indicating the mean amount of intact RET-LDL within a cell (C), were calculated from dual-parameter histograms. In C, values are expressed as percentages of the value at time zero of chase. The mean of two experiments is shown.
Images showing heterogeneous vesicles in close contact with each other were frequently observed (Fig. 4B). Other organelles such as mitochondria, endoplasmic reticulum, and the Golgi apparatus were fairly absent from the aggregate (Fig. 4B). In addition, these non-endocytic organelles, and other structures such as coated pits showed normal morphology in LEX1 cells (Fig. 4B and data not shown). Immunoelectron microscopy showed that these aggregated vesicles were positive for lgp-B with varying strength (Fig. 4C). They were also positive for rab7 with more distally distributed staining (Fig. 4D). These staining patterns make a good agreement with the immunofluorescence observations and strongly suggest that, in LEX1 cells, heterogeneous lgp-B-positive vesicles were gathered around the MTOC to form a vesicle aggregate.

LEX1 is a recessive mutant

We performed dominance tests with LEX1 cells. The phenotypic differences between homotypically fused wild-type cells and homotypically fused LEX1 cells (Fig. 5, WThph × WTbsr, and LEX1hph × LEX1bsr, respectively) were essentially identical to the differences between their parental wild-type and LEX1 cells (Fig. 2A,B and data not shown). LEX1-wild-type hybrids (Fig. 5, WThph × LEX1bsr, and LEX1hph × WTbsr) exhibited the wild-type phenotypes.
similar to those shown by homotypically fused wild-type cells (Fig. 5, WThph × WTbsr), both in the RET-LDL flow cytometry (Fig. 5A) and in the morphological analysis for the distribution of lgp-B (Fig. 5B). Essentially identical results were obtained using another set of selectable dominant markers (bsr and neo) for cell fusion (data not shown). These results show that LEX1 cells are recessive to wild-type cells.

R18-LDL was accumulated in the perinuclear rab7-positive vesicles in LEX1 cells
To investigate to which intracellular compartment LDL is delivered in LEX1 cells, the transport of R18-LDL was examined by fluorescence microscopy. In wild-type cells, R18-LDL was observed in scattered particulate patterns at any time point of chase (Ohashi et al., 1992). In LEX1 cells, a similar particulate pattern was observed after a short period of chase (Fig. 6A,B). However, at about 30 minutes of chase, R18-LDL started to show a distinct perinuclear pattern, which became more evident after longer chases (45 minutes) (Fig. 6C-E). At 45 minutes chase, this perinuclear distribution of R18-LDL was substantially colocalized with rab7 (Fig. 6F), whereas in wild-type cells, most R18-LDL was not colocalized with rab7.

Fig. 4. Electron microscopic analysis of LEX1 cells. (A,B) Conventional electron micrographs of a wild-type cell (A) and a LEX1 cell (B). Aggregation of vesicles of heterogeneous appearance was observed around the MTOC (mt) in LEX1 cells (B), but not in wild-type cells (A). Arrowheads in B indicate vesicles in close contact with each other. The arrow, the open arrow, and the open arrowhead in B indicate vesicles containing filamentous material, particulate material, and both filamentous and particulate materials, respectively. cp: coated pit, g: Golgi apparatus. mb: multivesicular body, mt: MTOC, n: nucleus. Bar, 500 nm. (C,D) Silver enhancement immunogold localization of lgp-B (C) and rab7 (D) in LEX1 cells. Arrowheads indicate aggregation of vesicles. v: vacuole, m: mitochondria, n: nucleus. Bar, 500 nm.
The enhanced colocalization of R18-LDL with rab7 in LEX1 cells was found statistically significant by image quantification (Fig. 7). In addition, at 45 minutes chase, much of R18-LDL was localized within the perinuclear lgp-B-positive vesicles in LEX1 cells; however, the perinuclear R18-LDL-positive structures constituted only part of the lgp-B-positive vesicles (Fig. 6H). In contrast, in wild-type cells, R18-LDL showed nearly identical distribution to lgp-B at 45 minutes chase (data not shown). We did not recognize any changes in the colocalization extent of R18-LDL with lgp-B or lgp-B.

Fig. 5. LEX1 mutant is recessive to wild-type cells. Wild-type cells and LEX1 cells resistant to hygromycin B (WTph and LEX1ph, respectively), or to blastcidin S (WTbsr and LEX1bsr), were fused in combinations indicated in each panel as described in Materials and Methods. (A) The obtained fused cells were analyzed by flow cytometry using RET-LDL. Three thousand cells were analyzed for each determination and results are shown as dual parameter histograms in contour expression. (B) The obtained fused cells were analyzed by immunofluorescence for lgp-B localization. A cell profile is drawn in each panel. Bar, 10 μm.
rab7 at longer chase periods (60, 90 minutes) in LEX1 cells (Fig. 7 and data not shown).

The perinuclear distribution of R18-LDL was clearly distinct from the distributions of the other markers mentioned in the previous section. CI-MPR was in the neighborhood of R18-LDL after 45 minutes of endocytosis, but their colocalization was hardly seen (data not shown). At earlier time points (about 10-15 minutes of chase), R18-LDL and CI-MPR were found colocalized, yet only partially, in LEX1 cells (data not shown). The perinuclear TfR staining was close to, yet did not show any colocalization with perinuclear R18-LDL after 45 minutes of endocytosis (data not shown).

Antigenicity of endocytosed LDL was lost in the perinuclearly aggregated vesicles in LEX1 cells

We also immunolocalized endocytosed LDL in LEX1 cells. R18 fluorescence derived from R18-LDL (LDL-derived R18) stays in lysosomes even at a chase time when LDL is expected to be mostly degraded (Ohashi et al., 1992). Thus the R18 fluorescence observed represents the structures where either undegraded R18-LDL or R18 released from degraded R18-LDL resides. In contrast, the distribution of immunoreactive LDL should represent compartments where LDL retains its antigenicity. As chase time progressed, the intensity of immunofluorescent LDL became weaker in wild-type cells, and to a lesser extent in LEX1 cells (data not shown). After 45 minutes of uptake in LEX1 cells, some immunofluorescent LDL was observed at the perinuclear region, but it did not very much colocalize with rab7 (Fig. 6I) or lgp-B (data not shown).

In addition, the perinuclear immunoreactive LDL constituted only a minor population of compartments positive for R18 derived from R18-LDL endocytosed simultaneously (data not shown). These observations suggest that, in the perinuclearly aggregated compartments in LEX1 cells, the antigenicity of endocytosed LDL was already lost.

LEX1 cells had differences in the activity and subcellular distribution of lysosomal enzymes

The specific activities of several lysosomal enzymes in LEX1 cells were determined, and were compared with those in wild-type cells (Fig. 8A). The activities of two enzymes (β-hexosaminidase and acid phosphatase) in LEX1 cells were similar to wild-type cells (Fig. 8A). However, the activities of two other enzymes (β-galactosidase and β-glucuronidase) were markedly less in LEX1 cells (Fig. 8A). On the other hand, the activity of a Golgi marker enzyme, α-mannosidase, did not show any significant difference (data not shown).

Although the specific activities of β-hexosaminidase and acid phosphatase in LEX1 cells were normal, the intracellular distribution of these two enzymes in LEX1 cells over a Percoll gradient was not exactly the same as in wild-type cells (Fig. 8B-E). While β-hexosaminidase was concentrated in the characteristic heavy lysosomal fractions in both LEX1 and wild-type cells (Fig. 8B,C, bars), the recovery in these heavy fractions (25% from the bottom) of this enzyme in LEX1 cells was significantly lower than in wild-type cells (mean value 54% of the wild type from 6 independent experiments similar to that shown in Fig. 8B,C; P<0.001 by t-test). The same was
The case with acid phosphatase; this enzyme showed a biphasic distribution in wild-type cells with the lighter peak codistributing better with Golgi α-mananosidase than with pinocytic HRP (Fig. 8E); the recovery of this enzyme in the heaviest 25% fractions (Fig. 8D,E, bars) in LEX1 cells was 60% of that in wild-type cells (n=6, P<0.02).

Endocytosed R18-LDL and HRP were not efficiently chased into heavy lysosomal fractions in LEX1 cells

The distribution of endocytosed R18-LDL and HRP (a pinocytic marker) was investigated by subcellular fractionation using Percoll to assess endocytic processing. As previously observed (Ohashi et al., 1992), in wild-type cells, a substantial amount of LDL-derived R18 was chased into the heavy lysosomal fractions codistributing with β-hexosaminidase activity during the chase period at between 15 and 30 minutes (Fig. 9A,C). In contrast, the accumulation of R18 in the heavy fractions was less obvious in LEX1 cells during this chase period (Fig. 9B,D). Also in an HRP pulse (60 minutes)-chase (60 minutes) experiment, the recovery of HRP activity in the heavy lysosomal fractions was substantially lower in LEX1 cells (Fig. 9E). These results indicate that the endosome-lysosome processing of both LDL and HRP is perturbed in LEX1 cells.

The recycling pathway to the plasma membrane in LEX1 cells was normal

The recycling pathway of the LDL receptor in LEX1 cells was assessed by measuring the pulse uptake (10 minutes) of DiO-LDL after the uptake of unlabeled LDL for various times (Fig. 10). If recycling of LDL receptors to the plasma membrane is impaired, the uptake of unlabeled LDL would cause less internalization of DiO-LDL due to the intracellular trapping of LDL receptors coendocytosed with unlabeled LDL. However, no such reduction in DiO-LDL internalization was observed in LEX1 cells (Fig. 10, LEX1). The same was observed in wild-type cells (Fig. 10, WT). When similar experiments were done in the presence of monensin, known to interrupt the recycling pathway of the LDL receptor in human fibroblasts (Basu et al., 1981), the pulse uptake of DiO-LDL was reduced both in LEX1 cells and in wild-type cells (Fig. 10, WTmon and LEX1mon). This observation indicates that monensin interrupts LDL receptor recycling in LEX1 cells as effectively as in wild-type cells. These results show the normal recycling property of LDL receptors in LEX1 cells.

The recycling pathway of transferrin in LEX1 cells was assessed by indirect immunofluorescence staining of human transferrin in pulse-chase experiments, and was found to be indistinguishable from that in wild-type cells (data not shown). LDL is internalized together with transferrin and subsequently sorted from recycling transferrin in CHO cells (Mayor et al., 1993). When simultaneous endocytosis of LDL and transferrin was performed and both were immunolocalized, the staining pattern of transferrin after a 10 minute pulse uptake was distinct from that of LDL in both LEX1 and wild-type cells (data not shown). These observations indicate that transferrin diverges from the lysosomal pathway and recycles back to the plasma membrane normally in LEX1 cells.

DISCUSSION

We have devised a systematic three-step screening procedure to specifically isolate mutant cells defective in the intracellular degradation of endocytosed LDL based on a novel rationale. By this screening method, a constitutive mutant cell line, LEX1, was selected and established.

Specific late endosome/lysosome aggregation formed on microtubules in LEX1 cells

One prominent characteristic of LEX1 cells was the
perinuclear coaggregate of heterogeneous vesicles formed around the MTOC. The effect of nocodazole suggests that the aggregate is formed along microtubules. At least two types of vesicles were identified in the aggregate. One population consisted of the perinuclear vesicles positive for both lgp-B and cathepsin D, yet negative for rab7, CI-MPR, or TfR. These vesicles clearly represent conventional lysosomes, positive for both lysosomal enzymes and lysosomal glycoproteins, and negative for late or early endosomal markers (Kornfeld and Mellman, 1989; Mellman, 1996; Mukherjee et al., 1997). The other population was positive for rab7 and lgp-B, yet negative for TfR, suggesting that this population represents a late endocytic compartment (Mellman, 1996; Mukherjee et al., 1997). Interestingly, the rab7-positive late endosome population in LEX1 cells was fairly negative for CI-MPR, another late endosome marker, expected to be present also in the late endosomes together with rab7 (Chavrier et al., 1990; Mellman, 1996; Mukherjee et al., 1997). Despite the absence of CI-MPR, these vesicles are likely to be late endocytic compartments with digestive activities, since the rab7-positive aggregated vesicles seemed to be hydrolytic at least to some extent as observed by immunofluorescence for LDL.

Furthermore, the rab7-positive vesicles accumulated R18 derived from endocytosed LDL starting at about 30 minutes chase, suggesting that the rab7-positive aggregated vesicles are also kinetically late. Taken together, LEX1 cells seem to exhibit an intermediate complex showing arrested interaction between the lysosomes and the rab7-positive, CI-MPR-negative late endosomes (LEX1 late endosomes) that are in close contact with each other along on microtubules around the MTOC. The association of these vesicles is likely to be fairly specific, since the aggregated vesicles seemed to avoid contacts with other possible nonspecific targets such as the Golgi and early endosomes.

Although the accumulation of such an intermediate complex in LEX1 cells is a novel observation, the accumulation of CI-MPR-negative late endosomes interacting with lysosomes is with precedence. In HEp-2 cells, late-endocytic mature multivesicular bodies, negative for CI-MPR, were observed in direct contact with lysosomes containing preactivated HRP/diaminobenzidine (Futter et al., 1996). It has recently been shown that CI-MPR enters and rapidly leaves multivesicular endosomes without accumulating in a prelysosomal compartment in HEp-2 cells (Hirst et al., 1998).
Thus the colocalization of rab7 and CI-MPR in certain cells could merely be a kinetic coincidence. In this view, it is possible that the rab7-positive vesicles in LEX1 cells are late endosomes matured from CI-MPR-positive endosomes by removal of CI-MPR containing vesicles. In agreement with this, R18-LDL appeared to be best (but only partially) colocalized with CI-MPR at 10 to 15 minutes of chase in LEX1 cells, and accumulated in the vesicle aggregate thereafter. The CI-MPR-positive endosomes in LEX1 cells seem to be equivalent to the wild-type CI-MPR-positive endosomes en route to the lysosomes, since no recognizable abnormality in CI-MPR distribution was observed in LEX1 cells and since, also in wild-type cells, R18-LDL appeared to be best (but only partially) colocalized with CI-MPR at 10 to 15 minutes of chase (I. Miwako et al., unpublished observations).

Equilibrium is shifted towards the late endosomes from the lysosomes in LEX1 cells

Lysosomes are highly dynamic structures, thought to undergo fusion with endosomes as well as with other lysosomes (Deng et al., 1991; Deng and Storrie, 1988; Ferris et al., 1987; Ward et al., 1997). Complete intermixing of contents and membrane of lysosomes originating from different cells was observed in a heterokaryon (Deng et al., 1991; Deng and Storrie, 1988; Ferris et al., 1987). Based on such exchange and overlapping distribution of both membrane and contents between late endosomes and lysosomes, it has been suggested that there is a dynamic ‘equilibrium’ between late endosomes and lysosomes (Griffiths, 1996).

In LEX1 cells, intermixing among late endosomes and lysosomes seemed to be defective. LDL-derived R18 was accumulated in the rab7-positive perinuclear late endosomes, and was partially colocalized with lgp-positive vesicles at 45 minutes at 37°C, and were chased for 60 minutes at 37°C. The post-nuclear supernatants were prepared from the cells and subjected to 20% isoosmotic Percoll fractionation. The distribution of R18 over the gradient was determined (closed circles). The gradient density is shown in broken lines. Bars in C and D indicate the heavy lysosomal fractions. (E) LEX1 cells and wild-type cells endocytosed 1 mg/ml HRP for 60 minutes at 37°C, and were chased for 60 minutes at 37°C. The post-nuclear supernatants were prepared from the cells and were subjected to 20% isoosmotic Percoll gradient fractionation. The distribution of HRP over the gradients in LEX1 cells (open circles) and in wild-type cells (closed circles) was determined. The gradient density is shown in a broken line. The bar indicates the heavy lysosomal fractions.
flow cytometry and expressed as percentage of that at preincubation (WTmon). The amount of endocytosed DiO-LDL was determined by monensin with LEX1 cells (LEX1mon) and wild-type cells (WT) endocytosed 10 μg/ml unlabeled LDL for various times as indicated (Preincubation time). The cells were then washed with ice-cold Hanks-Hepes, and received prewarmed (37°C) LPDS/F12 containing 10 μg/ml DiO-LDL and were incubated for 10 minutes at 37°C. The same treatment was done in the presence of 25 mM monensin with LEX1 cells (LEX1mon) and wild-type cells (WTmon). The amount of endocytosed DiO-LDL was determined by flow cytometry and expressed as percentage of that at preincubation time zero.

Fig. 10. LDL receptors recycled back to the plasma membrane normally in LEX1 cells. LEX1 cells (LEX1) and wild-type cells (WT) endocytosed 10 μg/ml unlabeled LDL for various times as indicated (Preincubation time). The cells were then washed with ice-cold Hanks-Hepes, and received prewarmed (37°C) LPDS/F12 containing 10 μg/ml DiO-LDL and were incubated for 10 minutes at 37°C. The same treatment was done in the presence of 25 mM monensin with LEX1 cells (LEX1mon) and wild-type cells (WTmon). The amount of endocytosed DiO-LDL was determined by flow cytometry and expressed as percentage of that at preincubation time zero.

abnormalities, confined to the late endocytic stages, late endosome-lysosome equilibrium might be coupled with the presentation of endocytosed materials to lysosomal enzymes. Alternatively, the equilibrium might be related to the targeting, retention or processing of lysosomal enzymes. These possibilities remain to be tested to understand the coupling of the late endocytic transport and the biosynthetic pathway of lysosomal enzymes. There could also be a converse possibility that the reduced lysosomal enzyme activities resulting in the accumulation of undigested materials within vesicles have somehow caused the aggregation of vesicles. Although this possibility cannot be ruled out, no other mutants we have so far isolated with reduced lysosomal enzyme activities have shown the phenotype of aggregated late endosome-lysosome vesicles (I. Miwako et al., unpublished observations).

What is the relationship between the late endosomes and the lysosomes in the intermediate observed in LEX1 cells?

There are at least three models concerning the interaction between late endosomes and the lysosomes (Holzman, 1989; Storrie and Desjardins, 1996). Here we consider which of these models most simply explains the relationship between the lysosomes and the LEX1 late endosomes. The first model is the maturation model. If this model is applicable, the late endosomes making direct interaction with the lysosomes in an arrested intermediate observed in LEX1 cells may indicate an essential role for this small GTP-binding protein in the regulation of a late endocytic process that determines the late endosome-lysosome equilibrium. The existence of a rab protein regulating lysosome fusion has been suggested by the observation that purified GDP-dissociation inhibitor inhibited in vitro lysosome fusion in macrophages (Ward et al., 1997).
Conclusions
A number of manipulations have been shown to lead to the accumulation of transport intermediates and these manipulations have been used to dissect the requirements for late endocytic transport. For example, Luzio and colleagues studied the CI-MPR-positive late endosome-dense-core lysosome fusion requirements by monitoring the accumulation and consumption of sucroses (Bright et al., 1997). They have observed the reformation of dense core lysosomes from sucroses after digestion of sucrose; it is possible that this re-formation process is equivalent to, or largely overlapping with the sorting process taking place during lysosomal delivery of endocytosed materials and/or lysosome biogenesis. Hopkins and colleagues demonstrated that the inactivation of lysosomes via HRP/diaminobenzidine treatment caused late endocytic structures to accumulate, apparently docked to the inactive lysosomes (Futter et al., 1996). LEX1 cells might have defects related to these inhibitory manipulations, and might help, together with the studies with these manipulations, to understand the mechanisms, including roles for rab7 and microtubules, of membrane dynamics at the late endocytic stages and its relationship to lysosome biogenesis. In particular, it should be pointed out that there would be a fair chance that LEX1 cells have the arrested intermediate structure resulting from (a) specific gene defect(s) because the defective phenotypes appeared specific to the late endocytic pathway and were corrected by fusion with wild-type cells as revealed by a dominance test. The recessive nature of LEX1 cells might allow identification of gene(s) involved in late endocytic transport by expression cloning or by using the mutant cells as a arrested starting material for a cell-free (or semi-intact cell) system reconstituting a stage of the late endocytic membrane dynamics.

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REFERENCES


