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Antiviral Effects of a Thiol Protease Inhibitor on Foot-and-Mouth Disease Virus

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The thiol protease inhibitor E-64 specifically blocks autocatalytic activity of the leader protease of foot-and-mouth disease virus (FMDV) and interferes with cleavage of the structural protein precursor in an in vitro translation assay programmed with virion RNA. Experiments with FMDV-infected cells and E-64 or a membrane-permeable analog, E-64d, have confirmed these results and demonstrated interference in virus assembly, causing a reduction in virus yield. In addition, there is a lag in the appearance of virus-induced cellular morphologic alterations, a delay in cleavage of host cell protein p220 and in shutoff of host protein synthesis, and a decrease in viral protein and RNA synthesis. The implications of using E-64-based compounds as potential antiviral agents for FMDV are discussed.

Foot-and-mouth disease virus (FMDV) is a pathogen that affects a wide range of cloven-hoofed animals of agricultural and economic importance throughout the world. It is a member of the *Aphthovirus* genus of the *Picornaviridae* family of single-stranded, positive-sense RNA viruses. The RNA is translated into a polyprotein which is processed by virus-encoded proteases (18, 23, 32, 34, 40). The virus genome codes for two known proteases. The leader (L) protease, which is located at the amino terminus of the viral polyprotein, autocatalytically cleaves itself from the structural protein precursor, P1 (41), exposing the amino-terminal myristoylation site on VP4 (5). The second protease, 3C, processes most of the other viral proteins including the capsid proteins (47). Unlike in other picornaviruses, the putative 2A protease of FMDV is a peptide containing only 16 amino acids, but it has been implicated in the cleavage between itself and 2B (3, 38, 39). The final steps in virus maturation occur when viral RNA is inserted into the capsid and VP0 is cleaved into VP4 and VP2 by unknown mechanisms.

Within the picornaviruses, only the aphthovirus and cardiovirus genera encode L proteins. Unlike the L of FMDV, the cardiovirus L protein is processed from the P1 precursor by the virus-encoded 3C protease (35). The amino acid sequences of the two L proteins are very different, but limited homology does exist at their amino and carboxy termini. Among the serotypes of FMDV, L proteases share greater than 90% homology at the amino acid level (20a, 37).

The L protease of FMDV is also involved in the cleavage of p220, a subunit of the cap-binding protein complex involved in the initiation of translation at the 5′ end of most eukaryotic mRNAs (6). It is unclear whether FMDV L plays a direct or indirect role in the cleavage of p220. This is in contrast to the poliovirus 2A protease, which has been shown to indirectly induce p220 cleavage via alteration or activation of a second protease of cellular origin (22, 27, 28). Cleavage of p220 results in shutoff of cap-dependent host protein synthesis (8). Since picornavirus mRNA does not contain a cap structure, its translation is not affected by cleavage of p220; this leaves the host translation machinery dedicated solely to the synthesis of viral proteins. The cardioviruses, which contain L and 2A proteins, do not induce cleavage of p220 but are able to shutoff host protein synthesis by outcompeting host mRNA for the protein synthesis machinery (27, 31).

The picornavirus 2A and 3C proteases share structural homology with the trypsin-like family of serine proteases (2). L does not share in this structural homology (49), even though poliovirus 2A and FMDV L share functional similarity in their involvement with p220 cleavage. L has been implicated by amino acid sequence alignment to be a thiol protease related to the papain family of proteases (9, 26).

The compound L-*trans*-epoxysuccinyl-leucylamido(4-guanidino)butane (E-64) is a specific inhibitor of thiol proteases, including the papain family of cysteine proteases (15–17). It is a natural product isolated from *Aspergillus japonicus* and has been successfully synthesized in the laboratory (15, 16). The mode of action of this compound involves the covalent and irreversible binding of the C-2 carbon of the epoxysuccinyl group to the cysteine residue at the active site of the protease (17, 30).

The E-64 compound is charged and has a molecular weight of 357. A number of analogs of E-64, which are more membrane permeable, have also been synthesized (16, 17, 30). The partially charged analog E-64c is more reactive than the parent compound in vivo and is believed to be the actual active form (43, 44). The uncharged analog, E-64d, is membrane permeable (30). The E-64 compound and its derivatives are nontoxic in animal studies when given in daily doses of up to 400 mg/kg of body weight successively for 1 month by various administration routes (14, 20, 21). We describe our studies to characterize the effect of E-64 and related compounds on the L protease of FMDV and viral replication. In addition, we discuss the potential of L as a target of antiviral drug therapy.

MATERIALS AND METHODS

Viruses and cells. FMDV types A12 strain 119ab and O1 strain Campos were used to infect a bovine kidney cell line, LF-BK (43).

Antiserum. Rabbit anti-L serum (aL serum) was a gift from Ewald Beck (42). The production of rabbit anti-VP2 (aVP2),

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anti-VP3 (aVP3), and FMDV bovine convalescent-phase serum has been described previously (12). The sera were used to immunoprecipitate proteins from in vitro translations and infected-cell cytoplasmic extracts (12, 13). Rabbit anti-p220 serum (ap220 serum) was a gift from Rick Lloyd, University of Oklahoma Health Science Center, Oklahoma City.

Protease inhibitors. Most protease inhibitors were purchased from Boehringer Mannheim. E-64c was obtained from Sigma Chemical Company, and E-64d was a gift of Thomas Detwiler, SUNY Health Science Center, Brooklyn, N.Y. MDL,28170 (CbzValPhe) was a gift of Shujaath Mehdi, Merrill Dow Pharmaceuticals (30). Protease inhibitors were dissolved in aqueous solution when possible. MDL,28170 and E-64d were dissolved in dimethyl sulfoxide.

In vitro translation. FMD virion RNA (A12, A5, O1 Caseros, C3 Resende, SAT-2, SAT-3, and Asia-1), purified by sucrose gradient centrifugation, was translated in a rabbit reticulocyte lysate in the presence of 10 μCi of [^{35}S]methionine (10, 11, 36). The products were analyzed by polyacrylamide gel electrophoresis (PAGE) on 15% gels run overnight at 9 to 10 mA (24) and processed for fluorography.

In vivo assays. LF-BK cells were grown at 37°C in 60-mm dishes containing minimal essential medium plus 10% calf serum. The cells were infected at a multiplicity of infection of 10 to 20 in the absence or presence of E-64 or E-64d. E-64 was used in the experiment at a concentration of 5 mg/ml. E-64d was used at a range of concentrations between 50 and 500 μg/ml and was added either at the time of infection or 1 h postinfection. When cytopathic effects (CPE) were observed in 50% of the infected, non-inhibitor-treated (control) cells, all cultures were washed and incubated for 1 h in medium without methionine and then radiolabeled with 50 to 100 μCi of [^{35}S]methionine for 1 to 1.5 h. Cytoplasmic extracts were prepared (13) and directly examined by PAGE. For immunoprecipitation, cytoplasmic extracts were preincubated with *Staphylococcus aureus* bearing protein A to minimize nonspecific protein binding. Following centrifugation, supernatants were immunoprecipitated with FMDV-specific antisera as previously described (12, 47). For analysis of capsid structures, infected cells were radiolabeled with [^{35}S]methionine or [^{3}H]uridine in the presence of 5 μg of actinomycin D per ml, and cytoplasmic extracts were prepared (12, 48). The samples were centrifuged on 10 to 50% (wt/vol) sucrose gradients in NET buffer (150 mM NaCl, 5 mM EDTA, 10 mM Tris-HCl [pH 7.5]) in an SW41 rotor at 18,000 rpm for 17 h at 4°C. Fractions were collected, and aliquots were precipitated with trichloroacetic acid and then counted in a liquid scintillation counter.

Plaque assays. Plaque assays were performed in duplicate in 35-mm six-well plates containing confluent LF-BK cells. Serial dilutions of cytoplasmic extracts or tissue culture supernatants from cells infected in the absence or presence of inhibitor were made in minimal essential medium. Aliquots of 100 μl were allowed to adsorb onto confluent LF-BK cells for 1 h, and the cells were washed twice with phosphate-buffered saline (PBS), covered with 2× minimal essential medium and 1% gum tragacanth (1:1), and incubated for 24 h at 37°C. Plates were stained and fixed with 0.2% crystal violet–7.4% formaldehyde for 1 h and washed with water, and the plaques were counted.

p220 assays. Following Western immunoblotting, the host cell protein p220 was detected immunologically as previously described (6, 46) by using a semidry blotter and nitrocellulose membranes. Blots were blocked with 3% nonfat dry milk in PBS and incubated overnight at room temperature with a 1:100 dilution of p220 antiserum in 3% dry milk–PBS. Antibody was detected by autoradiography after incubation with ^{125}I-labeled protein A/G.

TABLE 1. Protease inhibitors tested for interference with FMDV L autocatalytic activity[a]

Inhibitor[b]	Concn range[c]	Specificity	Processing of L[d]
Antipain	25–100 μg/ml	Papain and trypsin	−
Aprotinin	0.6–2.0 μg/ml	Serine proteases	−
Cystatin	1.0–125 μg/ml	Cysteine proteases	−
E-64	0.1–2.0 mg/ml	Thiol proteases	+
E-64c	0.1–1.0 mg/ml	Thiol proteases	+
E-64d	50–500 μg/ml	Ester of E-64c	±
Iodoacetamide	10–500 μg/ml	Thiol proteases	−
Leupeptin	0.1–10 μg/ml	Serine and thiol proteases	−
MDL,28170	4.0–400 μg/ml	Cysteine proteases	±
Pepstatin A	0.1–1.0 μg/ml	Acid proteases	−
PMSF	100–200 μg/ml	Serine and thiol proteases	−
TLCK	25–100 μg/ml	Serine proteases	−
TPCK	10–500 μg/ml	Serine proteases	−

[a] FMD A12 virion RNA was translated in an *in vitro* translation system in the presence of a series of protease inhibitors, and the products were examined by PAGE for the inhibition of L cleavage.

[b] PMSF, phenylmethylsulfonyl fluoride; TLCK, *N*-tosyl-L-lysine-chloromethyl ketone; TPCK, *N*-tosyl-L-phenylalanine chloromethyl ketone.

[c] Inhibitors were used at concentration ranges suggested by the supplier.

[d] −, did not inhibit L autocatalytic cleavage; +, completely blocked L autocatalytic cleavage; ±, partially blocked L autocatalytic cleavage (both processed L and unprocessed precursor, L/P1, were observed).

RESULTS

In vitro protease inhibitor studies. Thirteen protease inhibitors were tested in an in vitro rabbit reticulocyte lysate translation system programmed with FMDV A12 RNA (Table 1). Figure 1 shows that E-64, at a concentration of 0.5 mg/ml, inhibited both A12 and O1 Caseros L autocatalytic activity. This is suggested by the absence of a band corresponding to the size of L, a decrease in the level of VP0, and the presence of two new bands corresponding to L/P1 and L/VP0/VP3. Identical results were obtained by in vitro translation of FMDV A5, C3 Resende, SAT-2, SAT-3, and Asia-1 virion RNAs (data not shown). E-64c completely inhibited autocatalytic processing of L, whereas E-64d and MDL,28170 only partially inhibited this activity (Table 1).

To confirm immunologically the identity of the new proteins synthesized in the presence of inhibitor, we immunoprecipitated O1 Caseros RNA in vitro translation products with aVP2, aVP3, and aL serum. Both L/P1 and L/VP0/VP3 were precipitated by all three antisera (Fig. 2, lanes 4, 6, and 8). The absence of VP0 and L in E-64-treated translation reactions was also confirmed (Fig. 2, compare lane 3 with lane 4 and lane 7 with lane 8). The immunoprecipitation of VP0 by aVP3 serum reflects the presence of altered capsid structures in the cell-free system as previously demonstrated (11).

Effects on protein processing in vivo. LF-BK cells were infected with FMDV A12 in the absence or presence of 5.0 mg of E-64 per ml. Infected cells were radiolabeled with [^{35}S]methionine, and proteins were examined by PAGE. E-64 inhibited processing of L from P1; L and VP0 were absent; and a new band corresponding to the size of L/VP0/VP3 was observed (Fig. 3, lanes 2 and 3). The identity of

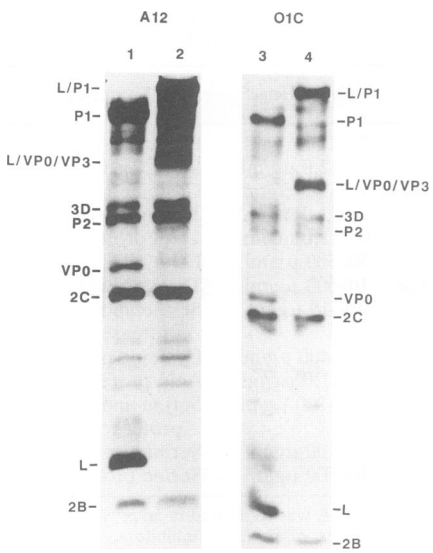

FIG. 1. Inhibition of FMDV L by E-64 in vitro. FMDV RNA was translated in a rabbit reticulocyte cell-free system in the absence (lanes 1 and 3) or presence (lanes 2 and 4) of 0.5 mg of E-64 per ml. Lanes: 1 and 2, FMDV A12 RNA; 3 and 4; FMDV O1 Caseros RNA. Lysates were analyzed by PAGE on a 15% polyacrylamide gel.

L/VP0/VP3 was confirmed by immunoprecipitation with aVP2 and aL serum (lanes 8 to 13).

A more membrane-permeable analog of E-64, E-64d, was used to determine whether similar effects on L autocatalytic

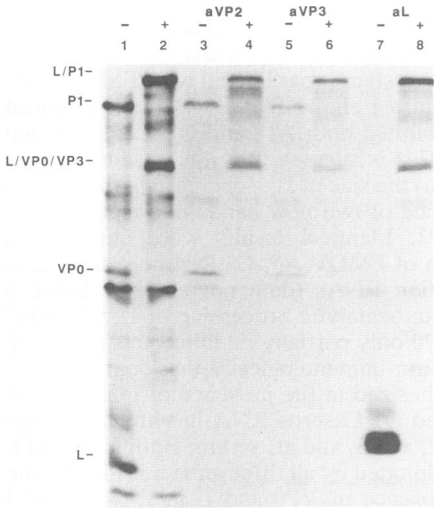

FIG. 2. Immunoprecipitation of in vitro FMDV translation products in the absence (−) or presence (+) of E-64. Radiolabeled in vitro translation reactions programmed with FMDV O1 Caseros RNA with and without E-64 were immunoprecipitated with aVP2, aVP3, or aL serum. The immunoprecipitated products were analyzed by PAGE on a 15% polyacrylamide gel. Lanes: 1, in vitro translation in the absence of E-64; 2, in vitro translation in the presence of 0.5 mg of E-64 per ml; 3 and 4, immunoprecipitation of translation reactions in the absence or presence, respectively, of E-64 with aVP2 serum; 5 and 6, immunoprecipitation of translation reactions in the absence or presence, respectively, of E-64 with aVP3 serum; 7 and 8, immunoprecipitation of translation reactions in the absence or presence, respectively, of E-64 with aL serum.

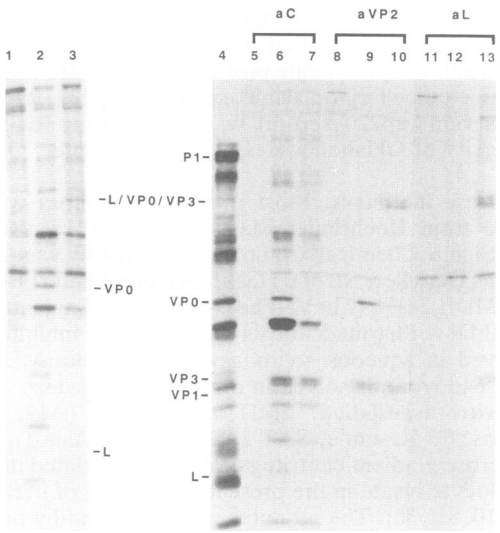

FIG. 3. Immunoprecipitation of cytoplasmic extracts from FMDV-infected LF-BK cells in the absence or presence of E-64. LF-BK cells were infected with FMDV A12 and radiolabeled with [^{35}S]methionine in the absence or presence of 5 mg of E-64 per ml. Cytoplasmic extracts were immunoprecipitated with bovine convalescent-phase, aVP2, and aL serum, and the immunoprecipitated products were analyzed by PAGE on a 15% polyacrylamide gel. Lanes: 1, mock-infected cell extract; 2, FMDV-infected cell extract; 3, extract from cells infected with FMDV in the presence of E-64; 4, in vitro translation programmed with FMDV A12 RNA; 5 to 7, extracts from mock-infected, FMDV-infected, and E-64-treated FMDV-infected cells, respectively, immunoprecipitated with bovine convalescent-phase (aC) serum; 8 to 10, extracts from mock-infected, FMDV-infected, and E-64-treated FMDV-infected cells, respectively, immunoprecipitated with aVP2 serum; 11 to 13, extracts from mock-infected, FMDV-infected, and E-64-treated, FMDV-infected cells, respectively, immunprecipitated with aL serum.

activity could be achieved with lower concentrations (50 to 500 μg/ml) of this compound. Cells were infected with FMDV A12 or O1 Campos. At 200 or 500 μg of E-64d per ml, there was a significant decrease in the amount of L and VP0 present and a new band corresponding to L/VP0/VP3 appeared (Fig. 4). Identical results were obtained with O1 Campos and with A12 if inhibitor was added at 1 h postinfection. Cytoplasmic extracts from O1 Campos-infected cells were immunoprecipitated with aL, aVP2, and bovine convalescent-phase serum. As with E-64-treated extracts, L and VP0 were absent or significantly reduced. The protein band corresponding to L/VP0/VP3 and a new band corresponding to L/P1 were precipitated by aL and aVP2 serum (Fig. 5). The overall level of viral protein synthesis was qualitatively reduced in infected cells treated with 200 or 500 μg of E-64d per ml when compared with the level in cells infected with FMDV in the absence of the compound (Fig. 4). However, correctly processed viral proteins 3D, 2C, VP1, and 2B were observed by immunoprecipitation with convalescent-phase serum (Fig. 5). Identical results were obtained with FMDV A12 (data not shown).

Inhibition of host shutoff. We consistently observed that infected cells treated with either E-64 or E-64d showed a qualitative decrease in virus-induced CPE compared with infected, non-inhibitor-treated cells (data not shown). At an E-64d concentration of 500 μg/ml, no CPE were observed by 5 to 6 h postinfection. At lower E-64d concentrations (50 to

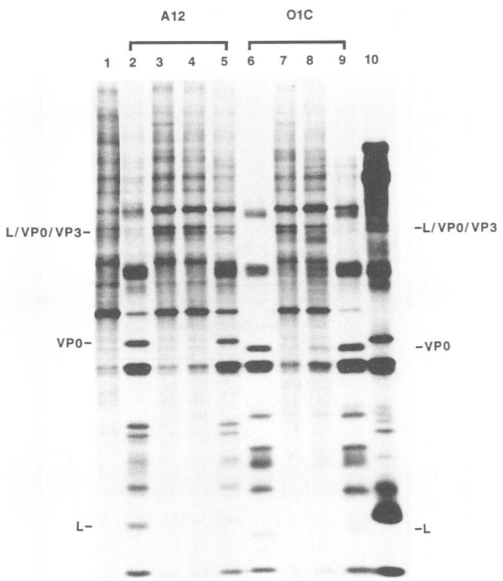

FIG. 4. Protein synthesis in FMDV-infected LF-BK cells in the absence or presence of different concentrations of E-64d. LF-BK cells were infected with FMDV A12 (lanes 2 to 5) or O1 Campos (lanes 6 to 9) in the absence or presence of different concentrations of E-64d and radiolabeled with [^{35}S]methionine. The cytoplasmic extracts were analyzed by PAGE on a 15% polyacrylamide gel. Lanes: 1, mock-infected cell extract; 2, FMDV A12-infected cell extract; 3 to 5, FMDV A12 in the presence of 500, 200, or 50 μg of E-64d per ml, respectively; 6, FMDV O1 Campos-infected cell extract; 7 to 9, FMDV O1 Campos in the presence of 500, 200, or 50 μg of E-64d per ml, respectively; 10, in vitro translation reaction programmed with FMDV A12 RNA. Markers on the left and right side of the figure refer to A12 and O1 Campos proteins, respectively.

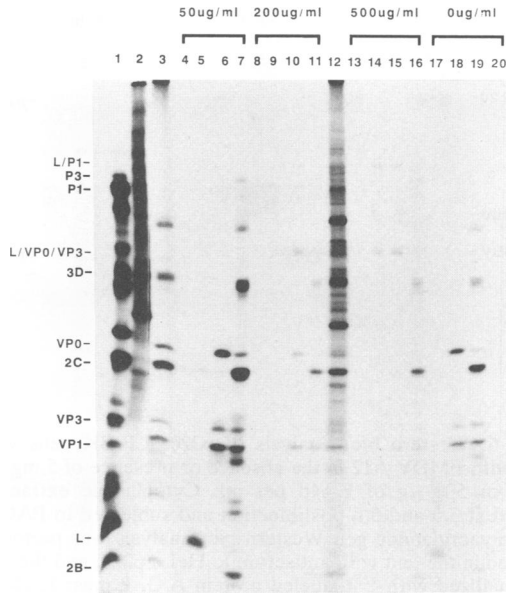

FIG. 5. Immunoprecipitation of cytoplasmic extracts from LF-BK cells infected with FMDV grown in the absence or presence of E-64d. LF-BK cells were infected with FMDV O1 Campos in the absence (lanes 17 to 20) or presence of E-64d at 50 μg/ml (lanes 4 to 7), 200 μg/ml (lanes 8 to 11), and 500 μg/ml (lanes 13 to 16) and radiolabeled with [^{35}S]methionine. The cytoplasmic extracts were immunoprecipitated with normal (lanes 4, 8, 13, and 20), aL (lanes 5, 9, 14, and 17), aVP2 (lanes 6, 10, 15, and 18), and bovine convalescent-phase serum (lanes 7, 11, 16, and 19). The immunoprecipitation products were subjected to PAGE on a 15% polyacrylamide gel. Lane 1, in vitro translation reaction programmed with FMDV A12 RNA; lane 2, mock-infected cell extract; lane 3, O1 Campos-infected cell extract; lane 12, O1 Campos-infected cells in the presence of 200 μg of E-64d per ml.

200 μg/ml) and at 5 mg of E-64 per ml, reduced levels of CPE were observed.

The characteristic picornavirus-induced shutoff of host protein synthesis is inhibited in infected cells treated with E-64d (Fig. 4). The protein pattern observed by PAGE in infected cells treated with 500 μg of E-64d per ml appeared almost identical to that in mock-infected cells (Fig. 4, compare lane 1 with lanes 3 and 7). Since virus-induced degradation of p220 is implicated in shutoff of host protein synthesis (8), we wanted to determine the extent of degradation of p220 in infected, inhibitor-treated cells. Western blot analysis of cytoplasmic extracts at 3.5 h postinfection showed that, in contrast to complete degradation of p220 in control infected cells (Fig. 6, lane 4), the majority of p220 was still intact in E-64d (500 μg/ml)-treated cells (lane 6) and a small percentage was still intact in E-64 (5 mg/ml)-treated cells (lane 5). By 6 h postinfection, only a small amount of intact p220 was observed in E-64d-treated cells (lane 9). The p220 degradation products present in E-64- and E-64d-treated cells appeared to be different from those in control infected cells (Fig. 6). In a control experiment, E-64d (500 μg/ml) had no effect on the integrity of p220 or the pattern of protein synthesis in mock-infected cells (data not shown).

Virus assembly. Since processing of P1 was inhibited in E-64-treated cells, it was of interest to examine the effect of this inhibitor on virus assembly. LF-BK cells were infected with FMDV A12 and radiolabeled with [^{35}S]methionine in the absence or presence of E-64, and the cytoplasmic extracts were analyzed by sucrose gradient centrifugation. In control infected cells, 140S viral and 70S empty capsid peaks were present (Fig. 7A). Comparison of profiles from repeated experiments showed the consistent absence of the 70S peak in extracts from E-64-treated cells. A variable result was obtained in the region encompassing the 140S peak. Sometimes a broad peak that spanned the 140S region was observed (Fig. 7A); at other times only a small peak of radioactivity that migrated in the gradient between the 140S and 70S peaks of the control was observed (data not shown). Analysis of the protein composition of this peak region from inhibitor-treated cells revealed a mixture of viral structural and host proteins (data not shown). In the presence of E-64d, only a single peak migrating between 140S and 70S was observed (data not shown). It, too, contained a mixture of viral structural and host proteins.

To determine whether virion RNA was incorporated into structures found in E-64-treated infected cells, we prepared cytoplasmic extracts from cells radiolabeled with [^{3}H]uridine and examined them by sucrose gradient centrifugation (Fig. 7B). In contrast to results with the control infected cells, little, if any, RNA was incorporated into structures in the presence of E-64 (Fig. 7B) or E-64d at 200 or 500 μg/ml (data not shown). A reduced level of virion RNA was found in 140S particles in cells treated with 50 μg of E-64d per ml (data not shown). To determine whether the absence of virion RNA was the result of the inability to encapsidate RNA or of the inhibition of synthesis of viral RNA, we extracted [^{3}H]uridine-labeled RNA from infected cells and

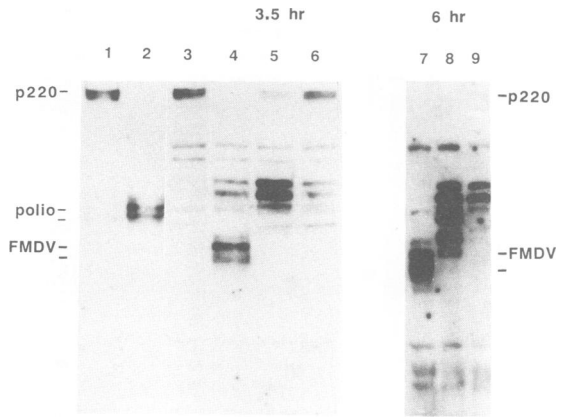

FIG. 6. Western blot analysis of p220. LF-BK cells were infected with FMDV A12 in the absence or presence of 5 mg of E-64 per ml or 500 μg of E-64d per ml. Cytoplasmic extracts were prepared at 3.5 and 6 h postinfection and subjected to PAGE on a 7.5% polyacrylamide gel. Western blot analysis was performed as described in the text with antiserum to HeLa p220, and the reaction was visualized with ^{125}I-labeled protein A/G. Lanes: 1, HeLa cell cytoplasmic extract; 2, HeLa cell cytoplasmic extract from poliovirus-infected cells; 3, cytoplasmic extract from mock-infected LF-BK cells; 4 and 7, extracts from FMDV-infected cells harvested at 3.5 and 6 h, respectively; 5 and 8, extracts from cells infected with FMDV in the presence of E-64 and harvested at 3.5 and 6 h, respectively; 6 and 9, extracts from cells infected with FMDV in the presence of E-64d and harvested at 3.5 and 6 h, respectively. Polio and FMDV, on the left and right side of the figure, refer to p220 degradation products in the two systems.

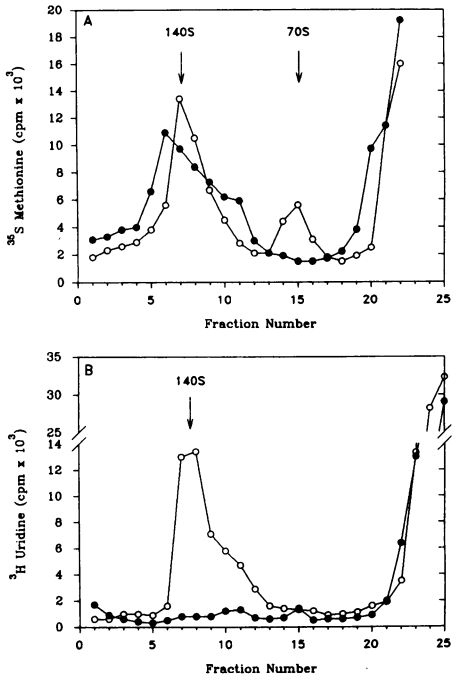

FIG. 7. Sucrose gradient analysis of capsid structures assembled in LF-BK cells infected with FMDV in the absence or presence of E-64. Cells were infected with FMDV A12 in the absence or presence of 5 mg of E-64 per ml and radiolabeled with either [^{35}S]methionine (A) or [^{3}H]uridine (B). Cytoplasmic extracts were centrifuged on 10 to 50% sucrose gradients in NET buffer in an SW41 rotor. The gradients were fractionated, and aliquots were assayed for acid-insoluble radioactivity. Symbols: ○, infected, untreated samples; ●, E-64-treated samples.

examined it by sucrose gradient centrifugation. In comparison with control infected cells, significantly reduced levels of FMDV RNA was synthesized in E-64d (150 μg/ml)-treated cells by 6 h postinfection (data not shown).

Effect of E-64d on virus yield. LF-BK cells were infected with FMDV A12 in the absence or presence of E-64d. Samples of extracellular and intracellular virus were collected at 1, 6, and 24 h postinfection. In the presence of E-64d, virus yield was decreased up to 1,000-fold (Fig. 8). At E-64d concentrations of 200 and 500 μg/ml, minimal and essentially no CPE were observed, respectively. The cell monolayers were still intact at 24 h, while cells were completely lysed in control plates. In the presence of E-64 there was a 30-fold decrease in virus yield compared with that in the control infection in both the extra- and intracellular fractions (data not shown).

DISCUSSION

Our results show that L protease activity is specifically and irreversibly inhibited by E-64 and its analogs. Inhibition of L processing initiates a cascade of events that affect the replication of FMDV. Processing of the structural protein precursor is blocked, leading to improper assembly of the viral capsid. Delay of p220 degradation allows continuation of cap-dependent translation and direct competition between host and viral mRNA. This results in a drastic reduction in viral protein synthesis and subsequently in viral RNA synthesis.

Inhibition of autocatalytic cleavage by L results in the appearance in vitro (Fig. 1) and in cell culture (Fig. 3 and 4) of polyproteins L/P1 and L/VP0/VP3. The presence of L attached to the amino terminus of P1 may modify the conformation of the capsid precursor such that the 3C cleavage sites are masked or altered. In addition, because L remains covalently attached to P1, N-terminal myristoylation of the capsid precursor is probably blocked. This event requires a free amino-terminal glycine on VP4 (5). Myristoylation is required for efficient assembly of poliovirus at membranes (5, 29) and may play a similar role in FMDV (25). We have demonstrated that in E-64- or E-64d-treated cells, capsid assembly is disrupted (Fig. 7), leading to a 500- to 1,000-fold reduction in virus yield (Fig. 8).

Our results suggest that L may belong to the papain family of proteases. The 3C and the 2A proteases of picornaviruses are members of the trypsin-like family of serine proteases (2). E-64 has not been shown to inhibit any serine proteases examined (1), and it does not affect either FMDV 3C or the putative 2A protease, since both in vitro and in vivo the P2 and P3 precursors are correctly processed (Fig. 1 and 5). The 3C and 2A proteases do not share any homology with L. Computer alignments show, instead, a similarity between L, papain, and the papain family of thiol proteases, all of which are inhibited by E-64 (9, 14, 26). This is a novel observation for the picornaviruses and raises questions about the evolutionary origins of L.

The presence of either E-64 or E-64d in FMDV-infected cells results in a qualitative reduction in classic virus-induced CPE, including morphological alterations and a delay in shutoff of host protein synthesis. In the presence of 200 or 500 μg of E-64d per ml, CPE are virtually absent at 6 h postinfection and are present at only limited levels after 24 h.

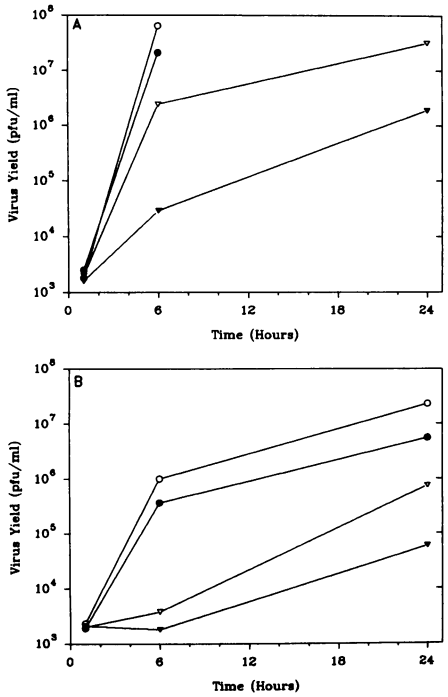

FIG. 8. Effect of E-64d on virus yield. LF-BK cells, in six-well plates, were infected with FMDV A12 at a multiplicity of infection of 10 for 1 h, and then the cell sheet was washed twice and incubated with medium in the absence or presence of different concentrations of E-64d. Viral titers in intracellular (A) and extracellular (B) fractions were determined at 1, 6, and 24 h postinfection. Symbols: ○, no E-64d; ●, 50 μg of E-64d per ml; ▽, 200 μg of E-64d per ml; ▼, 500 μg of E-64d per ml.

In FMDV-infected cells, shutoff of host protein synthesis correlates with cleavage of host cell protein p220. In E-64d-treated (500 μg/ml) infected cells, only a small percentage of p220 is cleaved by 3.5 h postinfection, in direct contrast to the complete cleavage of p220 in infected, non-inhibitor-treated cells (Fig. 6). At 6 h postinfection in E-64d-treated cells, there is still some intact p220 (Fig. 6). The inability to cleave p220 requires that viral mRNA compete directly with host mRNA for the cellular translation machinery. FMDV mRNA cannot effectively compete in E-64d-treated cells, as demonstrated by the efficient synthesis of host proteins and the low level of synthesis of viral proteins (Fig. 4). Similarly, poliovirus mRNA does not successfully compete against cap-dependent host mRNAs. In cells infected with poliovirus containing mutations in the 2A gene, p220 is not cleaved and host protein synthesis is not inhibited (4, 33). In contrast, encephalomyocarditis virus is able to efficiently outcompete cellular mRNAs and shut off host protein synthesis during infection without cleaving p220 (31).

The precise role of L in p220 degradation is unclear. Inhibition of p220 cleavage in infected, inhibitor-treated cells may be the result of the inability of the E-64-inactivated L protease to directly cleave p220 or to activate a cellular protein required for cleavage. If the latter process is correct, this would imply that FMDV uses a mechanism of p220 cleavage similar to that used in the poliovirus system, whereby 2A indirectly cleaves p220 by activating a cellular protease, p220ase (22, 28). Although this possibility cannot be conclusively ruled out, the mechanism by which L cleaves p220 differs intrinsically from that of poliovirus 2A because the p220 degradation products in the two systems are not the same (27) (Fig. 6) and the proteases are structurally distinct (2).

Some viral protein synthesis and replication occur even in the presence of 500 μg of E-64d per ml (Fig. 4 and 8). At 24 h postinfection there is a 50- to 100-fold increase in virus yield over that at 6 h. This is presumably the result of both a low level of viral protein synthesis occurring in the presence of the inhibitor and the metabolism of E-64d. When the level of viral protein synthesis exceeds the amount of inhibitor necessary to block L autocatalytic activity, L is processed from P1 and a complete cycle of virus replication begins. Furthermore, L can cleave in *trans*, thereby increasing the availability of P1 that can be processed by 3C (data not shown). This hypothesis suggests that subsequent addition of E-64d to infected cells at later times after infection might further delay the onset of virus replication.

The properties of an antiviral compound include specificity for the target molecule, disruption of some aspect of virus replication, and lack of toxicity to the host cell. We have presented evidence from both in vitro and cell culture experiments demonstrating that E-64 and, in particular, its derivative E-64d are potential antiviral agents because they are specific for L protease and their presence results in inhibition of viral assembly. In addition, toxicity studies have shown a 50% lethal dose of at least 2,000 mg/kg of body weight for these compounds in rats and mice by several administration routes; the compounds have also been used with no toxic effects in studies on muscular dystrophy in a number of animal species (7, 14, 19–21, 45). The compounds have been tested as antiviral agents only against rotavirus infection in mice (7). FMDV L protease is an ideal candidate target molecule for antiviral compounds. E-64 blocks L autocatalytic activity in all serotypes tested. Thus, an antiviral agent that attacks L would circumvent the problems of serotype-specific reactions observed with conventional FMDV vaccines.

We are initiating studies to determine whether E-64d can effectively inhibit viral infection in animal model systems. More effective, related analogs must also be screened. Further studies on the L protease itself, including expression, site-specific mutagenesis, and biochemical purification, are essential, since determination of the three-dimensional structure and molecular modeling would allow us to rationally design or select other compounds which directly fit in the active site of this enzyme.

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