Persistent reovirus infections of murine L929 (L) fibroblast cells select mutant (LX) cells that do not support proteolytic disassembly of reovirus virions within the endocytic pathway. To better understand the function and regulation of endocytic proteases, we conducted experiments to define the block to reovirus disassembly displayed by LX cells. In contrast to parental L cells, mutant LX cells harbor defects that interfere with the maturation and activity of cathepsin B and cathepsin L but not cathepsin H. The cDNAs encoding cathepsin B and cathepsin L in L cells are identical to those in LX cells, indicating that LX cells manifest an extrinsic block to the function of these enzymes. Mixed lysates of L cells and LX cells lack activity of both cathepsin B and cathepsin L, suggesting the presence of an inhibitor of cathepsin function in LX cells. A cathepsin B-green fluorescent protein (GFP) fusion protein expressed in L cells and purified by immunoprecipitation retains cathepsin B activity, whereas cathepsin B-GFP expressed in LX cells does not. However, activity of cathepsin B-GFP expressed in LX cells can be recovered by incubating the immunoprecipitate with L cell lysate followed by immunoprecipitation, providing further evidence that LX cells express a cathepsin inhibitor. Native-gel electrophoresis and gel filtration chromatography demonstrate that, in both cell lines, the double-chain form of cathepsin B is sequestered in a large molecular weight complex that renders this form of the enzyme inactive. Alteration of this sequestration complex appears to be responsible for inhibition of cathepsin B in LX cells. These findings suggest that cathepsins can be regulated within the endocytic pathway. Moreover, this regulation influences host cell susceptibility to intracellular pathogens.

The protease cascades required for complement activation, hemostasis, programmed cell death, and replication of some viruses illustrate that protease activity must be tightly regulated. Mechanisms of regulation include synthesis aszymogens, blockade by specific inhibitors, kinetic instability, and sequestration in localized environments. Endocytic proteases play important roles in propagating signals from the cell surface, hydrolysis of phagocytosed substrates, and generation of antigen-specific immune responses (1,2). Endocytic proteases also act on internalized microorganisms, in some cases mediating their destruction but in others removing surface components that allow subsequent steps in the infectious cycle (3). This study analyzes the defects in endocytic protease activity exhibited by mutant (LX) cells selected during persistent reovirus infection to provide new insights into regulation of these enzymes.

Studies of persistent viral infections have contributed significantly to an understanding of viral replication and viral pathogenesis (reviewed in Ref. 4). Persistent reovirus infections of murine L929 (L) cells select viral and cellular mutations that affect acid-dependent proteolysis of viral outer-capsid proteins during viral entry (5–8). Following attachment to cell surface receptors sialic acid (9,10) and junctional adhesion molecule 1 (11), reovirus virions are internalized into cells by receptor-mediated endocytosis (12–15). Within the endocytic pathway, host proteases catalyze the conversion of virions to infectious subviral particles (ISVPs) by cleavage of viral outer-capsid proteins (13,15–17). In murine fibroblasts, either of the endocytic proteases cathepsin B or cathepsin L are required for viral disassembly (3). Reovirus entry is completed as ISVPs penetrate endosomal membranes, releasing the transcriptionally active viral core particle into the cytoplasm (18–22).

The mutant LX cells studied here were derived from L cells persistently infected with reovirus strain type 3 Dearing for 230 days (8). During the first 4 days in culture, the persistently infected cell line underwent an intense period of crisis in which most of the cells in the culture were lysed. Over the next 2–3 weeks, small colonies of cells became apparent, and these colonies eventually reached sufficient density to permit passage. After stabilization, the cell line produced titers of infectious virus between 10^6 and 10^8 plaque forming units/ml throughout its maintenance. By passage in medium containing a reovirus-specific antiserum, the cultures were cured of viral infection as documented by plaque assay, infectious center assay, viral antigen staining, and reverse transcription-polymerase chain reaction for viral RNA (5,23–25). LX cells were cloned from the cured culture by two cycles of limiting dilution (8). These cells are not infected with virus and do not contain viral proteins or RNA (8).

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1 The abbreviations used are: LX cells, mutant L cells selected during persistent reovirus infection; DTT, dithiothreitol; GFP, green fluorescent protein; ISVP, infectious subviral particle; L cells, L929 cells; mAb, monoclonal antibody; PBS, phosphate-buffered saline; TBS, Tris-buffered saline; MEF, mouse embryo fibroblasts; Z, benzoyloxy carbonyl.
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L cells support growth of reovirus after infection by intact virions or ISVPs, generated by protease treatment of virions in vitro. In contrast, LX cells do not support growth of reovirus after infection by virions but do so after infection by ISVPs (5, 8). This observation suggests that mutant cells do not support entry steps leading to formation of ISVPs. Virions and ISVPs have identical requirements for binding to reovirus receptors (26); however, ISVPs do not require acid-dependent proteolysis to penetrate into the cytoplasm (5, 7, 15). L cells and LX cells do not differ in the capacity to internalize virions or distribute them to a perinuclear compartment (8). Intravesicular pH is equivalent in both cell types, and virions colocalize with an acid-sensitive fluorophore in both L cells and LX cells (8). However, LX cells do not support the proteolytic disassembly of the viral outer capsid following internalization into the endocytic pathway (8). Ultrastructurally, LX cells accumulate electron-dense, membrane-bound vesicles that resemble large lysosomes (8).

Preliminary characterization of mutant LX cells indicates that these cells manifest a defect in the expression of endocytic protease cathepsin L (8). By immunoblot analysis, LX cells express procathepsin L. However, unlike parental L cells, LX cells lack the mature, enzymatically active forms of this enzyme (8). Neither overexpression of cathepsin L nor treatment with cysteine protease inhibitor E64, which leads to an accumulation of the single-chain and double-chain forms of cathepsin L in wild-type (wt) cells (27), can rescue the defect in cathepsin L maturation in LX cells (8). Cathepsin L cDNA recovered from LX cells contains no mutations, indicating that the mutation in LX cells responsible for alterations in cathepsin L expression is extrinsic to the enzyme (8).

The cysteine endocytic proteases include cathepsins B, C, F, H, L, O, and X, which are expressed ubiquitously, and cathepsins K, S, and V, which are expressed in a restricted subset of tissues (28). Cathepsins B, H, and L are the most abundant endocytic cysteine proteases in fibroblasts (29–31). These proteases are first synthesized as preproenzymes with the signal peptide directing entry into the endoplasmic reticulum where the signal peptide is removed and the enzymes are glycosylated. Mannose 6-phosphate receptors in the Golgi bind the glycosylated proenzymes, leading to their transport to the endocytic pathway. As prelysosomal compartments acidify, the proenzymes are released from mannose 6-phosphate receptors (34, 35) and proteolytically cleaved to single-chain forms, liberating the propeptides. Both autocatalytic and intermolecular cleavage have been proposed for cathepsin B (36). For cathepsin L, it has been suggested that initial cleavage of the propeptide is not autocatalytic (27). For both cathepsin B and cathepsin L, subsequent cleavage of the single-chain form results in heavy and light chains. The heavy and light chains are linked by a disulfide bond, forming the double-chain form of the enzyme. Both the single-chain and double-chain forms of cathepsin B and cathepsin L are enzymatically active, whereas the proenzyme forms of these enzymes are not (34, 35, 37, 38).

To better understand the function and regulation of endocytic proteases, cysteine endocytic proteases in LX cells were characterized. Expression of cathepsin B, H, and L was assessed by immunoblot analysis, and activity was tested using fluorogenic substrates and substrate analogs. Based on these studies, we focused on determining how mature forms of cathepsin B could be present yet lack activity. The findings provide new insights into mechanisms underlying the regulation of endocytic proteases and illuminate the molecular basis of virus-cell coevolution during persistent reovirus infection.

EXPERIMENTAL PROCEDURES

Cells—Murine L cells were grown in either suspension or monolayer cultures in Joklik’s modified Eagle’s minimal essential medium (Irvine) supplemented to contain 100 units of penicillin/ml, 100 μg of streptomycin/ml, and 0.25 μg of amphotericin/ml. Mouse embryo fibroblasts (MEFs), derived from cathepsin L−/− mice (39) or cathepsin B−/− and cathepsin B−/− mice (40, 41), were grown in monolayer cultures in Dulbecco’s minimal essential medium (Invitrogen) supplemented to contain 10% fetal bovine serum, 100 units of penicillin/ml, and 100 μg of streptomycin/ml.

Immunoblot Analysis of Cathepsin B, L, and H—L, L cells and LX cells were collected by scraping and washed in phosphate-buffered saline (PBS). Cells were lysed with detergent (PBS, 1% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS, 1 mM EDTA) supplemented with EDTA-free protease inhibitor mixture (Roche Applied Science) at 4 °C for 5 min. Insoluble material was collected by centrifugation at 12,000 × g. Supernatants were mixed 1:1 with 2× sample buffer (125 mM Tris, 10 mM 2-mercaptoethanol, 4% SDS, 20% glycerol, and 0.01% bromphenol blue) and incubated at 100 °C for 5 min. Alternatively, monolayers of cells were washed with PBS and incubated in serum-free medium at 37 °C for 6 h. The medium was collected and centrifuged at 500 × g for 5 min to remove remaining cells. Proteins in the supernatant were precipitated with 20% trichloroacetic acid containing salmon sperm DNA (25 μg/ml), centrifuged at 15,000 × g for 10 min, and resuspended in 1× sample buffer (62.5 mM Tris, 5% 2-mercaptoethanol, 4% SDS, 10% glycerol, and 0.005% bromphenol blue).

Protein samples, normalized for cell number, were loaded into lanes of 12% polyacrylamide gels and electrophoresed at 200 constant voltage for 50 min. Following equilibration of the gel in transfer buffer (25 mM Tris, 192 mM glycine, 20% methanol) for 20 min, proteins were transferred to nitrocellulose membranes at 100 V for 1 h. After removal from the transfer apparatus, membranes were air-dried for 5 min and treated for 1 h with agitation in Tris-buffered saline (TBS) (50 mM Tris, pH 7.5, 150 mM NaCl) containing 0.05% Tween 20 and 5% low-fat dry milk. Membranes were incubated with rabbit antiserum raised against human cathepsin B (Athens Research and Technology, Athens, GA) diluted 1:500 in TBS plus Tween 20 and milk at 37 °C for 2 h, murine cathepsin L (42) diluted 1:10,000 at room temperature for 1 h, or human cathepsin H (Athens Research and Technology) diluted 1:1,000 at room temperature for 1 h. After three washes with TBS plus Tween 20, the membranes were incubated with horse radish peroxidase-conjugated goat anti-rabbit secondary antibody (Amersham Biosciences) diluted 1:2500 in TBS plus Tween 20 and milk. Membranes were washed three times in TBS plus Tween 20, incubated with enhanced chemiluminescent reagent (Amersham Biosciences) for 1 min, and exposed to Bio-Max MR film (Eastman Kodak Co., Rochester, NY).

Cysteine Protease—Active-site Labeling—Cysteine protease labeling reagent benzoyloxycarbonyl (Z)-Tyr-Ala-CHN2 (Peptides International, Louisville, KY) was dissolved in 25% ethanol to generate a 1 mM stock that was aliquoted and frozen at −20 °C. The cathepsin B-specific inhibitor CA-074Me (Peptides International) and the cathepsin L-specific inhibitor Z-Phe-Tyr(tBu)diazomethylketone (Calbiochem, San Diego, CA) were dissolved in Me2SO to generate 10 mM stocks that were aliquoted and frozen at −20 °C. The cysteine protease labeling reagent was iodinated by incubating 25 μl of 1 mM Z-Tyr-Ala-CHN2, 10 μl of 50 mM sodium phosphate, pH 7.5, and 1 μCi (10 μl) of Na125I in NaOH (Amersham Biosciences) in an IODO-GEN tube (Pierce) at room temperature for 10 min with mixing every 30 s. The reaction was terminated by the addition of 455 μl of 50 mM sodium phosphate, pH 7.5, and removal from the IODO-GEN tube for 5 min. The 50 μM Z-Tyr-Ala-CHN2 solution was aliquoted and frozen at −70 °C.

Cell suspensions (1 × 106 cells in 1 ml of cell culture medium) were incubated with 0.5 μM Z-Tyr-Ala-CHN2 at 37 °C for 1 h. Cells were washed once with PBS and lysed in 50 μl of lysis buffer (20 mM Tris, pH 7.4, 10 mM EDTA, 0.2% SDS, 1% Triton X-100) (43). Lysates were centrifuged at 12,000 × g, and 30 μl of supernatant was mixed with 6 μl of 6× sample buffer (0.535 mM Tris, pH 6.8, 30% glycerol, 10% SDS, 0.6 M DTT, and 0.012% bromphenol blue), boiled, and loaded into lanes of 12% polyacrylamide gels. Alternatively, cell suspensions were preincubated with 0–10 μM CA-074Me or with 10 μM Z-Phe-Tyr(tBu)diazomethylketone at 37 °C for 1 h prior to addition of 10 μl of 50 μM...
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Z-121H-Tyr-Ala-CN₂₅, for a final concentration of 0.5 μM, to the cell suspension.

For cell lysate experiments, cells were first lysed with detergent (100 mM sodium acetate, pH 5, 1 mM EDTA, 1% Triton X-100, 4 mM DTT) and then incubated with 0.5 μM Z-121H-Tyr-Ala-CN₂₅ at 30 °C for 30 min (43). For experiments using mixed lysates, L cell and LX cell lysates were mixed and incubated at 30 °C for 10 min before adding 0.5 μM Z-121H-Tyr-Ala-CN₂₅. Samples were mixed 1:1 with 6x sample buffer and resolved by SDS-PAGE using 12% polyacrylamide gels. Dried gels were exposed to film for autoradiography. For the mixed lysate experiments, dried gels were exposed to an imaging plate, and band intensity was quantitated by determining photoemulsion luminescence units using a Fujifilm Phosphorimager (Fujix Medical Systems, Inc., Stamford, CT). Densitometry of the autoradiograms corresponding to cathepsin B and cathepsin L and quantitated, and background density, calculated from an identically sized box positioned directly below the bands, was subtracted. Band densities were divided by the density for either cathepsin B or cathepsin L in the 1 L cell equivalent lane to give a ratio. An average ratio and standard deviation were calculated for two independent experiments.

**Construction of a Plasmid Encoding a Cathepsin B-GFP Fusion Protein**—To generate a 3’ Sall site in place of the stop codon, oligonucleotide primers 5’-GCTCGGT-GAGTGCCAGATCCGACG-3’ and 5’-GGACTGGAATGAAGATCGAACC-3’, which correspond to the 5’ and 3’ non-translated regions of the preprocathepsin B cDNA, were used to generate PCR products from isolated mRNA. Purified mRNA was melted in 90% Me₂SO at 95 °C for 5 min. Ice-cold primers were annealed to the melted template, and cDNA was generated using avian myeloblastosis virus reverse transcriptase (Roche Applied Sciences). PCR was performed using Taq polymerase (PerkinElmer Life Sciences) for 30 cycles, using a program of template denaturation at 94 °C for 2 min, primer annealing at 55 °C, and polymerase synthesis at 72 °C for 1 min. PCR was completed by a synthesis step at 72 °C for 1 h. Resultant cDNAs were cloned into the pCR1 vector (Invitrogen). Unambiguous sequences of preprocathepsin B cDNA, including the entire open reading frame, were determined by dideoxy chain termination at the DNA Sequencing Shared Resource at Virginia Commonwealth University. Sequencing of two cDNA clones derived from independent reverse transcriptase-PCR were determined.

**Cloning and Sequencing of Procathepsin B-encoding cDNA**—Cellular mRNA was purified from cell lysates using oligo(dT) magnet beads (Dynal, Oslo, Norway). Oligodeoxynucleotide primers 5’-GCTCGGT-GAGTGCCAGATCCGACG-3’ and 5’-GGACTGGAATGAAGATCGAACC-3’, which correspond to the 5’ and 3’ non-translated regions of the preprocathepsin B cDNA, were used to generate PCR products from isolated mRNA. Purified mRNA was melted in 90% Me₂SO at 95 °C for 5 min. Ice-cold primers were annealed to the melted template, and cDNA was generated using avian myeloblastosis virus reverse transcriptase (Roche Applied Sciences). PCR was performed using Taq polymerase (PerkinElmer Life Sciences) for 30 cycles, using a program of template denaturation at 94 °C for 2 min, primer annealing at 55 °C, and polymerase synthesis at 72 °C for 1 min. PCR was completed by a synthesis step at 72 °C for 1 h. Resultant cDNAs were cloned into the pCR1 vector (Invitrogen). Unambiguous sequences of preprocathepsin B cDNA, including the entire open reading frame, were determined by dideoxy chain termination at the DNA Sequencing Shared Resource at Virginia Commonwealth University. Sequencing of two cDNA clones derived from independent reverse transcriptase-PCR were determined.

**Subcellular Fractionation Using Ball-bearing Homogenization and Differential Centrifugation**—Cells were washed twice with PBS, collected by scraping, washed once with ST buffer (10 mM Tris, pH 7.4, 300 mM sucrose), and resuspended in ST buffer to a final volume of 1 ml. Cells were lysed by 20 passes through a ball-bearing homogenizer using 8.012-mm ball bearing until greater than 90% of the cells were lysed. Lysates were centrifuged at 1,000 × g for 10 min, the supernatant was reserved, and the pellet mitochondrial and 320,000 × g were mixed and incubated at 30 °C for 30 min. Supernatants (20 μl) that had been prewashed with lysis buffer were collected by centrifugation, and the supernatant was washed five times with lysis buffer, pelleting by centrifugation at 500 × g between washes. The immunoprecipitate was resuspended in 250 μl of reaction buffer (100 mM sodium acetate, pH 5, 1 mM EDTA, 4 mM DTT) and 250 μl of substrate solution (100 μM Z-Phe-Arg-MCA-diluted in 0.1% Brij 35). Samples were protected from light and rotated at 37 °C for 2 h. Samples were divided into two equal aliquots and added to wells of a black 96-well plate. Fluorescence was measured using a FLUOstar 403 fluorometer with excitation of 390 nm and emission of 460 nm. The readings in the two wells were averaged for each sample. Experiments were performed in triplicate.

For immunoblot analysis of cathepsin B-GFP immunoprecipitates, cells were either transfected with cathepsin B-GFP or mock transfected, lysed, and incubated with GFP-specific mAb B-2 (Santa Cruz Biotechnology, Santa Cruz, CA) was pre-bound to protein A-Sepharose beads (20 μl) by rotation at 4°C for at least 1 h. The preclear batch was added to the antibody-conjugated beads and rotated at 4°C for 4 h. The immunoprecipitate was washed five times with lysis buffer, pelleting by centrifugation at 500 × g between washes. The immunoprecipitate was resuspended in 250 μl of reaction buffer (100 mM sodium acetate, pH 5, 1 mM EDTA, 4 mM DTT) and 250 μl of substrate solution (100 μM Z-Phe-Arg-MCA-diluted in 0.1% Brij 35). Samples were protected from light and rotated at 37 °C for 2 h. Samples were divided into two equal aliquots and added to wells of a black 96-well plate. Fluorescence was measured using a FLUOstar 403 fluorometer with excitation of 390 nm and emission of 460 nm. The readings in the two wells were averaged for each sample. Experiments were performed in triplicate.

For immunoblot analysis of cathepsin B-GFP immunoprecipitates, cells were either transfected with cathepsin B-GFP or mock transfected, lysed, and incubated with GFP-specific mAb B-2 conjugated to protein A-Sepharose. Immunoprecipitates were washed five times with lysis buffer. Beads were collected by centrifugation, mixed with 50 μl of 1× sample buffer, boiled for 5 min, loaded into wells of 10% polyacrylamide gels, and electrophoresed at 200 V. Following transfer, membranes were incubated at 25°C for 1 h with rabbit polyclonal GFP-specific antisera FL (Santa Cruz Biotechnology) diluted 1:1,000 in TBS plusTween 20 and milk.

For experiments in which cathepsin B-GFP was incubated with L cell lysates, cathepsin B-GFP or GFP alone were expressed in LX cells. Following incubation of recombinant protein with GFP-specific antibody-conjugated beads at 4°C for 4 h, the immunoprecipitates were washed once with lysis buffer and resuspended in lysis buffer or L cell lysate. L cell lysate was prepared by collecting untransfected L cells by scraping, washing with PBS, and lysing with lysis buffer. Following centrifugation in a microcentrifuge, the supernatant was pre-clear prewashed with preprocathepsin B on the N-terminal end of the fusion protein. Immunoprecipitation of Cathepsin B-GFP—L cells (4 × 10⁶) and LX cells (3 × 10⁶) were plated on 100-mm plates. On the following day, the cells were transfected with 4 μg of plasmid encoding either cathepsin B-GFP or GFP alone using LipofectAMINE Plus (Invitrogen) according to the manufacturer’s instructions. After 24 h, cells were collected by scraping and washed three times with PBS. GFP fluorescence was measured using a FLUOstar 403 fluorometer with excitation of 485 nm and emission of 510 nm. Cells were resuspended in lysis buffer (50 mM sodium acetate, pH 6, 0.5% Triton X-100), normalizing for GFP expression. Insoluble material was collected by centrifugation, and the supernatant fraction was pre-clear prewashed with 4°C for 15 min with protein A-Sepharose (20 μl) that had been prewashed with lysis buffer. Cathepsin B-GFP immunoprecipitates were washed five times with lysis buffer. Beads were collected by centrifugation, mixed with 50 μl of 1× sample buffer, boiled for 5 min, loaded into wells of 10% polyacrylamide gels, and electrophoresed at 200 V. Following transfer, membranes were incubated at 25°C for 1 h with rabbit polyclonal GFP-specific antisera FL (Santa Cruz Biotechnology) diluted 1:1,000 in TBS plus Tween 20 and milk.
EDTA, 1% Triton X-100) adjusted to pH 3, 4, 5, or 6.5 and supplemented with protease inhibitors (1× protease inhibitor mixture tablets). After incubation at 4°C for at least 5 min, P320 lysates were centrifuged at high speed in a microcentrifuge, generating lysed P320 supernatant and lysed P320 pellet fractions. Twenty μl of the lysed P320 supernatant fraction was mixed with 4 μl of 6× sample buffer, boiled for 5 min, and loaded into wells of 12% polyacrylamide gels. The lysed P320 pellet fraction was resuspended in 100 μl of 1× sample buffer and boiled for 5 min. Twenty-μl aliquots of the lysed P320 pellet were loaded into wells of 12% polyacrylamide gels. Immunoblot analysis was performed using cathepsin B-specific antiserum.

**Analysis of Cathepsin B by Native-gel Electrophoresis**—L cells and LX cells were collected by scraping and washed with PBS. Cells were incubated in lysis buffer (100 mM sodium acetate, pH 6, 1 mM EDTA, 1% Triton X-100) at 4°C for 5 min. Insoluble material was collected by centrifugation at 12,000 × g. Supernatants were mixed with 3× sample buffer (50% glycerol, 0.1% bromphenol blue, 2× M Tris-HCl, pH 6.8), loaded into wells of 4–20% Tris-HCl pre-cast native-gels (Bio-Rad), and electrophoresed with running buffer (12× M Tris-HCl, 192× mM glycine) that did not contain SDS. Purified cathepsin B (Sigma) was used as a control. Proteins were transferred to nitrocellulose, and immunoblot analysis was performed using cathepsin B-specific antiserum.

**Analysis of Cathepsin B by Gel Filtration Chromatography**—Cells (6 × 10⁶) were collected by scraping, washed three times with PBS, and resuspended in 400 μl of lysis buffer (100 mM sodium acetate, pH 6, 1 mM EDTA, 1% Triton X-100). After incubation, the samples were centrifuged at 4°C for 5 min, lysates were pelleted at high speed in a microcentrifuge, and saved for immunoblot analysis. The supernatants were passed through 20-μm Spin-X filters (Costar) and loaded using a 0.5-ml loop onto a Superdex 75 column (Amersham Biosciences Corp.) that had been equilibrated in column buffer (50 mM sodium acetate, pH 6). The column was eluted with column buffer at a flow rate of 0.5 ml/min, and 0.5-ml fractions were collected with fraction 1 corresponding to loading of the sample. To detect cathepsin B, 50 μl of each fraction was mixed with 50 μl of 6× sample buffer, boiled for 5 min, and loaded into wells of 12% polyacrylamide gels. Immunoblot analysis was performed using cathepsin B-specific antiserum. To measure cathepsin B activity, 10 μl of each fraction was added to 90 μl of reaction buffer (100 mM sodium acetate, pH 5, 1 mM EDTA, 4 mM DTT) and 100 μl of substrate solution (100 μM Z-Arg-Arg-MCA in 0.1% Brij 35). Samples were incubated at room temperature for 30 min, and fluorescence was measured using a fluorometer. Alternatively, the P320 subcellular fraction was isolated from 6 × 10⁶ cells using ball-bearing homogenization and differential centrifugation. The P320 fraction was incubated in lysis buffer (100 mM sodium acetate, pH 4, 1 mM EDTA, 1% Triton X-100), fractionated using a Superdex 75 column, eluted with column buffer (50 mM sodium acetate, pH 6), and assessed for the presence of cathepsin B activity.

RESULTS

**Cathepsin Expression in LX Cells**—To determine whether LX cells manifest alterations in endocytic cysteine proteases, steady-state expression levels of cathepsin B, cathepsin H, and cathepsin L, the most abundant cysteine proteases in the endocytic pathway of fibroblast cells (29–33), were determined by immunoblot analysis of lysed cells and secreted proteins. Cathepsin B is synthesized as a 43–46-kDa inactive proenzyme precursor that is either secreted from cells or processed to a 31-kDa single-chain intermediate form, which is subsequently cleaved in lysosomes to yield a double-chain mature form consisting of a 24–25-kDa heavy chain and a 5-kDa light chain (48–50). Cathepsin B in lysates and medium of L cells and LX cells was detected using an antiserum raised against human cathepsin B (Fig. 1A). Equivalent levels of the double-chain form of cathepsin B were detected in lysates derived from L cells and LX cells. The predominant species observed at steady state under the lysis conditions used was the double-chain form in both cell lines. The single-chain form of cathepsin B was detected in L cell lysates but not in LX cell lysates. In additional experiments, using a larger number of cell equivalents and longer exposures to the chemiluminescence reaction, a trace amount of cathepsin B single-chain form was observed in LX cells (data not shown and Fig. 7B). No secreted cathepsin B was detected in the medium of L cells or LX cells. The secreted protein lanes were loaded with sample derived from a number of cells equivalent to that loaded in the lysate lanes, and the same resuspension of precipitated proteins was used for each immunoblot probed with different antibodies. Thus, the majority of the cathepsin B synthesized in these fibroblast cells is transported to intracellular compartments and processed to the double-chain form.

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Inhibitory activity of the mature form of cathepsin B is because of mutations in the enzyme, cDNAs encoding preprocathepsin B were generated from L cells and LX cells and sequenced. The cDNAs encoding cathepsin B derived from both cell types were identical and contained no mutations in comparison to the sequence of murine cathepsin B (63) (data not shown). Similarly, the cDNAs encoding procathepsin L derived from L cells and LX cells also were identical and contained no mutations in comparison to the sequence of murine cathepsin L, as reported previously (8). Therefore, like the defect in cathepsin L, the defect in cathepsin B cannot be ascribed to an intrinsic alteration of the enzyme.

Results reported thus far indicate that LX cells express mature forms of cathepsin B that are inactive, yet the cathepsin B-encoding cDNA contains no mutations. These observations are consistent with the hypothesis that LX cells express an inhibitor of cathepsin B activity. To test this hypothesis, we mixed lysates of L cells and LX cells and assessed cathepsin B activity using Z-Arg-Arg-MCA (Fig. 3A). As increasing amounts of LX cell lysate were added to 1 L cell equivalent
FIG. 2. Cysteine protease active-site labeling of L cells and LX cells. A, untreated cells. Cathepsin B +/+ (cat B +/+), cathepsin B −/− (cat B −/−), cathepsin L +/+ (cat L +/+), and cathepsin L −/− (cat L −/−) MEFs, L cells (L), and LX cells (LX) were incubated with 0.5 μM cysteine protease labeling reagent Z-[125I-Tyr]Ala-CN$_2$ at 37 °C for 1 h. Cells were lysed with detergent, and cellular proteins, normalized for cell number, were resolved by SDS-PAGE and exposed to film. Bands corresponding to cathepsin B (cat B) and cathepsin L (cat L) are indicated (A–D). A longer exposure of each gel to film is shown below the full-length gel and the dotted line to highlight the band corresponding to cathepsin L (A–D). B, cells treated with specific protease inhibitors. L cells were preincubated with untreated medium or medium supplemented to contain 1 or 10 μM cathepsin B inhibitor, CA-074Me (B$_1$), or 3.3 or 10 μM cathepsin L inhibitor, Z-Phe-Tyr(t-Bu)-diazomethylketone (L$_1$), at 37 °C for 1 h. Cells were then labeled with 0.5 μM Z-[125I-Tyr]Ala-CN$_2$ at 37 °C for 1 h, lysed with detergent, and resolved by SDS-PAGE. C, lysates. Cathepsin B +/+ , cathepsin B −/− , cathepsin L +/+ , and cathepsin L −/− MEFs, L cells, and LX cells were lysed with detergent (100 mM sodium acetate, pH 5, 1 mM EDTA, 1% Triton X-100, 4 mM DTT) and then incubated with 0.5 μM Z-[125I-Tyr]Ala-CN$_2$ at 30 °C for 30 min. D, mixed lysates. Cells were lysed with detergent and normalized for cell number. Lysates were mixed at ratios of 1 unit of L cell lysate to either 3 (1L:3LX) or 10 units (1L:10LX) of LX cell lysate and then incubated with Z-[125I-Tyr]Ala-CN$_2$. Alternatively, 1 unit of L cell lysate (1L), 1 unit of LX cell lysate (1LX), or 10 units of LX cell lysate (10LX) were incubated with the labeling reagent. Proteins in the reaction were resolved by SDS-PAGE. A representative gel is shown. E and F, quantitation of cathepsin B and cathepsin L. The densities of bands corresponding to active cathepsin B (E) and active cathepsin L (F) were determined following electrophoresis of cell lysates incubated with labeling reagent as shown in Fig. 2D. The density of each band was divided by the density of the corresponding band in the number 1 L lane to provide an assessment of enzyme activity relative to L cells. The results are expressed as the mean ratios for two independent experiments. Error bars indicate standard deviations.
lysat, cathepsin B activity decreased in a dose-dependent manner. As a control for dilutional effects, the addition of bovine serum albumin in lieu of LX cell lysate, at the same protein concentration, volume, and buffer, to L cell lysate equivalent resulted in no diminution of cathepsin B activity (Fig. 3A). These results suggest that the absence of cathepsin B activity in LX cells is due to the presence of an inhibitor.

To corroborate these findings, lysates were incubated with the cysteine protease active-site labeling reagent Z-[125I-Tyr]-Ala-CN2 (Fig. 2, C and D). Lysates of cathepsin B+/+, cathepsin B−/−, cathepsin L+/+, and cathepsin L−/− MEFs were used to confirm the identity of bands after labeling lysed cells (Fig. 2C). The 31-kDa band in the labeled lysates is cathepsin B, as it is absent in cathepsin B−/− MEFs and present in cathepsin L−/− MEFs, whereas the 22–24-kDa band is cathepsin L, as it is present in cathepsin B−/− MEFs and absent in cathepsin L−/− MEFs. Similar to results obtained using intact cells, L cell lysates contained cathepsin B and cathepsin L binding activity, whereas LX cell lysates did not, even when a 10-fold excess of cell lysate was used (Fig. 2, C and D). To determine whether LX cell lysates inhibit the activity of cathepsin B and cathepsin L, lysates of L cell and LX cells were mixed, at ratios of 1:3 and 1:10, prior to incubation with the active-site labeling reagent (Fig. 2D). As an increasing amount of LX cell lysate was added to a fixed amount of L cell lysate, the cathepsin B binding activity decreased. With 10-fold excess LX cell lysate, little cathepsin B binding activity was detected. The cathepsin L binding activity also decreased in the mixed lysates in comparison to untreated L cell lysate. To quantitate the loss of binding in mixed lysates, band intensities were measured using a PhosphorImager and normalized to the density of the corresponding bands in 1 L cell lysate as a relative index of enzyme activity (Fig. 2, E and F). Mixing lysates of L cells and LX cells resulted in a dose-dependent decrease in the activity of cathepsin B (Fig. 2E) and a substantial decrease in the activity of cathepsin L (Fig. 2F). These findings provide additional evidence that LX cells express an inhibitor that blocks the activity of cathepsin B and cathepsin L.

To determine whether the inhibition of cathepsin B and cathepsin L in LX cells extends to other cysteine endocytic proteases, we measured the activity of cathepsin H in LX cells using the substrate Arg-MCA, which fluoresces after cathepsin H cleavage (31, 44, 45). Because aminopeptidases also can cleave Arg-MCA, these experiments were performed in the presence of 1 μM puromycin, which is a potent inhibitor of aminopeptidases (31). To define the specificity of the assay, we tested the capacity of the cysteine protease inhibitor E64, the cathepsin B inhibitor CA-074, and the cathepsin L inhibitor Z-Phe-Tyr(t-Bu)-diacetylketone to inhibit cleavage of Arg-MCA (Fig. 3B). Incubation of L cell and LX cell lysates with 100 μM E64 resulted in a substantial decrease in Arg-MCA cleavage. However, incubation of the lysates with either 1 μM CA-074 or 3.3 μM Z-Phe-Tyr(t-Bu)-diacetylketone did not decrease cleavage of Arg-MCA. As cleavage is inhibited by the cysteine protease inhibitor but not by the specific cathepsin B or cathepsin L inhibitors, the E64-sensitive cleavage of Arg-MCA is likely measuring specific cathepsin H activity. The subset of proteolytic activity resulting in cleavage of Arg-MCA that can be inhibited by E64 is equivalent in L cell and LX cell lysates, suggesting that cathepsin H activity is equivalent in L cells and LX cells. In contrast to results obtained in studies of cathepsin B activity, mixing L cell and LX cell lysates resulted in a doubling of cathepsin H activity (data not shown). Thus, these findings suggest that LX cells express an activity that inhibits cathepsin B and cathepsin L but not cathepsin H.

For cathepsin L, the lack of single-chain and double-chain forms in LX cells corresponds, as expected, with a lack of activity. For cathepsin H, a decrease in the double-chain form and slight increase in the single-chain form in LX cells results in wild-type levels of activity. Cathepsin B in LX cells is more puzzling. The double-chain form of this protease is present and contains no mutations, yet it is not active. Experiments with mixed lysates suggest that LX cells express an inhibitory function that blocks the activity of cathepsin B.
cathepsin B-GFP fusion protein was engineered and tested for cathepsin B activity following immunoprecipitation. Cathepsin B-GFP expression constructs have been used previously to study cathepsin transport (40, 64). Preprocathepsin B-GFP was expressed in L cells and LX cells by transient transfection, and the recombinant protein was captured by immunoprecipitation using a GFP-specific mAb. Expression levels of the recombinant protein in L cells and LX cells were normalized by measuring GFP fluorescence. Following washing of antibody-coated beads, immunoprecipitates were resuspended in reaction buffer containing cathepsin B-sensitive substrate Z-Phe-Arg-MCA, and fluorescence was measured. The results are presented as the mean cathepsin B activity for three independent experiments. Error bars indicate standard deviations. B, LX cells were transfected with cathepsin B-GFP or GFP alone. Recombinant protein was collected from lysed cells by immunoprecipitation using GFP-specific mAb B-2. Immunoprecipitated protein was incubated with lysate buffer or with lysed, untransfected L cells at 4 °C for 3 h. After washing in lysis buffer, cathepsin B activity was measured using Z-Phe-Arg-MCA. The results are presented as the mean cathepsin B activity for three independent experiments. Error bars indicate standard deviations. Measurements were obtained in the linear range (data not shown).

To ascertain whether differences in the capacity of L cells and LX cells to generate active cathepsin B are because of differences in processing of cathepsin B-GFP to enzymatically active forms, the immunoprecipitates were analyzed by immunoblot analysis. L cells and LX cells were transiently transfected with cathepsin B-GFP or mock-transfected. Recombinant protein was captured by immunoprecipitation using a murine GFP-specific mAb, resolved by SDS-PAGE, and immunoblotted with rabbit GFP-specific polyclonal antiserum FL. Bands corresponding to procathepsin B fused to GFP (procat B-GFP), single-chain form of cathepsin B fused to GFP (SC cat B-GFP), and double-chain form of cathepsin B fused to GFP (DC cat B-GFP) are indicated. Molecular weight standards (in kilodaltons) are shown.

FIG. 5. Processing of cathepsin B-GFP in L cells and LX cells. L cells and LX cells were either transfected with preprocathepsin B-GFP (+) or mock transfected (−). Transfected cells were normalized for GFP fluorescence and lysed with detergent (100 mM sodium acetate, pH 6, 1 mM EDTA, 0.5% Triton X-100). Recombinant protein was immunoprecipitated (IP) using murine GFP-specific mAb B-2. Immunoprecipitated protein was boiled in sample buffer and resolved by SDS-PAGE. Immunoblot (IB) analysis was performed using rabbit GFP-specific polyclonal antiserum FL. Bands corresponding to procathepsin B fused to GFP (procat B-GFP), single-chain form of cathepsin B fused to GFP (SC cat B-GFP), and double-chain form of cathepsin B fused to GFP (DC cat B-GFP) are indicated. Molecular weight standards (in kilodaltons) are shown.
pressed in LX cells were mixed with untransfected L cell lysate in an attempt to elute or neutralize the inhibitory activity (Fig. 4B). LX cells were transiently transfected with cathepsin B-GFP, and the recombinant protein was immunoprecipitated using a GFP-specific mAb. As observed previously (Fig. 4A), this immunoprecipitate did not display cathepsin B activity (Fig. 4B). The immunoprecipitate of cathepsin B-GFP expressed in LX cells was then incubated with untransfected L cell lysate, immunoprecipitated a second time using GFP-specific antibody, washed with lysis buffer, and resuspended in cathepsin B substrate reaction buffer. This treatment resulted in dramatic recovery of cathepsin B activity of cathepsin B-GFP expressed in LX cells (Fig. 4B). As a control for nonspecific effects, GFP alone was expressed in LX cells, immunoprecipitated using GFP-specific antibody, and incubated with L cell lysate. In contrast to results obtained using cathepsin B-GFP, immunoprecipitates of GFP do not contain cathepsin B activity following incubation with L cell lysate (Fig. 4B). Thus, the dramatic increase in cathepsin B activity of cathepsin B-GFP expressed in LX cells following incubation with L cell lysate is not a consequence of nonspecific precipitation of cathepsin B activity. To test whether recovery of cathepsin B activity following incubation of LX cell-expressed cathepsin B-GFP with L cell lysate is because of the presence of a reducing agent, cathepsin B-GFP was incubated in lysis buffer supplemented with 4 mM DTT. Following incubation in the presence and absence of 4 mM DTT, L cell-expressed cathepsin B-GFP had equivalent cathepsin B activity in the two conditions, whereas LX cell-expressed cathepsin B-GFP did not have activity in either condition (data not shown). Thus, recovery of LX cell-expressed cathepsin B-GFP activity cannot be attributed to incubation with a reducing agent. Together, these findings provide strong evidence that the altered activity of cathepsin B in LX cells is because of inhibition of the enzyme.

Solubilization of the Mature Forms of Cathepsin B—To carefully probe the properties of the single-chain and double-chain forms of cathepsin B expressed in L cells and LX cells, we lysed cells using lysis buffers titrated to different pH values. The insoluble material was collected by centrifugation, and the soluble fraction was resolved by SDS-PAGE and immunoblotted using cathepsin B-specific antiserum (Fig. 6A). Cathepsin B activity in the soluble fraction was assessed using the fluorogenic substrate Z-Arg-Arg-MCA (Fig. 6B). A pH-dependent increase in the single-chain form of cathepsin B was detected following lysis of L cells. However, under no condition of pH was the single-chain form of cathepsin B observed following lysis of LX cells. The cathepsin B double-chain form was present in lysates of both L cells and LX cells following lysis at pH 6, but bands corresponding to the double-chain form were barely visible following lysis at pH 5. In L cells, the double-chain form of cathepsin B was not detected at any pH less than 5. However, following lysis of LX cells at progressively lower pH, the double-chain form of cathepsin B reappeared (Fig. 6A). These findings suggest that the biochemical environment of the cathepsin B double-chain form differ in L cells and LX cells. Similar band patterns were observed when lysis was performed using 1% Nonidet P-40 buffers titrated to different pH values or in lysis buffers supplemented with protease inhibitors (data not shown).

Cathepsin B activity in the soluble fractions of L cells and LX cells was not altered under any of the lysis conditions used in these experiments (Fig. 6B). Following lysis of L cells at pH 5 or less, the single-chain form of the enzyme was most prominent and likely contributes the majority of cathepsin B activity. Comparing lysis of L cells at pH 6 to lysis at pH 5, there was a substantial gain of the cathepsin B single-chain form and loss of cathepsin B double-chain form, yet enzyme activity did not change. Thus, it seems likely that the double-chain form contributed significant enzyme activity following lysis of L cells at pH 6 but not at pH 5 or less. The double-chain form of cathepsin

![Fig. 6](image_url) Immunoblot analysis of cathepsin B following cell lysis with buffers titrated to different pH values. A, L cells and LX cells were lysed in lysis buffers (100 mM sodium acetate, 1 mM EDTA, 1% Triton X-100) ranging in pH from 6 to 3 as indicated. Lysates were centrifuged at high speed in a microcentrifuge. The resulting supernatants were mixed with 6× sample buffer, boiled, and loaded into wells of 12% polyacrylamide gels. Immunoblot analysis was performed using a cathepsin B-specific antiserum. B, lysate supernatants were tested for cathepsin B activity using the substrate Z-Arg-Arg-MCA. Equal volumes of lysates were mixed with reaction buffer (100 mM sodium acetate, pH 5, 1 mM EDTA, 4 mM DTT) and substrate solution (100 μM Z-Arg-Arg-MCA in 0.1% Brij 35). Samples were normalized for cell number. The results are presented as the mean cathepsin B activity for three independent reactions. Error bars indicate standard deviations. Measurements were obtained in the linear range (data not shown). C, the P320 subcellular fraction, which includes the endocytic compartment, was isolated by ball-bearing homogenization and differential centrifugation and lysed in 100 μl of lysis buffers (100 mM sodium acetate, 1 mM EDTA, 1% Triton X-100, 1× protease inhibitors) ranging in pH from 6.5 to 3 as indicated. The lysed P320 fractions were centrifuged at high speed in a microcentrifuge to generate lysed P320 supernatant and lysed P320 pellet fractions. The lysed P320 supernatant, 20 μl, was mixed with 4 μl of 6× sample buffer, boiled, and loaded into wells of 12% polyacrylamide gels. D, the lysed P320 pellets were resuspended in 100 μl of 1× sample buffer and boiled, and 20-μl aliquots were loaded into wells of 12% polyacrylamide gels. Immunoblot analysis was performed using a cathepsin B-specific antiserum. Bands corresponding to the single-chain (SC) and double-chain (DC) forms of cathepsin B are indicated.
B also was present following lysis of LX cells at pH 6 but was not active. In fact, none of the conditions used to lyse LX cells yielded active cathepsin B.

To determine whether the altered solubilization of mature forms of cathepsin B was present in the subcellular fraction that includes the endocytic compartment, cells were lysed by ball-bearing homogenization and fractionated by differential centrifugation (46, 47). Centrifugation at 1,000 × g and 2,300 × g pelleted nuclei, larger vesicles, and mitochondria. The remaining intracellular membranes, including the endocytic compartment, were pelleted at 320,000 × g to generate the P320 subcellular fraction. The supernatant following centrifugation at 320,000 × g, which contains the cytosolic fraction, was removed. The P320 subcellular fraction was resuspended in lysis buffers titrated to different pH values containing 1% Triton X-100 and protease inhibitors. The detergent-lysed P320 was centrifuged to generate lysed P320 supernatant and lysed P320 pellet fractions. These fractions were resolved by SDS-PAGE and immunoblotted using a cathepsin B-specific antiserum (Fig. 6, C and D). Following lysis at pH 6.5, the double-chain form of cathepsin B was detected in both the supernatant and pellet fractions of lysed P320 derived from L cells and LX cells. However, following lysis at pH 5, the cathepsin B double-chain form was not detected in the supernatant but instead was found in the pellet fraction of lysed P320 from both cell types. Indeed, the intensity of the band in the pellet corresponding to the double-chain form increased in comparison to that observed following lysis at pH 6.5. Lysis at pH 4 solubilized substantially larger amounts of the double-chain form from LX cell-derived P320 in comparison to L cell-derived P320. More double-chain form was solubilized following pH 3 lysis of L cell-derived P320, although there was still less than that observed following lysis of LX cell-derived P320. Following lysis at pH 3, the double-chain form no longer was detected in the pellet of lysed P320 derived from either cell type. Consistent with previous results (Fig. 6A), the single-chain form of cathepsin B was detected only in L cells, and its band intensity peaked in the supernatant produced by lysis of P320 at pH 5 (Fig. 6C). The supernatant of the lysed P320 subcellular fraction revealed the same general band pattern of mature forms of cathepsin B that was observed following immunoblot analysis of unfractionated lysed cells. However, the pattern was shifted to higher pH values in the subcellular fractionation experiments (compare Fig. 6, A and C). For example, lysis at pH 4 of the P320 subcellular fraction derived from LX cells resulted in detection of large amounts of cathepsin B double chain in the supernatant, whereas lysate of unfractionated cells required a decrease to pH 3 in the lysis buffer to solubilize large amounts of double-chain form. This discrepancy is likely explained by the greater buffering capacity of the whole cell extract in comparison to small vesicles, which would alter the actual pH during lysis. Nonetheless, these observations suggest that the double-chain form of cathepsin B in LX cells resides in an altered environment within the endocytic compartment.

Analysis of Cathepsin B by Native-gel Electrophoresis—Findings made in experiments using mixed cell lysates and cathepsin B-GFP suggest that LX cells express an activity that inhibits cathepsin B. One possibility is that cathepsin B is inactive because it is sequestered in a complex with other cellular components. To test this hypothesis, the mobility of cathepsin B in lysates of L cells and LX cells was compared with that of purified cathepsin B by native-gel electrophoresis using non-denaturing and non-reducing conditions. L cells and LX cells were lysed with 1% Triton X-100 at pH 6, a condition in which the majority of cathepsin B derived from L cells and LX cells is in the double-chain form (Fig. 6). The insoluble material was collected by centrifugation, and the soluble fraction was resolved by native PAGE (Fig. 7A) or by SDS-PAGE (Fig. 7B). Following SDS-PAGE, the mobility of the single-chain and double-chain forms of cathepsin B expressed in L cells and LX cells approximated that of the single-chain and double-chain forms of purified cathepsin B (Fig. 7B). However, following native-gel electrophoresis, the mobility of bands from both cell lines that immunolabel with cathepsin B antisera was significantly slower than those corresponding to purified cathepsin B (Fig. 7A). These findings suggest that cathepsin B is sequestered in a complex with other cellular components in both L cells and LX cells. However, in contrast to cathepsin B expressed in LX cells, a substantial portion of cathepsin B expressed in L cells migrated at a faster rate that was similar to purified cathepsin B. This difference in native-gel electrophoretic mobility exhibited by cathepsin B expressed in L cells and LX cells may account for the difference in cathepsin B activity.

Analysis of Cathepsin B by Gel Filtration Chromatography—The retarded mobility of cathepsin B following native-gel electrophoresis but not SDS-PAGE suggested that cathepsin B exists in a large molecular weight complex. To further test whether cathepsin B resides in such a complex, cell lysates were fractionated by gel filtration chromatography. L cells were lysed with detergent at pH 6 and separated by gel filtration using a Superdex 75 column. Fractions from the column were resolved by SDS-PAGE and immunoblotted using a cathepsin B-specific antisera (Fig. 8A). Fractions also were tested for cathepsin B activity using Z-Arg-Arg-MCA (Fig. 8B). The single-chain form of cathepsin B was observed in fractions 20–23, peaking in intensity in fraction 21. These fractions also contained robust cathepsin B activity, which also peaked in fraction 21, correlating precisely with the band intensity of the
single-chain form. The faint band corresponding to the proenzyme form peaked in fraction 20, indicating a slightly larger size than the single-chain form, as expected. In contrast to the cathepsin B single-chain form, the double-chain form was detected in earlier fractions, peaking in fractions 15 and 16. However, there was little cathepsin B activity in these fractions, which were near the void volume of the column used in these experiments. In additional studies using a Superdex 200 column and standards of known molecular mass, the double-chain form peaked in fractions of ~400 kDa or greater; fractions containing most of the double-chain form also were near the void volume in this column (data not shown). These findings indicate that the majority of the cathepsin B double-chain form expressed in L cells resides in a large molecular weight complex and is inactive in this state.

To determine whether cathepsin B expressed in LX cells is similarly contained in a large molecular weight complex, LX cells were lysed in detergent at pH 6 and fractionated by gel filtration using a Superdex 75 column. Fractions eluting from the column were analyzed for cathepsin B expression (Fig. 9B). In contrast to findings made using L cells, no single-chain form of cathepsin B was detected following fractionation of LX cells. A trace amount of proenzyme was evident in LX cells, peaking in fractions 20 and 21. As in L cells, the majority of the double-chain form was detected in early fractions, peaking in fractions 15 and 16, and the double-chain form found in these fractions exhibited no activity (Fig. 9B). These results suggest that the double-chain form of cathepsin B expressed in LX cells resides in a large molecular weight complex and that enzyme retained in this complex is completely inactive. A modest amount of cathepsin B double-chain form was present in fractions 20 and 21, yet this enzyme was also inactive. This small amount of double-chain form in LX cells eluted in a slightly heavier fraction than the L cell-derived single-chain form, which peaked in fractions 20 to 22 (compare Figs. 8A and 9A). Thus, the inactive double-chain form of cathepsin B expressed in LX cells may be bound to a small molecule, which in turn may explain the lack of enzyme activity in these fractions.

To test whether the large complex that sequesters the double-chain form of cathepsin B resides within the endocytic compartment, LX cells were lysed by ball-bearing homogenization and fractionated by differential centrifugation. The P320 subcellular fraction was resuspended in lysis buffer at pH 4. Insoluble material was collected by centrifugation, and the
Figure 10. Analysis of cathepsin B following gel filtration chromatography of lysed P320 subcellular fraction derived from LX cells. The P320 subcellular fraction, which includes the endocytic compartment, was isolated by ball-bearing homogenization and differential centrifugation. The P320 fractions were lysed with detergent (100 mM sodium acetate, pH 4, 1 mM EDTA, 1% Triton X-100) and centrifuged. The supernatant was resolved by gel filtration using a Superdex 75 column. Fractions of 0.5 ml were collected following elution with column buffer (50 mM sodium acetate, pH 6). A, 50-μl aliquots of each fraction were mixed with 10 μl of 6 × sample buffer, boiled, and loaded into wells of 12% polyacrylamide gels. Immunoblot analysis was performed using a cathepsin B-specific antiserum. Bands corresponding to proenzyme (Pro) and the double-chain form (DC) of cathepsin B are indicated. B, 10-μl aliquots of each fraction were added to 190-μl reactions using the cathepsin B-specific substrate Z-Arg-Arg-MCA. Fluorescence was measured after 30 min incubation. Measurements were obtained in the linear range (data not shown).
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in LX cells did not. Processing of cathepsin B-GFP was equivalent in both L cells and LX cells, indicating that the defect in cathepsin B activity is not because of a block in formation of mature forms of the enzyme. In fact, the predominant mature form of cathepsin B-GFP appeared to be the single-chain form fused to GFP in both L cells and LX cells. Importantly, activity of cathepsin B-GFP following expression in LX cells was recovered by incubating the cathepsin B-GFP fusion protein with L cell lysate and immunoprecipitating a second time using a GFP-specific antibody. We thought it possible that this recovery of activity was either because of loss of an inhibitor from L cells or gain of an activator from L cells. The presence of an activator in L cells predicts that mixing L cell lysate with LX cell lysate would result in gain of cathepsin B activity. The absence of an inhibitor in L cells predicts that mixing cell lysates would result in loss of cathepsin B activity, which is exactly what we found. Therefore, these experiments argue strongly that LX cells express an activity that inhibits proteolysis by mature forms of cathepsin B.

In both L cells and LX cells, the mature double-chain form of cathepsin B was found to reside in a large molecular weight complex. Interestingly, the cathepsin B sequestered in this complex was inactive. This cathepsin B-sequestration complex was detected in both L cells and LX cells; however, the complex exhibited different biochemical properties in each cell type. Following lysis of LX cells at low pH, the cathepsin B-sequestration complex was solubilized, whereas identical treatment of L cells did not solubilize the complex. These findings suggest that the sequestration complex is altered in LX cells.

The basis for the altered cathepsin B-sequestration complex in LX cells may involve mutations in components of the complex other than cathepsin B, substitution of different complex components with similar molecular weight, or post-translational modification of complex components that do not significantly change the size of the complex. If exchange factors exist that shuttle complex-bound, inactive cathepsin B with unbound, active cathepsin B, LX cells may have alterations in these exchange factors that result in constant sequestration of cathepsin B in the complex without shuttling to the unbound state. Clarification of the mechanism of cathepsin B sequestration will require the identification of complex constituents in L cells and LX cells.

Comparing the results presented in Figs. 6 and 8 may provide insight into the function of the cathepsin B sequestration complex. In the experiments shown in Fig. 6, cathepsin B activity in L cells lysed at pH 6 approximates that in L cells lysed at pH 5, despite the relative loss of the cathepsin B single-chain form and gain of the double-chain form. These observations suggest that, under the conditions used for the experiment shown in Fig. 6, the double-chain form contributes significantly to total enzyme activity. In the experiment shown in Fig. 8, the double-chain form of cathepsin B in L cells resides in a large molecular weight complex and is inactive in this complex. There are several possible explanations for the seemingly discordant observations in Figs. 6 and 8. The double-chain form in the large molecular weight complex may be modestly active, although much less active than the free single-chain form of cathepsin B. In comparing pH 6 lysis to pH 5 lysis, the reduced amount of the very active single-chain form and greatly increased amount of the modestly active double-chain form may result in equivalent total levels of cathepsin B activity. Alternatively, a more active subset of the single-chain form may be released at pH 6 than at pH 5, although this seems less likely. Experiments shown in Figs. 6 and 8 were not performed under identical conditions. For Fig. 6, the interval between cell lysis and assessment of cathepsin B activity was much shorter than the gel filtration experiment shown in Fig. 8 for which a longer interval was required for sample processing. It seems possible that separation of lysate components by gel filtration influences the half-life of the single-chain and double-chain forms of cathepsin B. If the large molecular weight complex acts as a chaperone to protect mature forms of cathepsin B from degradation, separation of the complex by gel filtration may lead to rapid degradation of isolated mature forms of the enzyme. A compelling explanation for the results in Figs. 6 and 8 is that in unfractionated lysates and cells, double-chain forms of cathepsin B may be capable of dissociating from the large molecular weight complex to cleave fluorogenic substrates. In the fractionated lysates, the hypothesized exchange factors that mediate the shuttling of bound and free mature cathepsin B may be lost. Thus, the cathepsin B double-chain form would display activity in unfractionated lysates yet not in fractionated lysates where it is retained in the large molecular weight complex.

The activity that inhibits cathepsin B in LX cells, as demonstrated by experiments using cathepsin B-GFP fusion protein and mixed L cell and LX cell lysates, may involve the cathepsin B-sequestration complex. Alternatively, LX cells may manifest independent mechanisms for regulating cathepsin B activity: a de novo inhibitory activity and an altered sequestration complex. The processing of cathepsin B to single-chain and double-chain forms occurs during transport through the endocytic compartment. The double-chain form of cathepsin B and the single-chain and double-chain forms of cathepsin B-GFP expressed in LX cells are inactive. Following subcellular fractionation, the cathepsin B double-chain form is present in a large molecular weight complex in the P320 subcellular fraction, which includes endocytic vesicles and excludes the cytoplasm. These results suggest that cathepsin B is inhibited within endocytic compartments and that the cathepsin B-sequestration complex is found within the endocytic pathway.

Several mechanisms are known to regulate endocytic protease activity. The proenzyme is inactive as the enzyme active site is blocked by the pro-region (66–68). Proteolytic processing in the endocytic compartment activates these enzymes, which protects the endoplasmic reticulum and Golgi complex from unregulated protease activity (28). The pro-regions also serve to stabilize the enzymes at neutral pH (34). Mature forms of the cathepsins, including B, H, and L, are enzymatically active only at acidic pH and are irreversibly inactivated at neutral pH (34). The cystatins are a class of ~12-kDa proteins that nonspecifically inhibit cathepsins. Cystatins are largely restricted to the cytoplasm and extracellular space and not contained within endosomes and lysosomes (2, 69, 70). However, a subset of cystatins may be found within lysosomes. For example, cystatin F, whose expression is restricted to hematopoietic cells, co-fractionates with the lysosomal marker B-hexosaminidase during subcellular fractionation in density gradients (71). Therefore, it is possible that a cystatin localized to the endocytic compartment inhibits cathepsin B in LX cells.

Other data support the existence of endocytic cathepsin-sequestration complexes that serve to regulate cathepsin activity. In antigen-presenting cells, the p41 splice variant of the invariant chain inhibits cathepsin L (72, 73). In cells lacking p41, steady-state levels of the double-chain form of cathepsin L are decreased. Thus, the inhibitor p41 acts as a chaperone, maintaining a pool of mature cathepsin L in the endocytic compartment for antigen processing (73). In the absence of this chaperone, mature cathepsin L is degraded with faster kinetics (73). Moreover, the double-chain form of cathepsin L in some antigen-presenting cells is inactive (74), similar to that observed for cathepsin B in the studies reported here. Thus, the
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This study demonstrates a novel mechanism for the regulation of endocytic protease activity. LX cells inhibit mature forms of cathepsin B, and the double-chain form of cathepsin B is sequestered in an inactive state in a large molecular weight complex in the endocytic pathway. Alteration of this sequestration complex appears to be responsible for the diminished activity of cathepsin B in LX cells, leading in part to the inhibition of the proteolytic disassembly of the reovirus outer capsid. Thus, studies of the interplay of virus and cell during persistent reovirus infection have identified a new framework for understanding endocytic protease processing and activity.

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