

No Role for Pepstatin-A-Sensitive Acidic Proteinases in Reovirus Infections of L or MDCK Cells

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Strong evidence indicates that virions of mammalian reoviruses undergo proteolytic processing by acid-dependent cellular proteinases as an essential step in productive infection. Proteolytic processing takes the form of a series of cleavages of outer-capsid proteins $\sigma 3$ and $\mu 1/\mu 1C$. Previous studies showed an effect of both NH_4Cl and E-64 on these cleavages, indicating that one or more of the acid-dependent cysteine proteinases in mammalian cells (cathepsins B and L, for example) is required; however, these studies did not address whether acid-dependent aspartic proteinases in those cells (cathepsin D, for example) may also be required. To determine the role of aspartic proteinases in reovirus entry, studies with pepstatin A, a specific inhibitor of aspartic proteinases, were performed. The results showed that pepstatin A neither blocks nor slows reovirus infection of L or MDCK cells. Experiments using ribonuclease A and other proteins as cleavable substrates showed that cathepsin-D-like proteinases from these cells are inhibited within the tested range of pepstatin A concentrations both *in vitro* and within living cells. In other experiments, virion-bound $\sigma 3$ protein was shown to be a poor substrate for cleavage by cathepsin D *in vitro*, consistent with the findings with inhibitors. In sum, the data indicate that cathepsin-D-like aspartic proteinases provide little or no activity toward proteolytic events required for infection of L or MDCK cells with reovirus virions. © 1998 Academic Press

INTRODUCTION

The virions of mammalian orthoreoviruses (reoviruses) are nonenveloped and contain eight viral proteins arranged in two concentric icosahedral capsids surrounding the viral genome of 10 segments of double-stranded RNA. The earliest steps by which reovirus virions enter cells to initiate productive infection include attachment to cell-surface receptors, uptake by receptor-mediated endocytosis, and delivery into acidic endosomal and lysosomal vacuoles (Silverstein and Dales, 1968; Sturzenbecker *et al.*, 1987; Virgin *et al.*, 1994). Soon after infection with virions, proteolytic processing of outer-capsid proteins $\sigma 3$ and $\mu 1/\mu 1C$ can be demonstrated, yielding subvirion particles that resemble ones generated *in vitro* by digestion with alkaline proteinases (infectious subvirion particles, or ISVPs) (Silverstein *et al.*, 1970; Chang and Zweerink, 1971; Shatkin and LaFiandra, 1972). These particles are characterized by degradative removal of $\sigma 3$ and limited cleavage of $\mu 1/\mu 1C$ to yield defined, particle-bound fragments (Nibert and Fields, 1992). Weak bases like NH_4Cl that can raise the pH in acidic vacuoles and proteinase inhibitors like E-64 that are selective for cer-

tain cysteine proteinases (Seglen, 1983) have been shown to block productive infections with reovirus virions in a manner that correlates with the degree to which cleavages of $\sigma 3$ and $\mu 1/\mu 1C$ are blocked (Sturzenbecker *et al.*, 1987; Baer and Dermody, 1997). These and other findings suggest that the proteolytic processing of $\sigma 3$ and $\mu 1/\mu 1C$ are required events for infection by reovirus virions and are mediated at least in part by one or more of the acid-dependent cysteine proteinases that reside in late endosomes and lysosomes (Bohley and Seglen, 1992; Hasilik, 1992). Since recent work provided evidence that the cleavage of $\mu 1/\mu 1C$ during reovirus entry is dispensable for infection (Chandran and Nibert, 1998), attention might now be focused on cleavages of $\sigma 3$ as the required ones.

The extent of $\sigma 3$ cleavage that must occur prior to subsequent steps in infection, including membrane penetration and activation of the viral transcriptase (reviewed by Nibert and Fields, 1994), remains uncertain. This is true in terms of both the number and sites within each $\sigma 3$ molecule and the number and positions of different $\sigma 3$ molecules within the outer capsid that must be cleaved. Some evidence suggests that the proteolytic processing of $\sigma 3$ during reovirus entry is a stepwise process, involving cleavage of $\sigma 3$ at several, or even multiple, sites within its primary sequence before the virus particle can proceed with infection. For example, Virgin *et al.* (1994), using $\sigma 3$ -specific monoclonal anti-

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bodies that bind to reovirus virions and interfere with $\sigma 3$ processing both *in vitro* and *in vivo*, showed that certain antibodies blocked infection but allowed limited cleavage of $\sigma 3$ such that defined fragments of that protein were generated. The authors speculated that initial cleavages of $\sigma 3$ were free to occur in the presence of antibody but later cleavages of $\sigma 3$ (and $\mu 1/\mu 1C$) were blocked, suggesting that one or more of these later cleavages are also necessary for infection.

One hypothesis for the manner of $\sigma 3$ cleavage during reovirus entry into cells is that several of the acid-dependent proteinases in late endosomes and lysosomes, including the cysteine proteinases cathepsins B and L and the aspartic proteinase cathepsin D (Bohley and Seglen, 1992; Hasilik, 1992), act in concert to mediate whichever cleavages of $\sigma 3$ are necessary for infection. The participation of several proteinases might be redundant in that they might readily substitute for one another in cleaving $\sigma 3$. Alternatively, it might be that a particular proteinase is required to mediate cleavage at a specific site in $\sigma 3$. Such specificity would reflect elements of both $\sigma 3$ —its primary sequence at the cleavage site and the structural context in which that site is presented in virion-bound protein—and the proteinase—its cleavage specificity and the structure of the catalytic region defining whether the proteinase can access the cleavage site in $\sigma 3$. To have a better understanding of reovirus entry into cells, additional information is needed on how the different acid-dependent proteinases in cells might act in combination to mediate the required cleavages of $\sigma 3$.

Previous experiments with E-64 suggested that one or more of the lysosomal cysteine proteinases is required for infections of L cells by reovirus virions (Sherry *et al.*, 1996; Baer and Dermody, 1997). However, those experiments did not address whether a lysosomal aspartic proteinase like cathepsin D might also be required. To test this possibility we used a well-characterized, reversible inhibitor of cathepsin D, pepstatin A (Dean and Barrett, 1976), to determine its effects on reovirus infections.

RESULTS

Pepstatin A shows no effect on reovirus growth in L or MDCK cells

L cells were pretreated with different concentrations of pepstatin A for 2 h followed by absorption with virions or ISVPs of reovirus T1L. Infected cells were then incubated for 36 h to permit viral growth in the presence of the respective concentrations of pepstatin A. Determinations of the yields of infectious progeny (Fig. 1A) demonstrated that pepstatin A, even at the highest concentration tested (300 μM), had little or no effect on progeny yields after infection with either particle type, suggesting that a cathepsin-D-like proteinase activity is not required for these infections. These findings are in contrast to the

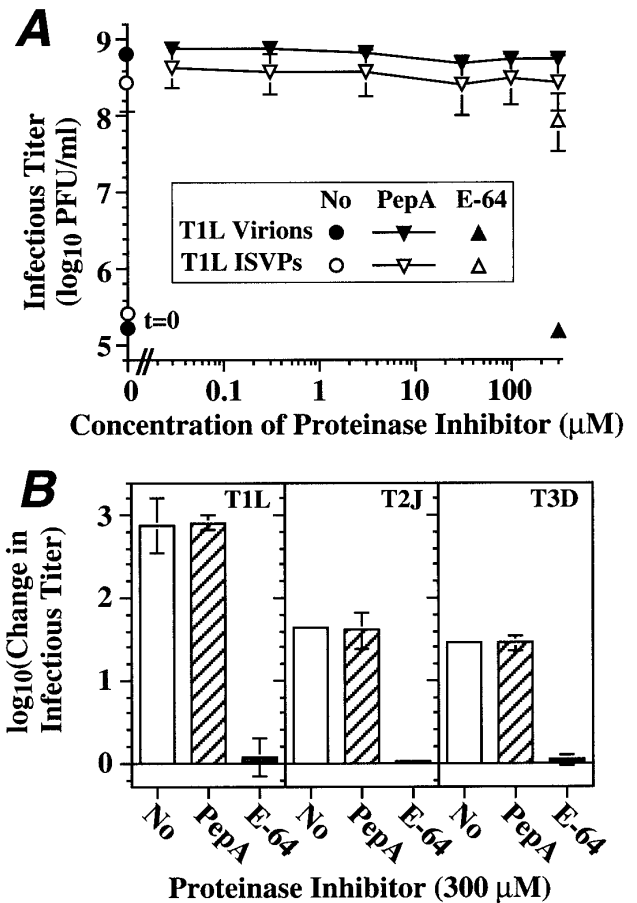


FIG. 1. Effect of pepstatin A (PepA) on yields of infectious viral progeny at 36-h p.i. of L or MDCK cells with reovirus virions or ISVPs. Each data point represents the mean (\pm standard deviation) for three independent trials. (A) L cells were pretreated with pepstatin A (30 nm to 300 μM), E-64 (300 μM), or no inhibitor for 2 h before infection with purified virions or ISVPs of reovirus T1L. Samples labeled $t = 0$ were harvested for titrating immediately after the adsorption period. (B) MDCK cells were pretreated with 300 μM pepstatin A, 300 μM E-64, or no inhibitor for 2 h before infection with purified virions of reoviruses T1L, T2J, or T3D. In (B), infectious titers are expressed relative to the mean titer obtained for samples of each strain that were harvested immediately after the adsorption period. Rodgers *et al.* (1997) previously showed that reovirus T3D grows to lower titers in MDCK cells than T1L.

nearly total inhibition of growth seen after infection with virions, but not ISVPs, in the presence of 300 μM E-64 (Fig. 1A), a cysteine proteinase inhibitor known to have those effects (Baer and Dermody, 1997; Chandran and Nibert, 1998). In related experiments, 300 μM pepstatin A was shown to have little or no effect on yields at 36 h p.i. of L cells with virions of reoviruses type 3 Dearing (T3D) or type 2 Jones (T2J) (data not shown). In addition, 300 μM pepstatin A was found to have little or no effect on progeny yields at 36 h after infection with these reoviruses in MDCK cells (Fig. 1B). The last two experiments demonstrate that the apparent dispensability of a cathepsin-D-like activity for infection is unique to neither reovirus T1L nor L cells.

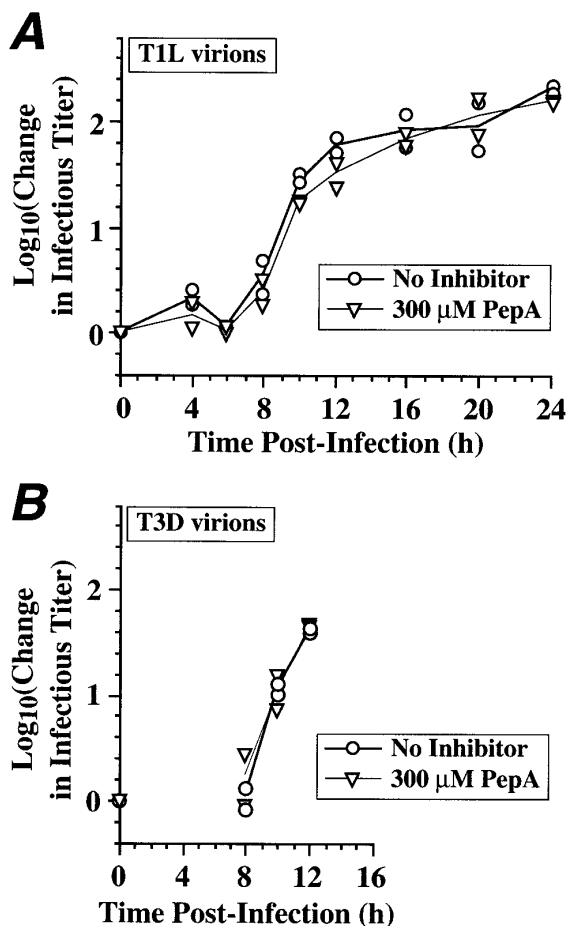


FIG. 2. Single-cycle growth curves for infection of L cells with reovirus virions in the presence or absence of pepstatin A (PepA). For each series, two independent samples were analyzed for each time point as shown, and lines are drawn through the means of the duplicate determinations. (A) Reovirus T1L virions were used for infection, and pepstatin A was added to cells immediately after the adsorption period. (B) Reovirus T3D virions were used for infection, and pepstatin A was added for a 2-h pretreatment before the adsorption period.

The preceding analyses of viral growth after 36 h in L or MDCK cells left open the possibility that pepstatin A might have an effect at earlier times postinfection, without affecting the later yields of infectious progeny. To test this possibility, we monitored the infectious titers of cultures at regular intervals of 2–4 h after infection of L cells with reovirus T1L virions in the presence or absence of 300 μM pepstatin A. The results showed little or no difference over 24 h in such single-cycle growth experiments whether or not pepstatin A was added (Fig. 2A). Similar results were obtained after infection of L cells with T3D virions, although in those experiments the only time points examined were ones surrounding the exponential phase of growth (Fig. 2B). Yields during the exponential phase of growth were also examined after infection of MDCK cells with either T1L or T3D virions and revealed little or no effects of 300 μM pepstatin A (data not shown).

Shields *et al.* (1991) found evidence that pepstatin A reaches maximum concentrations in cultured cells only after 24- to 48-h incubation. We were thus concerned that pepstatin A might not have blocked infection in the preceding experiments (which included 0- to 2-h pretreatments with pepstatin A; see Figs. 1 and 2, legends) because it might not yet have achieved an effective concentration in the cells. To rule out that possibility, we also performed experiments in which L cells were pre-treated with 30 or 300 μM pepstatin A for 24 h and then infected with T1L virions. In these experiments once again, pepstatin A was found to have little or no effect on the yields of infectious progeny either during the exponential phase of growth or at 24 h p.i. (data not shown). In sum, the results of these experiments indicate that pepstatin A neither reduces the final (36-h) yields of infectious progeny nor slows the rate at which these yields develop after reovirus infections of L or MDCK cells, suggesting that a cathepsin-D-like activity plays little or no role in these infections. The capacity of pepstatin A to achieve maximal inhibitory effects after short times in our experiments may reflect that it reaches maximal concentrations in the endo/lysosomal compartments more rapidly than in the whole cell as analyzed by Shields *et al.* (1991).

L- and MDCK-cell lysates contain a cathepsin-D-like proteinase that is inhibited by pepstatin A

Despite extensive literature that pepstatin A is an effective inhibitor of cathepsin D (Dean and Barrett, 1976; Bohley and Seglen 1992), it was conceivable that it was not inhibiting this enzyme in L and MDCK cells. To test this possibility, we employed a characterized assay for cathepsin D activity in acidified cell lysates (Bogitish *et al.*, 1992). The assay uses SDS-polyacrylamide gel electrophoresis (SDS-PAGE) to monitor cleavage of a protein, bovine serum albumin (BSA) in most of our experiments, that is added to the lysates *in vitro*. To eliminate activities of acidic cysteine proteinases like cathepsins B and L, E-64 was added to the lysates prior to BSA. Despite the presence of E-64, cleavage of BSA was observed in lysates prepared from either L or MDCK cells (Fig. 3A, lanes 2 and 4), demonstrating that some other type of acidic proteinase was present. Moreover, when 1 μM pepstatin A was added to the lysates, cleavage of BSA was strongly inhibited (data not shown), indicating that a cathepsin-D-like acidic proteinase was mediating most of the *in vitro* cleavage.

To determine whether pretreating cells with pepstatin A had any effect on the cleavage of BSA in the preceding assay, we pretreated the cells with 300 μM pepstatin A before generating lysates. In those cases the lysates showed no activity at cleaving BSA (Fig. 3A, lanes 3 and 5). Using different concentrations of pepstatin A for pretreatments and monitoring BSA cleavage by SDS-PAGE,

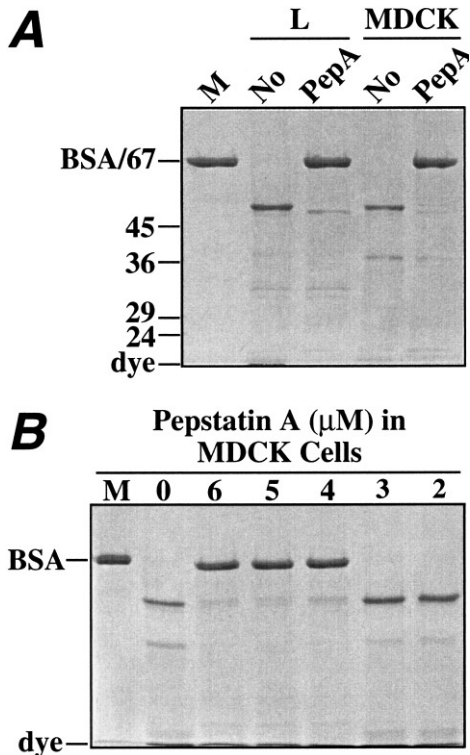


FIG. 3. Demonstration of cathepsin-D-like activity in acidified cell lysates. Ten percent SDS-polyacrylamide gels were used to monitor for cleavage of BSA that was added to the lysates in each experiment. (A) L or MDCK cells were pretreated for 2 h with (PepA) or without (No) 300 μM pepstatin A prior to preparation of acidified cell lysates. A mock-treated sample (M) containing BSA alone in reaction buffer (pH 3.8) was also included. Molecular weight markers are indicated at left. (B) An experiment was performed exactly as in (A) except that pepstatin A was added to the medium at different concentrations prior to preparation of acidified cell lysates. A mock-treated sample (M) as described in (A) was again included.

the concentration for 50% inhibition (IC_{50}) of the cathepsin-D-like proteinase activity in the lysates was estimated to be 0.3 μM for L cells (data not shown) and 3.5 μM for MDCK cells (Fig. 3B) for pepstatin A. These values are similar to ones reported in other studies of cathepsin D activity in cultured cells (Nishimura *et al.*, 1989). Experiments in which L cells were pretreated with different concentrations of pepstatin A for 24 h before preparation of lysates yielded a nearly identical IC_{50} for its effect on the cathepsin-D-like proteinase activity (data not shown), providing additional evidence that pepstatin A rapidly achieves its maximal inhibitory effects on this activity in L cells. Thus because the cathepsin-D-like activity in lysates of these cells was inhibited by pretreatment with pepstatin A at very low concentrations (IC_{50} 0.3 to 3.5 μM), whereas no effect on infections with reovirus virions was seen even at very high concentrations (300 μM), the findings suggest that pepstatin-A-sensitive acidic proteinases like cathepsin D are dispensable for reovirus infections in L or MDCK cells.

Pepstatin A inhibits cleavage of a protein substrate inside living cells

Experiments in the preceding section are limited in that they use cleavage of protein substrates mixed with cell lysates to correlate with cleavage of the virion-bound $\sigma 3$ protein inside living cells. To obtain evidence that a cathepsin-D-like proteinase is capable of cleaving proteins inside cells and that this cleavage can be blocked by adding pepstatin A, we performed the following experiments. A site-directed mutant of ribonuclease (RNase) A—which is known to undergo uptake and partial degradation inside cells (Hebert and Raines, unpublished data)—was covalently tagged with fluorescein at cysteine 19. The fluorescein-tagged RNase A was then added to cells, allowed to undergo uptake, and recovered in cell lysates for analysis by SDS-PAGE and fluorimaging. Decreasing recovery of full-length RNase A with increasing time was taken as evidence for its degradation (Fig. 4A). Nearly total inhibition of RNase A degradation was obtained by adding 20 mM NH_4Cl to the culture medium (Fig. 4B), consistent with involvement of the acid-dependent lysosomal proteinases. Partial inhibition of RNase A degradation was also obtained by adding either 30 μM pepstatin A or 30 μM E-64 to the culture medium (Fig. 4B), consistent with independent involvement of both aspartic and cysteine proteinases in the protein degradation. When pepstatin A and E-64 were added to cells together, nearly total inhibition of RNase A degradation, comparable to that seen with NH_4Cl , was observed (data not shown), indicating that the proteinases blocked by these inhibitors are the primary ones involved in degrading RNase A. When the effects of pepstatin A were examined in a concentration curve, the IC_{50} for its inhibition of RNase A degradation was shown to be near 0.2 μM (Fig. 4C), almost identical to that measured for L-cell lysates in the *in vitro* assay. Since the cathepsin D-like activity that cleaves RNase A in living cells was inhibited by treatment with pepstatin A at low concentrations (IC_{50} 0.2 μM), whereas no effect on infections with reovirus virions was seen even at very high concentrations (300 μM), the findings provide a firm demonstration that pepstatin-A-sensitive proteinases like cathepsin D are dispensable for reovirus infections in L cells.

The $\sigma 3$ protein in reovirus virions is a poor substrate for the cathepsin-D-like proteinases in cell lysates or for purified cathepsin D

There might be several reasons why the cathepsin-D-like activity that is demonstrable in L and MDCK cells plays no required role in reovirus entry. Two primary possibilities are that the reovirus $\sigma 3$ protein is a poor substrate for cathepsin D and that cathepsin D can cleave $\sigma 3$ but that its activity can be well substituted by a different proteinase, not sensitive to pepstatin A. To

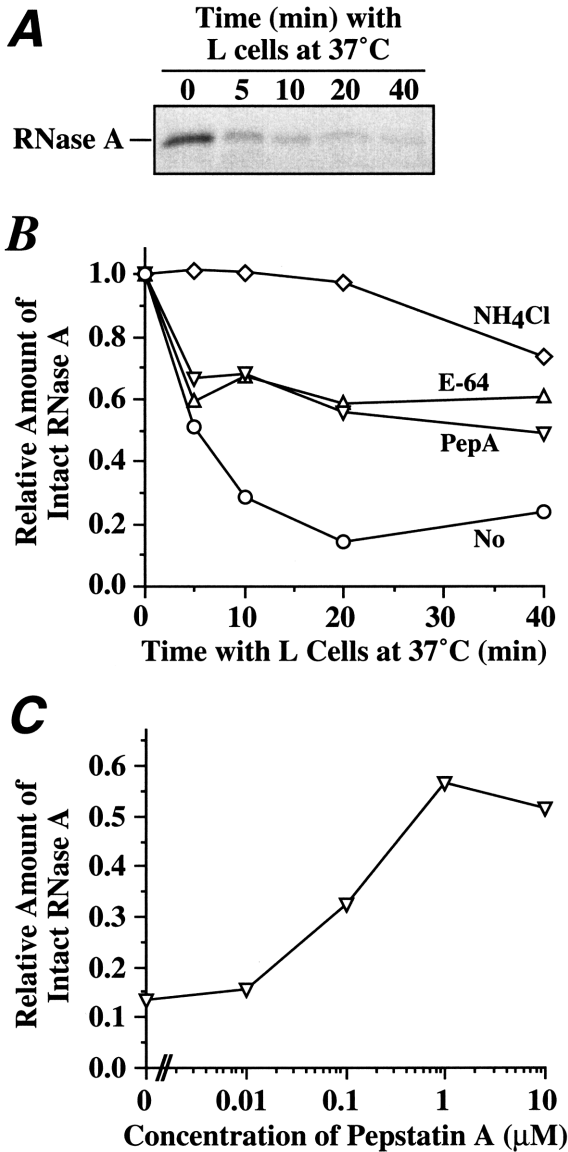


FIG. 4. Inhibition of cathepsin-D-like activity within L cells by pepstatin A. Fluorescently labeled RNase A was used for all experiments. (A) L cells were pretreated with 1% DMSO. RNase A was then added to the culture medium, and its time-dependent degradation was monitored. A representative fluor image is shown. (B) L cells were pretreated with 20 mM NH₄Cl, 30 μM E-64, 30 μM pepstatin A (PepA), or 1% DMSO (No) for 2 h at 37°C. Time-dependent degradation of RNase A was then monitored in the presence of the respective inhibitor. Each data point represents the mean value for three independent trials. The mean standard deviation for all data points was 0.12. (C) L cells were pretreated with different concentrations of pepstatin A, and degradation of RNase A was monitored at 30 min. The relative amount of intact RNase A was calculated as a percentage of that at the 0 min time point. Each data point represents the mean of two determinations.

address the first of these possibilities, we tested whether virion-bound $\sigma 3$ protein can be cleaved by the cathepsin-D-like proteinase(s) in acidified, E-64-treated lysates of either L or MDCK cells. We first demonstrated that two proteins tested in addition to BSA—hemoglobin (data not shown) and carbonic anhydrase (Fig. 5A)—were

cleaved in these lysates and that cleavage of each was inhibited by treating cells with 300 μM pepstatin A prior to lysis, indicating that a cathepsin-D-like proteinase was

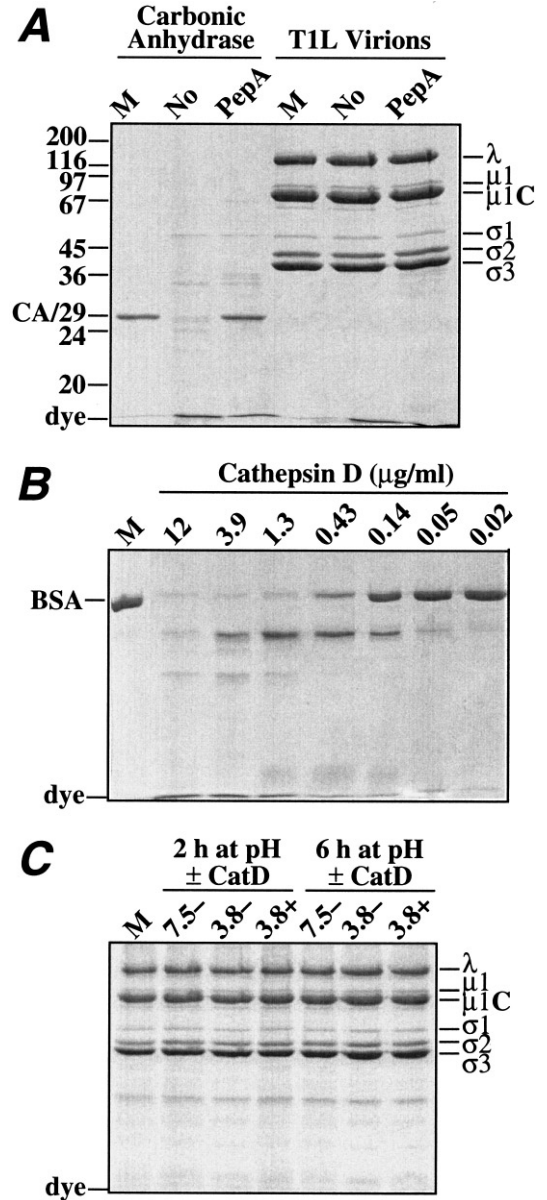


FIG. 5. Capacity of a cathepsin-D-like activity in cell lysates or in a commercial preparation of cathepsin D to cleave carbonic anhydrase, BSA, or the $\sigma 3$ protein in reovirus T1L virions. (A) An experiment with acidified cell lysates was performed exactly as in Fig. 3A except that either carbonic anhydrase or T1L virions were added as substrate. Proteins in the reaction mixtures were resolved in a 12% SDS-polyacrylamide gel. (B) BSA was mixed with different concentrations of cathepsin D (Sigma) in reaction buffer (pH 3.8) and incubated for 2 h at 37°C. A mock-treated sample (M) containing BSA alone in reaction buffer was also incubated for 2 h at 37°C. Proteins were visualized by Coomassie staining after electrophoresis. (C) Purified [³⁵S]methionine/cysteine-labeled T1L virions were mixed with (+) or without (-) 350 μg/ml cathepsin D (CatD) in virion buffer (pH 7.5) or reaction buffer and incubated at 37°C for 2 or 6 h. Viral proteins (labeled at right) were visualized by phosphor imaging after electrophoresis. A sample containing the same number of untreated virions was run on the gel as a marker (M).

the active agent. Nevertheless, when reovirus T1L virions were added to lysates from either L cells (data not shown) or MDCK cells (Fig. 5A), little or no cleavage of $\sigma 3$ was observed. The same results were obtained using T2J and T3D virions (data not shown). These findings suggest that pepstatin A has no effect on reovirus infections in L and MDCK cells at least partly because the intact, virion-bound $\sigma 3$ protein is a poor substrate for the cathepsin-D-like acidic proteinase(s) in these cells.

As an additional test of the preceding conclusion, we used purified cathepsin D for treatment of reovirus virions under acidic conditions *in vitro*. Cleavage of BSA at a rate comparable to that with L- or MDCK-cell lysates was seen with a 1.3- $\mu\text{g}/\text{ml}$ (40 nM) concentration of this enzyme (Fig. 5B), and the cleavage was fully inhibited with 1 μM pepstatin A (data not shown). Nevertheless, even with the highest concentration of cathepsin D tested (350 $\mu\text{g}/\text{ml}$, 10 μM), little or no cleavage of $\sigma 3$ in virions of reoviruses T1L (Fig. 5C), T2J (data not shown), or T3D (data not shown) was seen. These results provide direct evidence that virion-bound $\sigma 3$ is a poor substrate for cathepsin D.

DISCUSSION

Current conclusions regarding cellular proteinases required for reovirus entry

This paper provides new evidence that pepstatin-A-sensitive aspartic proteinases, including cathepsin D, play little or no role in the cleavages of virion-bound $\sigma 3$ that are required for reovirus entry into L or MDCK cells. The role of cathepsin-D-like proteinases in reovirus infection had not been resolved before these studies, in that previous evidence that lysosomal cysteine proteinases are required for $\sigma 3$ processing (Sherry *et al.*, 1996; Baer and Dermody, 1997; Chandran and Nibert, 1998) did not rule out the possibility that an aspartic proteinase like cathepsin D might also be required for particular cleavages of $\sigma 3$. In the current study we demonstrated that cathepsin-D-like proteinases in L and MDCK cells are blocked at concentrations of pepstatin A well below those that continue to show no effects on reovirus infection, indicating that such proteinases are not required for reovirus infection of these cells and that they in fact contribute little activity toward the necessary proteolytic cleavages of reovirus proteins. These findings can be combined with evidence that one or more cysteine proteinase is essential for $\sigma 3$ processing during reovirus entry in attempts to identify the full complement of proteinases and their sites of cleavage in $\sigma 3$ during the entry process. It remains to be determined which of the cysteine proteinases in mammalian lysosomes, including cathepsins B and L (Mason and Wilcox, 1993; Chapman *et al.*, 1997), are specifically required. In addition, it remains possible that participation of cathepsin D in $\sigma 3$ cleavage might be demonstrated with other cell types or

other reovirus strains, such as certain cells in host animals or cells and viruses that have undergone coevolution during persistent reovirus infection (Dermody *et al.*, 1993).

Analysis of results with proteinase inhibitors

Selective chemical inhibitors can be useful tools for determining the roles of particular proteinases in cellular or viral processes. Studies of the caspases involved in apoptosis provide good examples of the utility of such inhibitors (Villa *et al.*, 1997). Similarly, evidence for the role of particular lysosomal proteinases in degrading the invariant chain of class II MHC has been obtained using selective inhibitors (Villadangos *et al.*, 1997). To interpret the results obtained with a proteinase inhibitor, it is important to show that the inhibitor is affecting its intended target(s) inside cells (Villadangos *et al.*, 1997). In the current study, having found that pepstatin A had little or no effect on reovirus infections, we were concerned that the inhibitor might not be inhibiting the cathepsin-D-like proteinases in cells or that such proteinases might not be present, either of which possibility would confound our interpretation of the infection results. We thus performed auxiliary experiments to demonstrate the presence and activity of cathepsin-D-like proteinases in the cells and the concentration of pepstatin A at which they were inhibited. These experiments provide an example of how assays for proteinase activity and inhibition can be coupled with biological assays to permit results with proteinase inhibitors to be interpreted with confidence.

Why can't cathepsin D cleave $\sigma 3$?

Evidence in this study demonstrates that the intact $\sigma 3$ protein in virions of reoviruses T1L, T2J, and T3D is a poor substrate for cleavage by cathepsin D *in vitro* at conditions that approximate those inside lysosomes and that promote the rapid cleavage of other protein substrates by this enzyme. A likely explanation is that the primary sequences and three-dimensional structures of the proteinase-sensitive regions of virion-bound $\sigma 3$ and the active-site regions of cathepsin D are improperly matched for $\sigma 3$ to be cleaved. While we have not directly shown in this study that cathepsin D is incapable of mediating any required cleavages of $\sigma 3$ that occur later in the degradation pathway for this protein (after earlier cleavages by other proteinases) (Virgin *et al.*, 1994), the finding that pepstatin A neither blocks nor slows infection by these viruses suggests that the activity of cathepsin D at mediating any such later cleavages is readily substituted by that of other proteinases. Other factors that might contribute to the negligible effect of pepstatin A on reovirus infection, however, include the possibilities that neither cathepsin D nor any other aspartic proteinase is present at the appropriate levels or in the appro-

appropriate cellular compartments to contribute substantially to $\sigma 3$ cleavage during reovirus entry. In any case, the insensitivity of intact, virion-bound $\sigma 3$ protein to cleavage by cathepsin D *in vitro* is striking and begs a structure-based explanation.

MATERIALS AND METHODS

Cells

Spinner-adapted murine L929 cells (L cells) were maintained in suspension in Joklik's modified minimal essential medium (Irvine Scientific, Irvine, CA) supplemented to contain 2% fetal bovine serum (Hyclone Laboratories, Logan, UT), 2% neonatal bovine serum (Hyclone), 2 mM glutamine, 100 U/ml penicillin, and 100 $\mu\text{g/ml}$ streptomycin (Irvine). For monolayer cultures, 1% FungiBact (Irvine) was also added. MDCK cells were grown as monolayers in 10% fetal bovine serum, 2 mM glutamine, 100 U/ml penicillin, and 100 $\mu\text{g/ml}$ streptomycin. For experiments involving cell monolayers in two-dram vials, 5×10^5 cells were added per vial.

Viruses

Purified virus particles of reovirus strain T1L were used for most experiments, but strains T2J and T3D were also used where specified. Purified virions were obtained and stored in virion buffer as described (Nibert and Fields, 1992). Particle concentrations in purified virion preparations were measured as described (Smith *et al.*, 1969). To generate purified virions containing [^{35}S]methionine/cysteine-labeled proteins, Tran ^{35}S -label (12.5 $\mu\text{Ci/ml}$; ICN Biochemicals, Costa Mesa, CA) was added to the virus-cell suspension at the start of infection. ISVPs were obtained from purified virions by chymotrypsin treatment and were later purified by centrifugation in CsCl gradients as described (Nibert and Fields, 1992). Plaque assays to determine titers of infectious particles in purified and experimental cell lysate stocks were performed as described (Nibert and Fields, 1992).

Proteinase inhibitors

Proteinase inhibitors E-64 and pepstatin A (Sigma Chemical Co., St. Louis, MO) were dissolved in dimethyl sulfoxide (DMSO) for stock solutions. For samples receiving no inhibitor, DMSO was added to a final concentration of 1%.

SDS-PAGE

Samples were prepared for SDS-PAGE as described (Nibert and Fields, 1992). SDS-PAGE was carried out in 10 or 12% acrylamide gels, and proteins were visualized by staining with Coomassie brilliant blue R-250 (Sigma). Gels loaded with radiolabeled proteins were dried onto filter paper and visualized by phosphor imaging (Molecular Dynamics, Sunnyvale, CA).

End-point analyses for reovirus growth

L- or MDCK-cell monolayers in two-dram vials were pretreated with or without proteinase inhibitor at the specified concentrations for 2 or 24 h at 37°C, at which time the media were removed, the cells were chilled to 4°C, and purified reovirus virions or ISVPs were added at 2 PFU/cell. After 1-h incubation at 4°C to permit virus absorption, media containing the respective concentrations of inhibitors were added back to the monolayers, which were then moved to 37°C to permit virus entry and replication. At specified times postinfection, cultures were harvested, subjected to freezing and thawing to release virus, and used for plaque assays to measure titers of infectious progeny.

Growth curves

Complete single-cycle growth curves (e.g., Fig. 2A) were performed as described (Chandran and Nibert, 1998) except that 300 μM pepstatin A was added to the growth media for specified samples at the end of the absorption period. For single-cycle growth curves analyzing yields of infectious progeny during the exponential phase of growth only (e.g., Fig. 2B), samples were generated as for end-point analyses, including 2- or 24-h pretreatment of cells with the specified inhibitor, and were then harvested at different times in the range of 8–16 h.

Cleavage of proteins *in vitro* by acidified cell lysates

L- or MDCK-cell monolayers in two-dram vials were pretreated for 2 or 24 h at 37°C with different concentrations of pepstatin A (0–300 μM). The media were then removed, and the monolayers were washed extensively with phosphate-buffered saline (PBS) (8.4 mM Na_2HPO_4 , 1.6 mM KH_2HPO_4 , 137 mM NaCl; 2.9 mM KCl, 2 mM MgCl_2 ; pH 7.5) to remove excess inhibitor. Reaction buffer (100 μl ; containing 100 mM potassium acetate, 5 mM MgCl_2 , 5 mM cysteine; pH 3.8) was added to each vial and subjected to three cycles of freezing and thawing. Debris from the disrupted cells was removed by centrifugation at 16,000 *g* for 5 min at 4°C. To the resulting supernatant (20 μl), 1 μM E-64 was added to inhibit acid-dependent cysteine proteinases that may have been present. BSA (250 $\mu\text{g/ml}$), carbonic anhydrase (200 $\mu\text{g/ml}$), or reovirus T1L virions (1×10^{13} particles/ml) were added and incubated for 2 h at 37°C to permit cleavage of proteins. After incubation, proteins were resolved on a 10 or 12% SDS-polyacrylamide gel and visualized by Coomassie staining.

Purification of fluorescently labeled A19C RNase A

pBXR is a plasmid that directs the expression of wild-type RNase A in *Escherichia coli* (delCardayré *et al.*, 1995). Oligonucleotide-mediated site-directed mutagen-

esis was performed on single-stranded DNA to produce the gene encoding RNase A with an alanine-to-cysteine substitution at position 19 (A19C) (Hebert and Raines, unpublished). A19C RNase A was purified according to described methods (Kim and Raines, 1993; delCardayré *et al.*, 1995) except that solutions were kept under argon gas. The sulphhydryl group of cysteine 19 was protected from oxidation by treatment with 5,5-dithio-bis (2-nitrobenzoic acid) (DTNB; Sigma) (Messmore *et al.*, 1995) and then deprotected by the addition of dithiothreitol (fivefold molar excess; Fisher; Fair Lawn, NJ) at pH 9.0. Deprotected A19C RNase A was modified by reaction with 5-iodoacetamidofluorescein (5-IAF; Molecular Probes; Eugene, OR). In brief, 5-IAF (20-fold molar excess) was added to deprotected A19C RNase A, and the mixture was incubated at 25°C for 10 min. Excess 5-IAF was removed by gel filtration chromatography using a NICK column (Pharmacia Biotech; Piscataway, NJ).

RNase A degradation assay

L cells were added to two-dram vials in the presence of varying concentrations (0–30 μM) of proteinase inhibitor. Cells were incubated at 37°C for 2 h. The media were removed and fluorescently labeled A19C RNase A was added (final concentration 1 μM) for a 30-min incubation at 4°C. Cells were washed, and fresh media containing the respective inhibitor concentrations were added. Cells were incubated at 37°C, and samples were removed at specified times. Cells were washed and resuspended in 100 μl lysis buffer [100 mM potassium acetate, 5 mM MgCl_2 ; 5 mM cysteine; and 1 mM mammalian proteinase inhibitor cocktail (Sigma); pH 3.8]. Cell lysis was achieved by three cycles of freezing and thawing. The lysate was precipitated by addition of an equal volume of 10% (v/v) trichloroacetic acid. The protein pellet was analyzed by SDS-PAGE. Fluorescent A19C RNase A was detected by fluor imaging (Molecular Dynamics), and data were analyzed with the computer program IMAGEQUANT (Molecular Dynamics).

Cleavage of proteins *in vitro* with purified cathepsin D

Cathepsin D (bovine spleen, Sigma) was prepared at 3.5 mg/ml (100 μM) in water and stored in frozen aliquots. BSA (250 $\mu\text{g/ml}$) or purified [^{35}S]methionine/cysteine-labeled T1L virions (5×10^{12} particles/ml) were mixed with different concentrations of cathepsin D in virion buffer (pH 7.5) or reaction buffer (pH 3.8) and incubated at 37°C for 2 or 6 h. Proteins were then resolved in a 10% SDS-polyacrylamide gel and visualized by Coomassie staining or phosphor imaging.

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REFERENCES

- Baer, G. S., and Dermody, T. S. (1997). Mutations in reovirus outer-capsid protein $\sigma 3$ selected during persistent infections of L cells confer resistance to protease inhibitor E64. *J. Virol.* **71**, 4921–4928.
- Bogitish, B. J., Kirschner, K. F., and Rotmans, J. P. (1992). *Schistosoma japonicum*: Immunochemistry studies on hemoglobin digestion using heterologous antiserum to bovine cathepsin D. *J. Parasitol.* **78**, 454–459.
- Bohley, P., and Seglen, P. O. (1992). Proteases and proteolysis in the lysosomes. *Experientia* **48**, 151–157.
- Chandran, K., and Nibert, M. L. (1998). Protease cleavage of reovirus capsid protein $\mu 1/\mu 1\text{C}$ is blocked by alkyl sulfate detergents, yielding a new type of infectious subviral particle. *J. Virol.* **72**, 467–475.
- Change, C., and Zweerink, H. J. (1991). Fate of parental reovirus in infected cells. *Virology* **46**, 544–555.
- Chapman, H. A., Riese, R. J., and Shi, G. P. (1997). Emerging roles for cysteine proteases in human biology. *Annu. Rev. Physiol.* **59**, 63–88.
- Dean, R. T., and Barrett, A. J. (1976). Lysosomes. *Essays Biochem.* **12**, 1–40.
- delCardayré, S. B., Ribó, M., Yokel, E. M., Quirk, D. J., Rutter, W. J., and Raines, R. T. (1995). Engineering ribonuclease A: Production, purification, and characterization of wild-type enzyme and mutants at Gln 11. *Protein Eng.* **8**, 261–273.
- Dermody, T. S., Nibert, M. L., Wetzel, J. D., Tong, X., and Fields, B. N. (1993). Cells and viruses with mutations affecting viral entry are selected during persistent infection of L cells by mammalian reoviruses. *J. Virol.* **67**, 2055–2063.
- Hasilik, A. (1992). The early and late processing of lysosomal enzymes: Proteolysis and compartmentation. *Experientia* **48**, 130–151.
- Kim, J.-S., and Raines, R. T. (1993). Bovine seminal ribonuclease produced from a synthetic gene. *J. Biol. Chem.* **268**, 17392–17396.
- Mason, R. W., and Wilcox, D. (1993). Chemistry of lysosomal cysteine proteinases. *Adv. Cell Mol. Biol. Membr.* **1**, 81–116.
- Messmore, J. M., Fuchs, D. N., and Raines, R. T. (1995). Ribonuclease A: Revealing structure-function relationships with semisynthesis. *J. Am. Chem. Soc.* **117**, 8057–8060.
- Nibert, M. L., and Fields, B. N. (1992). A carboxy-terminal fragment of protein $\mu 1/\mu 1\text{C}$ is present in infectious subviral particles of mammalian reoviruses and is proposed to have a role in penetration. *J. Virol.* **66**, 6408–6418.
- Nibert, M. L., and Fields, B. N. (1994). Early steps in reovirus infection of cells. In "Cellular Receptors of Animal Viruses" (E. Wimmer, Ed.), pp. 341–364. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
- Nishimura, Y., Kawabata, T., Furuno, K., and Kato, K. (1989). Evidence that aspartic proteinase is involved in the proteolytic processing event of procathepsin L in lysosomes. *Arch. Biochem. Biophys.* **271**, 400–406.
- Rodgers, S. E., Barton, E. S., Oberhaus, S. M., Pike, B., Gibson, C. A., Tyler, K. L., and Dermody, T. S. (1997). Reovirus-induced apoptosis of MDCK cells is not linked to viral yield and is blocked by Bcl-2. *J. Virol.* **71**, 2540–2546.
- Seglen, P. O. (1983). Inhibitors of lysosomal function. *Methods Enzymol.* **96**, 737–764.
- Shatkin, A. J., and LaFiandra, A. J. (1972). Transcription by infectious subviral particles of reovirus. *J. Virol.* **10**, 698–706.
- Sherry, B., Baty, C. J., and Blum, M. A. (1996). Reovirus-induced acute myocarditis in mice correlates with viral RNA synthesis rather than generation of infectious virus in cardiac myocytes. *J. Virol.* **70**, 6709–6715.

- Shields, P. P., Gonazales, T. A., Charles, D., Gilligan, J. P., and Stern, W. (1991). Accumulation of pepstatin in cultured endothelial cells and its effect on endothelin processing. *Biochem. Biophys. Res. Commun.* **177**, 1006–1012.
- Silverstein, S. C., and Dales, S. (1968). The penetration of reovirus RNA and initiation of its genetic function in L-strain fibroblasts. *J. Cell Biol.* **36**, 197–230.
- Silverstein, S. C., Schonberg, M., Levin, D. H., and Acs, G. (1970). The reovirus replicative cycle: Conservation of parental RNA and protein. *Proc. Natl. Acad. Sci. USA* **67**, 275–281.
- Smith, R. E., Zweerink, H. J., and Joklik, W. K. (1969). Polypeptide components of virions, top component and cores of reovirus type 3. *Virology* **39**, 791–810.
- Sturzenbecker, L. J., Nibert, M., Furlong, D., and Fields, B. N. (1987). Intracellular digestion of reovirus particles require a low pH and is essential step in the viral infectious cycle. *J. Virol.* **61**, 2351–2361.
- Villa, P., Kaufmann, S. H., and Earnshaw, W. C. (1997). Caspases and caspase inhibitors. *Trends Biochem. Sci.* **22**, 388–393.
- Villadangos, J. A., Riese, R. J., Peters, C., Chapman, H. A., and Ploegh, H. L. (1997). Degradation of mouse invariant chain: Roles of cathepsins S and D and the influence of major histocompatibility complex polymorphism. *J. Exp. Med.* **186**, 549–560.
- Virgin, H. W., IV, Mann, M. A., and Tyler, K. L. (1994). Protective antibodies inhibit reovirus internalization and uncoating by intracellular proteases. *J. Virol.* **68**, 6719–6729.