Cathepsin L and Cathepsin B Mediate Reovirus Disassembly in Murine Fibroblast Cells*

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After attachment to receptors, reovirus virions are internalized by endocytosis and exposed to acid-dependent proteases that catalyze viral disassembly. Previous studies using the cysteine protease inhibitor E64 and a mutant cell line that does not support reovirus disassembly suggest a requirement for specific endocytic proteases in reovirus entry. This study identifies the endocytic proteases that mediate reovirus disassembly in murine fibroblast cells. Infection of both L929 cells treated with the cathepsin L inhibitor Z-Phe-Tyr(α-Bu)-diazomethyl ketone and cathepsin L-deficient mouse embryonic fibroblasts resulted in inefficient proteolytic disassembly of viral outer-capsid proteins and decreased viral yields. In contrast, both L929 cells treated with the cathepsin B inhibitor CA-074Me and cathepsin B-deficient mouse embryonic fibroblasts support reovirus disassembly and growth. However, removal of both cathepsin B and cathepsin L activity completely abrogates disassembly and growth of reovirus. Concordantly, cathepsin L mediates reovirus disassembly more efficiently than cathepsin B in vitro. These results demonstrate that either cathepsin L or cathepsin B is required for reovirus entry into murine fibroblasts and indicate that cathepsin L is the primary mediator of reovirus disassembly. Moreover, these findings suggest that specific endocytic proteases can determine host cell susceptibility to infection by intracellular pathogens.

Endocytic proteases play important roles in propagating signals from the cell surface, processing molecules for distribution to appropriate intracellular organelles, and generation of antigen-specific immune responses. Endocytic proteases also act on internalized microorganisms, in some cases mediating their destruction, but in others, removing components that allow subsequent steps in the infectious cycle. Although endocytic proteases engage in nonspecific bulk hydrolysis within lysosomes, there are several examples of specific biologic functions mediated by individual endocytic proteases (1). For example, in epithelial cells of the thymic cortex, cathepsin L plays a key role in cleaving the CLIP peptide, allowing for antigen presentation in the context of MHC class II molecules. As a result, cathepsin L-deficient mice are immunodeficient due to a defect in positive selection of T cells (2). In addition, cathepsin L is required for regulation of hair growth, and cathepsin L-deficient mice are “furless” (3). Cathepsin B can modulate pathological trypsinogen activation (4) and apoptosis induced by tumor necrosis factor-α (5). To better understand the role and specificity of endocytic proteases in virus-host interactions, we studied the proteolytic disassembly of reovirus.

Mammalian reoviruses are nonenveloped viruses that contain a segmented, double-stranded RNA genome. In comparison to enveloped viruses, less is known about cell entry mechanisms of nonenveloped viruses, a group that includes several important human pathogens. Mammalian reoviruses are a useful experimental system to study cell entry of nonenveloped viruses, as discrete steps in the viral entry pathway have been defined. Reovirus particles consist of two concentric protein shells, the outer capsid and core. After engagement of cell-surface receptors including junctional adhesion molecule-1 (6) and sialic acid (7–13), reovirus virions enter cells by receptor-mediated endocytosis (14–16). Within the endocytic compartment, virions are exposed to acid-dependent proteases, which catalyze formation of infectious subviral particles (ISVPs).1

Endocytic proteases degrade outer-capsid protein and cleave outer-capsid protein μ1/μ1C to form particle-associated fragments μ1/Δδ and φ (for review, see Ref. 17). ISVPs are capable of penetrating membranes, thereby delivering transcriptionally active core particles into the cytoplasm (18–22).

Previous studies suggest that reovirus disassembly is dependent on specific endocytic proteases. Treatment of cells with the cysteine protease inhibitor E64 (23) blocks steps in reovirus disassembly required for generation of ISVPs (24–26). Treatment of reovirus virions with purified cathepsin L leads to formation of particles that have the biochemical and growth properties of ISVPs generated in vitro by treatment of virions with intestinal proteases (27). In contrast, treatment of cells with the aspartic protease inhibitor pepstatin A (28) does not alter reovirus entry and growth (29), and treatment of virions with cathepsin D does not result in generation of ISVPs (26, 29). These findings suggest that endocytic cysteine proteases but not aspartic proteases are required for reovirus disassembly.

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1 The abbreviations used are: ISVP, infectious subviral particle; MEF, mouse embryonic fibroblast; PBS, phosphate-buffered saline; DTT, dithiothreitol; m.o.i., multiplicity of infection; pfu, plaque-forming unit; wt, wild type.
Persistent reovirus infection of murine L929 (L) cells selects mutant (LX) cells that do not support viral disassembly within the endocytic pathway. Studies of mutant LX cells provide further evidence that reovirus entry is dependent on specific proteases. Infection of LX cells with virions does not result in viral growth, whereas infection with ISVPs does (30). These findings indicate that LX cells have a defect in viral entry steps leading to formation of ISVPs. Parental L cells and mutant LX cells do not differ in the capacity to internalize reovirus virions, acidify intracellular compartments, or deliver virions to acidified organelles. However, in contrast to parental L cells, the activity of two cysteine endocytic proteases, cathepsin B and cathepsin L (27), is absent in mutant LX cells. The activity of a third cysteine protease, cathepsin H, is equivalent in parental L cells and mutant LX cells. These findings provide further support for the idea that cysteine proteases mediate disassembly of reovirus virions.

To identify the specific endocytic proteases that uncoat reovirus virions in fibroblast cells, L cells were treated with specific inhibitors of cathepsin B and cathepsin L and tested for the capacity to support reovirus disassembly and growth. Similar assays were performed using mouse embryo fibroblasts (MEFs) derived from cathepsin B-deficient mice and cathepsin L-deficient mice. Finally, the in vitro disassembly of virions to ISVPs by purified cathepsin B and cathepsin L was compared. These studies indicate that cathepsin B and cathepsin L mediate reovirus disassembly in cellular endosomes. Moreover, these studies delineate a new, physiologically relevant substrate for cathepsin B and cathepsin L.

**Experimental Procedures**

**Cells and Viruses—Murine L929 (L) cells were grown in monolayer cultures in Dulbecco’s modified Eagle’s minimal essential medium (Invitrogen, Scientific, Santa Ana, CA) supplemented with 5% fetal bovine serum (Intergen, Purchase, NY), 2 mM L-glutamine, 100 units of penicillin/ml, 100 µg of streptomycin/ml, and 0.25 µg of amphotericin/ml (Invitrogen). MEFs, derived from cathepsin B-deficient mice and cathepsin L-deficient mice, were grown in monolayer cultures in Joklik-modified Eagle’s minimal essential medium (Invitrogen, Scientific, Santa Ana, CA) supplemented with 5% fetal bovine serum (Intergen, Purchase, NY), 2 mM L-glutamine, 100 units of penicillin/ml, 100 µg of streptomycin/ml, and 0.25 µg of amphotericin/ml (Invitrogen). MEFs, derived from cathepsin B−/− and cathepsin L−/− mice (31, 32), were grown in monolayer cultures in Dulbecco’s modified Eagle’s minimal essential medium (Invitrogen) supplemented with 2 mM L-glutamine, 100 units of penicillin/ml, and 100 µg of streptomycin/ml. Reovirus strains type 1 Lang (TLL) and type 3 Dearing (T3D) are laboratory stocks. Purified virion preparations were made using second-passage L-cell lysate stocks of twice-plaque-purified reovirus (as previously described (33). Purified virions containing 35S-labeled proteins were obtained by adding Easy Tag Express-35S protein labeling kit (Promega) to cell suspensions (~12.5 µCi/ml) at the initiation of infection. ISVPs were prepared by treating purified TLL virions with Nε-succinimidylmaleimoid potassium-treated bovine α-chymotrypsin (Sigma) as previously described (24).

**Specificity of Inhibitors—**The cathepsin B inhibitor CA-074Me (B9) (Peptides International, Louisville, KY), the cathepsin L inhibitor Z-Phe-Tyr-(Bu)-diazomethyl ketone (L5) (Calbiochem-Novabiochem), the cathepsin B substrate Z-Arg-Arg-MCA (Peptides International), and the cathepsin L substrate (Z-Phe-Arg)-R110 (Molecular Probes, Eugene, OR) were dissolved in Me2SO to generate a 10 mM stock that was separated into aliquots, and frozen. CA-074Me specifically inhibits intracellular cathepsin B (34–36), whereas Z-Phe-Tyr-(Bu)-diazomethyl ketone specifically inhibits cathepsin L (37).

Cathepsin B activity was measured by preincubulating L cells (2 × 10⁶ in 1.5-ml microcentrifuge tubes in 1 ml of medium supplemented to contain 0–10 µM B9 or 0–10 µM L5 at 37 °C for 1 h. The medium was removed, and cells were adsorbed with reovirus at a multiplicity of infection (m.o.i.) of 2 plaque-forming units (pfu)/cell in gel saline (39). After a 1-h incubation at 4 °C, cells were washed once with PBS, and 1 ml of fresh medium supplemented with 0–10 µM protease inhibitors was added. Alternatively, MEFs (2 × 10⁶) in 24-well plates were preincubated with medium supplemented with 0–1 µM B9 or 0–1 µM L5 at 37 °C for 1 h. The medium was removed, and cells were adsorbed with reovirus at a m.o.i. of 2 pfu/cell. After incubation at room temperature for 30 min, cells were washed once with PBS, and 1 ml of fresh medium supplemented with 0–10 µM protease inhibitors was added. After incubation at 37 °C for 24 h, cells were frozen and thawed twice, and viral titers in cell lysates were determined by plaque assay (39). Independent experiments were performed using single wells of cells, which were titrated in triplicate.

**Growth of Reovirus in Cells Treated with Protease Inhibitors—**Monolayers of Cell wells (4 × 10⁴) in 24-well plates infected with T3D or TLL was treated with 0–10 µM protease inhibitors for 1 h in medium supplemented to contain 0–10 µM B9 or 0–10 µM L5. The medium was removed, and cells were adsorbed with reovirus at a multiplicity of infection (m.o.i.) of 2 plaque-forming units (pfu)/cell in gel saline (39). After a 1-h incubation at 4 °C, cells were washed once with PBS, and 1 ml of fresh medium supplemented with 0–10 µM protease inhibitors was added. Alternatively, MEFs (2 × 10⁶) in 24-well plates were preincubated with medium supplemented with 0–1 µM B9 or 0–1 µM L5 at 37 °C for 1 h. The medium was removed, and cells were adsorbed with reovirus at a m.o.i. of 2 pfu/cell. After incubation at room temperature for 30 min, cells were washed once with PBS, and 5 ml of fresh medium supplemented with 0–10 µM protease inhibitors was added. Alternatively, MEFs (3 × 10⁶) in 100-mm dishes were preincubated with 0–1 µM B9 or 0–10 µM L5 at 37 °C for 24 h. Cells were frozen and thawed twice, and viral titers in cell lysates were determined by plaque assay (39). Independent experiments were performed using single wells of cells, which were titrated in triplicate.

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**Fluorescent Focus Assay of Viral Infectivity—**Virus inocula were adsorbed to confluent MEF monolayers (1 × 10⁶ cells) in 24-well plates as growth experiments. After incubation at 37 °C for 18 h to permit completion of a single round of viral replication, cell monolayers were fixed with 1 ml of methanol at −20 °C for a minimum of 30 min. Fixed monolayers were washed twice with PBS, blocked with 5% immunoglobulin-free bovine serum albumin (Sigma) in PBS, and incubated at 37 °C for 30 min with protein A-affinity-purified polyclonal rabbit anti-reovirus serum at a 1:500 dilution in PBS, 0.5% Triton X-100. Monolayers were washed twice with PBS, 0.5% Triton X-100 and incubated with a 1:1000 dilution of anti-rabbit immunoglobulin serum conjugated with Alexa-488 (Molecular Probes). Monolayers were washed twice with PBS, 0.5% Triton X-100, and infected cells were visualized by indirect immunofluorescence. Infected cells were identified by the presence of intense cytoplasmic fluorescence that was excluded from the nucleus (41). Background staining in uninfected control monolayers was not detected.

**SDS-PAGE of Reovirus Structural Proteins—**Discontinuous SDS-PAGE was performed as previously described (42). Viral particles were solubilized by incubation in sample buffer (125 mM Tris, 2% 2-mercaptoethanol, 1% SDS, 0.01% bromphenol blue) and frozen at 20 °C.
endocytic proteases and reovirus disassembly

Effect of cathepsin B inhibitor CA-074Me (B₁) and cathepsin L inhibitor Z-Phe-Tyr(t-Bu)-diazoethyl ketone (L₁) on growth of reovirus strains T1L and T3D in L cells. A, cathepsin B activity in L cells treated with protease inhibitors. L cells were preincubated with either B₁ or L₁ for 1 h and lysed. Cathepsin B substrate Z-Arg-Arg-MCA was added to lysates, and then incubation at room temperature for 30 min, fluorescence was measured. Negative control (Neg) contained no cell lysate in the reaction. B, cathepsin L activity in L cells treated with protease inhibitors. After a 1-h preincubation with either B₁ or L₁, cells were incubated with Z-Phe-Arg(2)-R110 in the presence of either B₁ or L₁ for 90 min, and fluorescence was measured. Negative control (Neg) contained no substrate in the reaction. The results are presented as mean fluorescence for three independent experiments. Error bars indicate S.D. C, monolayers of L cells (4 × 10⁴) were preincubated for 1 h in medium supplemented with B₁, L₁, or both B₁ and L₁, and cathepsin B activity was measured by incubating intact cells with 25 μg of cathepsin L/ml at 37 °C for 1 h. Samples were loaded into wells of 14% polyacrylamide gels and electrophoresed at 200 V. After electrophoresis, viral proteins were transferred to polyvinylidene difluoride membranes (Bio-Rad) at 400 mA for 20 min. Membranes were stained with Coomassie Blue. The 13-kDa fragment was excised from the membrane, and the N terminus was sequenced using Edman degradation by the Vanderbilt Protein Chemistry Laboratory. Cathepsin L cleavage sites were modeled onto the T₁ structure (47) using Swiss PDB viewer 3.7 (Glaxo Wellcome) and POV-Ray (Persistence of Vision Development Team).

Results

Specificity of Protease Inhibitors—As a prelude to experiments to determine the effects of both an inhibitor of cathepsin B, Z-Phe-Tyr(t-Bu)-diazoethyl ketone (L₁), and an inhibitor of cathepsin B, CA-074Me (B₁), on reovirus disassembly and growth, we first tested the specificity of these protease inhibitors using fluorogenic protease substrates. De-esterification of the membrane-permeable proinhibitor CA-074Me inside cells generates the specific cathepsin B inhibitor CA-074 (36). Cathepsin B activity was measured by incubating cell lysates with the cathepsin B-specific substrate Z-Arg-Arg-MCA (38). Cathepsin L activity was measured by incubating intact cells with (Z-Phe-Arg(2)-R110), which is cleaved 820 times more efficiently by cathepsin L than cathepsin B (48, 49). In L cells treated with 1 μM B₁, cathepsin B activity was reduced to 1% of that in untreated cells (Fig. 1A), whereas cathepsin L activity was 87% that in untreated cells (Fig. 1B). In contrast, cathepsin B activity in L cells treated with 3.3 μM L₁ was 90% that in untreated cells, whereas cathepsin L activity was only 2.6% that in untreated cells. At 10 μM L₁, cathepsin L activity was further degraded during protease treatment of virions to generate ISVPs (11, 12, 14, 16, 43–45). To statistically compare o₂/₉ ratios between two different conditions, one-tailed, two-sample t tests assuming unequal variances were calculated using Excel 97 (Microsoft, Redmond, WA).

Identification of Cathepsin L Cleavage Sites in T₁L—Purified T₁L virions at a concentration of 8 × 10¹² particles/ml in reaction buffer (50 mM sodium acetate (pH 5.0), 100 mM NaCl, 3 mM DTT) were treated with 5 μl of 6X sample buffer (350 mM Tris (pH 6.8), 9.3% DTT, 10% SDS, 0.012% bromphenol blue) and incubated at 100 °C for 5 min. Samples were loaded into wells of 10% polyacrylamide gels and electrophoresed at 200-V constant voltage for 1 h. After electrophoresis, viral proteins were transferred to polyvinylidene difluoride membranes (Bio-Rad) at 400 mA for 20 min. Membranes were stained with Coomassie Blue. The 13-kDa fragment was excised from the membrane, and the N terminus was sequenced using Edman degradation by the Vanderbilt Protein Chemistry Laboratory. Cathepsin L cleavage sites were modeled onto the o₂ structure (47) using Swiss PDB viewer 3.7 (Glaxo Wellcome) and POV-Ray (Persistence of Vision Development Team).
reduced to 2% of untreated cells, whereas cathepsin B activity was reduced to 53% of untreated cells. Because cathepsin B is capable of cleaving the substrate (Z-Phe-Arg)_{2}R110, albeit with much less efficiency than cathepsin L (48), the residual fluorescence in cells treated with 10 μM L_{1} might be due to cathepsin B as opposed to uninhibited cathepsin L. Thus, concentrations of B_{1} and L_{1} can be defined that selectively inhibit cathepsin B and cathepsin L, respectively.

Viral Growth and Disassembly in L Cells Treated with Inhibitors of Cathepsin B and Cathepsin L—To test whether cathepsin B or cathepsin L is required for productive reovirus entry into cells, viral growth and disassembly were assessed using L cells, a murine fibroblast line, treated with B_{1} and L_{1}. L cells pretreated with 0–10 μM B_{1} or L_{1} were infected with reovirus at an m.o.i. of 2 pfu/cell, and yields were determined after 24 h of viral growth in the presence of 0–10 μM B_{1} or L_{1} (Fig. 1C). Yields of T1L and T3D in L cells treated with 1 μM B_{1}, which selectively inhibits cathepsin B, were approximately equivalent to yields in untreated L cells. In contrast, yields of T1L and T3D in L cells treated with 3.3 μM L_{1}, which selectively inhibits cathepsin L, were 30 and 13%, respectively, of yields in untreated cells. At 10 μM L_{1}, yields of T1L and T3D in L cells were 1.8 and 1.1%, respectively, of yields in untreated cells. Yields of T1L and T3D in L cells treated with 1 μM B_{1} and 10 μM L_{1}, which blocks the activity of both cathepsin B and cathepsin L, were 1.4- and 0.98-fold viral input, a 1300- and 2000-fold reduction in viral yield in comparison to untreated cells. These results indicate that inhibition of cathepsin B alone does not alter viral growth in L cells. However, inhibition of cathepsin L activity alone decreases growth of T1L from 3.3- to 56-fold and T3D from 7.6- to 91-fold. Moreover, these results demonstrate that viral growth is completely abolished in L cells when both cathepsin B and cathepsin L are inhibited.

To provide evidence that the observed inhibition of viral growth by treatment with B_{1} and L_{1} is due to inhibition of proteolytic disassembly of virions, cells were infected with ISVPs of strain T1L generated in vitro by chymotrypsin treatment of purified virions. ISVPs can grow in cells treated with inhibitors of virion-to-ISVP disassembly but not in cells treated with inhibitors of subsequent steps in the viral replication cycle (17). Viral yields after infection with ISVPs were equivalent in the presence and absence of B_{1} and L_{1} (Fig. 1C). These results suggest that the blockade of viral growth in L cells treated with inhibitors of cathepsin B and cathepsin L is due to blockade of virion-to-ISVP disassembly.

To demonstrate directly that differences in growth of virions in L cells treated with cathepsin B and cathepsin L inhibitors are linked to differences in viral disassembly, 35S-labeled virions of T1L were adsorbed to L cells that had been pretreated with 0–10 μM B_{1} and L_{1}. After 0 or 3 h of incubation in medium supplemented with or without B_{1} and L_{1}, viral structural proteins were resolved by SDS-PAGE and visualized by autoradiography (Fig. 2A). Degradation of outer-capsid protein α3 and cleavage of outer-capsid protein μ1C to δ, changes indicative of ISVP formation, were observed after 3 h of incubation in untreated cells. Degradation of α3 and generation of δ also occurred in cells treated with 1 μM B_{1}. In contrast, degradation of α3 and cleavage of μ1C occurred to a significantly lesser extent in cells treated with 3.3 μM L_{1} than in untreated cells. Because viral core protein α2 is not degraded during viral entry, band intensity of the α2 protein was used to normalize for potential discrepancies in sample preparation and loading (Fig. 2B). Using a one-tailed, two-sample t test assuming unequal variance, the difference in the α3/α2 ratio after infection of cells treated with 3.3 μM L_{1} and untreated cells was statistically significant (p < 0.002). Treatment of cells with 10 μM L_{1} resulted in further inhibition of α3 and μ1C cleavage, and the difference in the α3/α2 ratio after infection of cells treated with 10 μM L_{1} and 3.3 μM L_{1} was statistically significant (p < 0.0002). Treatment of cells with both 10 μM L_{1} and 1 μM B_{1} blocked all degradation of α3 and cleavage of μ1C. The α3/α2 ratio in cells treated with 10 μM L_{1} and 1 μM B_{1} approximated that observed at 0 h and was significantly greater than that seen after infection of cells treated with 10 μM L_{1} alone (p < 0.03). These results indicate that disassembly of reovirus virions in endosomes of murine L cells is dependent on either cathepsin B or cathepsin L. Moreover, these results suggest that cathepsin B activity is dispensable, whereas cathepsin L activity is not.

Viral Growth and Disassembly in Cathepsin L-deficient MEFs—Studies of L cells treated with L_{1} indicate a critical role for cathepsin L in reovirus entry into murine fibroblasts. To further test this hypothesis, MEFs generated from either cathepsin L+/+ or cathepsin L−/− mice were transfected with either a cathepsin L-expressing vector or an empty vector (mock) and infected with reovirus virions at an m.o.i. of 2 pfu/cell. Viral growth was assessed by a fluorescent focus assay in which a polyclonal anti-reovirus serum is used to detect newly synthesized reovirus protein. Infection of mock-transfected wt MEFs resulted in a majority of cells staining positive for reovirus protein, indicating productive viral infection (Fig. 3A). In contrast, infection of mock-transfected cathepsin L-deficient MEFs resulted in few reovirus-positive cells. Transfection of cathepsin L-deficient MEFs with the cathepsin L-encoding vector resulted in numerous cells that stained positive for reovirus protein. This result demonstrates that cathepsin L is required for productive reovirus infection in MEFs.
untreated. L-deficient MEFs (cathepsin L 

Fig. 3. Growth of reovirus virions in cathepsin L-deficient MEFs. A, monolayers of wt MEFs (cathepsin L +/−) and cathepsin L-deficient MEFs (cathepsin L −/−) (1 × 10⁶) were transfected with either an empty expression vector (Mock) or with a vector expressing murine procathepsin L. After a 24-h incubation, transfected cells were adsorbed with T1L at an m.o.i. of 2 pfu/cell. After incubation at 37 °C for 18 h, viral proteins were detected by indirect immunofluorescence using polyclonal rabbit anti-reovirus serum raised against T1L and antirabbit-immunoglobulin conjugated to Alexa488. B, monolayers of MEFs (1 × 10⁶) were preincubated for 1 h in medium supplemented with 0 or 1 μM B1. The medium was removed, and cells were adsorbed with T1L, T3D, or T1L ISVPs at an m.o.i. of 2 pfu/cell. After adsorption for 30 min, the inoculum was removed, fresh medium with or without B1 was added, and cells were incubated at 37 °C for 24 h. Viral titers in cell lysates were determined by plaque assay. The results are presented as the mean viral yield, calculated by dividing titer at 24 h by titer at 0 h, for three independent experiments. Error bars indicate S.D. UT, untreated.

positive, with an approximately equivalent percentage of cells staining for reovirus protein as seen after infection of wt MEFs transfected with the cathepsin L expression vector (Fig. 3A). These results suggest that the absence of cathepsin L renders MEFs significantly less permissive for reovirus infection and that this block to reovirus infection can be removed by complementing cathepsin L.

Infection of wt MEFs with either T1L or T3D results in robust viral growth, with viral yields of 47 and 435, respectively, after a single cycle of viral replication. In contrast, yields of T1L and T3D after infection of cathepsin L-deficient MEFs were 1.8- and 40-fold viral input, a reduction of 26- and 11-fold in comparison to yields after infection of wt MEFs. To test whether residual reovirus growth in cathepsin L-deficient MEFs is dependent on cathepsin B, MEFs were treated with B1 before infection with reovirus. Treatment of wt MEFs with B1 resulted in only a 2.9-fold decrease in yield of T3D in comparison to that after growth in untreated cells. However, treatment of cathepsin L-deficient MEFs with B1 completely abolished viral growth. As a control, infection of MEFs treated with 0–1 μM B1 with ISVPs resulted in essentially equivalent viral yields (Fig. 3B). These results indicate that genetic ablation of cathepsin L in conjunction with pharmacologic inhibition of cathepsin B renders MEFs incapable of supporting reovirus replication and suggest that the block to viral growth occurs before generation of ISVPs.

To determine whether cathepsin L is required for MEFs to support reovirus replication, wt MEFs and cathepsin L-deficient MEFs were adsorbed with 35S-labeled T1L virions, cells were lysed at various intervals, and viral proteins were resolved by SDS-PAGE. After infection of wt MEFs, α3 was rapidly degraded, and μ1C was cleaved to form δ. In contrast, infection of cathepsin L-deficient MEFs was associated with a significant delay of these cleavage events; α3 was degraded at a slower rate, whereas traces of δ generation were evident only at the latest time point (Fig. 4A). In concordance with these results, at each time point after viral adsorption of cathepsin L-deficient MEFs, the α3/δ2 ratio was significantly greater than that after adsorption of wt MEFs (p < 0.0009 for 3 h, p < 0.02 for 6 h, p < 0.009 for 9 h) (Fig. 4B). These results indicate that efficient proteolytic processing of the reovirus outer capsid requires cathepsin L.

To test whether cathepsin B is responsible for the residual capacity of cathepsin L-deficient MEFs to support reovirus disassembly, MEFs were pretreated with B1 and adsorbed with 35S-labeled T1L virions. Degradation of α3 and cleavage of μ1C to δ were only slightly diminished after infection of B1-treated wt MEFs in comparison to untreated MEFs (Fig. 5A). As anticipated, the α3/δ2 ratio after infection of wt MEFs treated with B1 was slightly higher than that after infection of untreated MEFs. However, after treatment of cathepsin L-deficient MEFs with B1, the modest degradation of α3 and cleavage of μ1C to δ observed 9 h after infection of untreated cells was

![Figure 3](http://www.jbc.org/...)

Fig. 3. Growth of reovirus virions in cathepsin L-deficient MEFs. A, monolayers of wt MEFs (cathepsin L +/−) and cathepsin L-deficient MEFs (cathepsin L −/−) (1 × 10⁶) were transfected with either an empty expression vector (Mock) or with a vector expressing murine procathepsin L. After a 24-h incubation, transfected cells were adsorbed with T1L at an m.o.i. of 2 pfu/cell. After incubation at 37 °C for 18 h, viral proteins were detected by indirect immunofluorescence using polyclonal rabbit anti-reovirus serum raised against T1L and antirabbit-immunoglobulin conjugated to Alexa488. B, monolayers of MEFs (1 × 10⁶) were preincubated for 1 h in medium supplemented with 0 or 1 μM B1. The medium was removed, and cells were adsorbed with T1L, T3D, or T1L ISVPs at an m.o.i. of 2 pfu/cell. After adsorption for 30 min, the inoculum was removed, fresh medium with or without B1 was added, and cells were incubated at 37 °C for 24 h. Viral titers in cell lysates were determined by plaque assay. The results are presented as the mean viral yield, calculated by dividing titer at 24 h by titer at 0 h, for three independent experiments. Error bars indicate S.D. UT, untreated.

![Figure 4](http://www.jbc.org/...)

Fig. 4. Proteolysis of reovirus outer-capsid proteins during viral disassembly in cathepsin L-deficient MEFs. A, monolayers of wt MEFs (cathepsin L +/−) and cathepsin L-deficient MEFs (cathepsin L −/−) (1.5 × 10⁶) were adsorbed with 1 × 10¹¹ purified 35S-labeled T1L virions. After incubation at room temperature for 30 min, the inoculum was removed, fresh medium with or without B1 was added, and cells were incubated at 37 °C for 0, 3, 6, or 9 h. Viral particles in cell lysates were subjected to SDS-PAGE. Viral proteins are labeled on the right. B, quantitation of α3 band intensity. The densities of bands corresponding to the α2 and α3 proteins were determined, and the results are expressed as the mean α3/α2 ratios for three independent experiments. Error bars indicate S.D.
not observed. At this late time point, δ formation was not evident, and the α3 band intensity and the α3/α2 ratio were equivalent to those at the 0-h time point in B1-treated cathepsin L-deficient MEFs (Fig. 5, A and B). Thus, inefficient proteolytic disassembly of reovirus virions in cathepsin L-deficient MEFs appears entirely dependent on cathepsin B.

Viral Growth and Disassembly in Cathepsin B-deficient MEFs—To more precisely define the role of cathepsin B in reovirus entry, cathepsin B-deficient MEFs were infected with reovirus virions, and viral yields were determined after 24 h of viral growth. Infection with either T1L or T3D resulted in approximately equivalent yields in wt MEFs and cathepsin B-deficient MEFs (Fig. 6). In concordance with studies using L cells treated with B1, these results indicate that the absence of cathepsin B does not alter the capacity of MEFs to support reovirus growth.

Because previous experiments suggested a requirement for either cathepsin B or cathepsin L in reovirus entry, we tested the effect of L1 on reovirus growth in cathepsin B-deficient MEFs. Treatment of wt MEFs with L1 resulted in 4–5-fold reductions in yields of T1L and T3D in comparison to those in untreated wt MEFs. In contrast, treatment of cathepsin B-deficient MEFs with L1 resulted in 55–600-fold lower viral yields in comparison to those after growth in untreated cathepsin B-deficient MEFs. In fact, viral yields in L1-treated cathepsin B-deficient MEFs were only 0.5–2-fold greater than viral input (Fig. 6). Thus, cathepsin B-deficient MEFs are much more sensitive than wt MEFs to the inhibitory effects of L1 on viral growth. Moreover, growth of reovirus in cathepsin B-deficient MEFs can be completely abrogated by treatment with an inhibitor of cathepsin L.

To determine whether cathepsin B-deficient MEFs can support disassembly of reovirus virions, 35S-labeled T1L virions were adsorbed to cathepsin B-deficient MEFs, cells were lysed at different times, and viral proteins were resolved by SDS-PAGE (Fig. 7A). In contrast to results obtained in experiments using cathepsin L-deficient MEFs, by 3 h after infection of cathepsin B-deficient MEFs, α3 was substantially degraded, and noticeable quantities of δ had accumulated. By 6 h after infection, more α3 was degraded, and greater amounts of δ were generated. However, in L1-treated cathepsin B-deficient MEFs, α3 and μ1C remained intact, even at 6 h after adsorption. The α3/α2 ratio after infection of cathepsin B-deficient MEFs treated with L1 approximated that observed at the 0-h time point and was significantly greater than the α3/α2 ratio after infection of wt MEFs at 6 h (p < 0.011) (Fig. 7B). These results indicate that cathepsin B-deficient MEFs are not altered in the capacity to disassemble reovirus virions and that inhibition of cathepsin L prevents virion disassembly in cathepsin B-deficient MEFs.

Treatment of Reovirus Virions with Purified Endocytic Proteases—Because reovirus disassembly in murine fibroblasts is dependent on cathepsin B and cathepsin L, with cathepsin L appearing to play a more important role in this process, we compared the relative capacities of purified cathepsin B and cathepsin L to mediate reovirus disassembly in vitro. Equivalent numbers of 35S-labeled virions of strain T1L were incubated with increasing concentrations of each enzyme. Outer-capsid protein α3 was degraded with increasing concentrations of both cathepsin B (Fig. 8A) and cathepsin L (Fig. 8B). However, degradation of α3 occurred with substantially lower concentrations of cathepsin L. At 1 μM enzyme, α3 was completely degraded by cathepsin L but minimally cleaved by cathepsin B. Even after incubation with 4 μM cathepsin B, substantial amounts of α3 remained. Generation of the δ fragment was evident after incubation with 1 μM cathepsin L but not 1 μM cathepsin B.
Identification of Cathepsin L Cleavage Sites in T1L Virions—Results presented thus far indicate that cathepsin L is the major mediator of reovirus disassembly in endosomes of murine fibroblasts. To identify sites in α3 cleaved by cathepsin L, T1L virions were treated with purified cathepsin L, and the C-terminal α3 cleavage fragment was subjected to peptide sequencing. Cathepsin L treatment of T1L α3 results in ∼13- and 29-kDa fragments after SDS-PAGE and Coomassie Blue staining, analogous to *Staphylococcus aureus* V8 protease treatment of T3D α3 (50, 51). In studies using V8 protease, the smaller fragment corresponds to the C-terminal fragment. Based on this finding, the 13-kDa fragment generated by cathepsin L treatment of T1L virions was transferred to a polyvinylidene difluoride membrane and subjected to N-terminal peptide sequencing by Edman degradation. Two amino acid sequences were evident from this analysis (Fig. 9A). The predominant sequence was HFGLS, which matches α3 amino acid residues 251–255. The minor sequence was TPARD, which corresponds to the 13-kDa fragment generated by cathepsin L.

During these experiments, we noted that cathepsin B cleavage of α3 produced a doublet band as full-length α3 was replaced by a fragment of slightly faster electrophoretic mobility (Fig. 8A). To determine whether cathepsin L degrades α3 by a similar mechanism, virions of T1L were treated with 1 μM cathepsin L over a time course, and viral structural proteins were resolved by SDS-PAGE (Fig. 8C). Degradation of α3 by cathepsin L also resulted in a doublet band that preceded complete degradation of the protein. After incubation for 1 h, a faint band appeared that migrated slightly faster than full-length α3. After incubation for 2–4 h, this lower band increased in intensity in direct proportion to the loss in intensity of the upper band. At times greater than 8 h, neither band was apparent (data not shown). After treatment of T1L virions with cathepsin L, the upper and lower bands were resolved by SDS-PAGE, excised from the gel, digested with trypsin, and analyzed by mass spectrometry. The trypptic fragments from the upper and lower bands had identical masses that matched predicted trypptic cleavage fragments of α3 (data not shown). These results suggest that an initial cleavage event by either cathepsin B or cathepsin L occurs near a terminus of the α3 protein.

Identification of Cathepsin L Cleavage Sites in T1L Virions—Results presented thus far indicate that cathepsin L is the major mediator of reovirus disassembly in endosomes of murine fibroblasts. To identify sites in α3 cleaved by cathepsin L, T1L virions were treated with purified cathepsin L, and the C-terminal α3 cleavage fragment was subjected to peptide sequencing. Cathepsin L treatment of T1L α3 results in ∼13- and 29-kDa fragments after SDS-PAGE and Coomassie Blue staining, analogous to *Staphylococcus aureus* V8 protease treatment of T3D α3 (50, 51). In studies using V8 protease, the smaller fragment corresponds to the C-terminal fragment. Based on this finding, the 13-kDa fragment generated by cathepsin L treatment of T1L virions was transferred to a polyvinylidene difluoride membrane and subjected to N-terminal peptide sequencing by Edman degradation. Two amino acid sequences were evident from this analysis (Fig. 9A). The predominant sequence was HFGLS, which matches α3 amino acid residues 251–255. The minor sequence was TPARD, which corresponds to α3 amino acid residues 244–248. Thus, sequence analysis of the 13-kDa α3 cleavage fragments generated by cathepsin L treatment of T1L virions indicates that cathepsin L cleaves α3 at two sites, between amino acids 250 and 251 and between amino acids 243 and 244.
Endocytic Proteases and Reovirus Disassembly

**Fig. 9. Cathepsin L cleavage sites in TIL α3.** A, the primary amino acid sequence of α3 from amino acids 241 to 255 is shown. The arrows point to identified cathepsin L cleavage sites. B, cathepsin L cleavage sites are highlighted in the crystal structure of α3. A ribbon diagram of the crystal structure of T3D α3 (47) is displayed. The cathepsin L cleavage sites in TIL are depicted in blue between amino acids 243 and 244 and between 250 and 251 and, within the surrounding amino acids from 241 to 253, in yellow. The C-terminal residues of α3, from amino acids 340 to 365, are colored red. Amino acid 354, which is a site mutated in PI and D-EA viruses, is colored green. The virion-distal end of α3 is at the top of the page, and the virion-proximal end and N terminus is at the bottom.

**DISCUSSION**

After receptor-mediated endocytosis, the reovirus outer capsid is subject to proteolysis by endocytic proteases, resulting in generation of ISVPs. These particles are obligate intermediates in reovirus disassembly that interact directly with vacuolar membranes leading to delivery of transcriptionally active viral cores into the cytoplasm (for review, see Ref. 17). Previous studies suggest that specific endocytic proteases mediate disassembly of reovirus virions to ISVPs. Cysteine protease inhibitor E64 blocks reovirus disassembly (24–26), but aspartic protease inhibitor pepstatin A does not (29). Mutant cells selected during persistent reovirus infection do not support reovirus disassembly despite internalizing and transporting virions to acidified, perinuclear compartments. These mutant cells have defects in the activity of cysteine proteases cathepsin B and cathepsin L but not cysteine protease cathepsin H (27). This study sought to identify the specific endocytic proteases that act on reovirus virions in endosomes of murine fibroblasts to generate functional ISVPs.

Studies using L cells and genetically deficient MEFs indicate that either cathepsin B or cathepsin L is required for reovirus virion-to-ISVP disassembly. Removal of cathepsin L activity alone, whether by active-site inhibitors or by genetic modification, significantly decreases the capacity of cells to support reovirus disassembly. In contrast, removal of cathepsin B activity alone does not alter the capacity of cells to support either disassembly or growth. However, removal of both cathepsin B and cathepsin L activity completely abrogates disassembly and growth of reovirus. In concordance with these data, cathepsin L mediates reovirus disassembly more efficiently than cathepsin B in vitro. Particles generated by cathepsin L treatment of TIL virions have biological hallmarks of ISVPs in that these particles bypass blocks to inhibitors of viral entry, such as E64 and ammonium chloride, and can infect mutant cells selected during persistent infection (27). Thus, murine fibroblasts have a requirement for either cathepsin B or cathepsin L to support reovirus disassembly, although cathepsin L is the major mediator of this process.

Why reovirus has evolved so that only specific proteases can effect its disassembly within cellular endosomes is an interesting question. The answer may lie in the virion stability/disassembly fulcrum. There is selective pressure for a virus to be stable in the environment yet disassemble and activate its genetic program in the appropriate intracellular compartment. Requirement by a nonenveloped virus for specific endocytic proteases to mediate capsid removal would allow it to be stable in the environment where these proteases are either not present or present in inactive forms. Upon delivery to the appropriate endocytic organelle, the resident proteases contained therein, such as cathepsin B and cathepsin L, would catalyze viral disassembly and facilitate delivery of the virus into the cytoplasm.

Results presented here implicate the reovirus outer capsid as a physiologically relevant substrate for cathepsin B and cathepsin L. The outer capsid consists of α3 and μ1 in 1:1 complexes, present at 600 copies/virion (52–54). The α3 protein acts as a cap to protect μ1, which is the viral protein that mediates membrane penetration (20–22). Thus, removal of α3 is essential to the disassembly process (16, 24). Reovirus α3s is a bilobed protein with its N terminus in a virion-proximal smaller lobe bound to μ1 and its C terminus in a virion-distal larger lobe (47, 55, 56). Studies using S. aureus V8 protease suggest that α3 is sensitive to proteolytic cleavage near amino acid 220 (50), which is approximately positioned between the two lobes (47).

We identified sites in TIL α3 cleaved by cathepsin L to provide additional evidence that α3 serves as a cathepsin L substrate and to gain insight into mechanisms of α3 cleavage in the endocyotic pathway. Two cleavage sites were identified in the smaller of two stable α3 cleavage fragments produced after cathepsin L treatment. The more predominant species was produced after cleavage between amino acids 250 and 251, and a lower abundance product was produced after cleavage between amino acids 243 and 244. It is possible that cleavage at one site precedes cleavage at the other, or both sites may be used, albeit with different efficiencies. Cathepsin L prefers large hydrophobic residues at the P2 positions for efficient cleavage (47). Cathepsin L cleavage sites in TIL α3 determined here conform to this preference. At the cleavage site between amino acids 250 and 251, phenylalanine is present at the P2 position; at the cleavage site between amino acids 243 and 244, the P2 position is occupied by leucine.

The first observed cleavage event after treatment of reovirus virions with either cathepsin B or cathepsin L in vitro is a α3 band of slightly faster electrophoretic mobility than full-length α3. Mass spectrometric analysis of the faster migrating species generated by cathepsin L treatment of TIL virions confirmed that it is indeed formed from α3. These findings suggest that an initial cleavage event occurs at a α3 terminus. Previous studies from our laboratory show that PI viruses isolated from L-cell cultures persistently infected with reovirus strain T3D (24, 30) and D-EA mutant viruses isolated from serial passage of T3D in the presence of cysteine protease inhibitor E64 (26) are more sensitive to proteolytic cleavage by either chymotrypsin or cathepsin L than parental strain T3D. Furthermore, PI and D-EA viruses, unlike wt T3D, are capable of growth in cells treated with E64. For all tested PI and D-EA viruses, the α3-encoding S4 gene is the primary genetic determinant of resistance to E64-mediated inhibition of viral growth. Remarkably, all PI and D-EA viruses subjected to genetic analysis have a tyrosine-to-histidine mutation at amino acid 354 in α3. For one of the PI
viruses and two of the D-EA viruses, histidine 354 is the only mutation in o3. These findings suggest that the C terminus of o3 plays an important role in determining susceptibility of the protein to proteolytic attack. In addition, strain-specific differences in o3 susceptibility to proteolysis have been mapped to the C-terminal third of o3 using chimeric o3 molecules (57). Based on these studies and the analysis of the previously determined crystal structure of T3D o3 (47), it seems most likely that the initial cleavage event in o3 occurs near the C terminus in the vicinity of amino acid 354. Importantly, the C-terminal region of o3 and the cathepsin L cleavage sites identified in this study are adjacent to each other in the T3D o3 crystal structure (Fig. 9B). Although the cathepsin L cleavage sites were identified in T1L o3, the deduced amino acid sequences of T1L and T3D differ at only 12 positions (58, 59), and thus, conclusions based on the structure of T3D o3 are likely to be applicable to T1L o3.

We hypothesize that the C-terminal region of o3 acts as a safety latch, controlling access to the more internal cleavage sites in o3 and subsequent conversion of viros to ISVPs. Because reovirus disassembly is an acid-dependent process, this latch might be primed for movement at acidic pH. The first o3 cleavage event may remove this safety latch and allow access to the cleavage sites identified in this study at residues 243–244 and 250–251. In viruses with mutations in the C-terminal region of o3, such as P1 and D-EA viruses, the safety latch may be altered by structural rearrangements. Alterations in structure have been observed by cryoelectron microscopy and three-dimensional image analysis of viruses with the tyrosine-to-histidine mutation at residue 354 in o3.3

Results presented in this report provide evidence that specific endocytic proteases play distinct roles in reovirus disassembly. To mediate virion-to-ISVP conversion, murine fibroblastic endocytic proteases play distinct roles in reovirus disassembly. Because reovirus disassembly is an acid-dependent process, the structure of T3D o3 is likely to be applicable to T1L o3.

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REFERENCES


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