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Coronavirus Protein Processing and RNA Synthesis Is Inhibited by the Cysteine Proteinase Inhibitor E64d

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Mouse hepatitis virus strain A59 (MHV-A59) encodes within the 22-kb gene 1 a large polyprotein containing three proteinase domains with proven or predicted cysteine catalytic residues. E64d, a specific, irreversible inhibitor of cysteine (thiol) proteinases, inhibits the processing of the gene 1 polyprotein. Specifically, E64d blocks the carboxy-terminal cleavage of p65. E64d also inhibits replication of MHV-A59 in murine DBT cells in a dose-dependent manner, resulting in reduced virus titers and viral syncytia formation. This inhibition of replication is associated with a rapid shutoff of new viral RNA synthesis, in a manner similar to that seen in the presence of cycloheximide. The E64d-associated inhibition of RNA synthesis likely results from E64d-specific inhibition of processing of the gene 1 polyprotein, resulting in inactive proteinase or replicase proteins. These results indicate that processing of the MHV-A59 gene 1-encoded polyprotein is required throughout infection to sustain RNA synthesis and virus replication. © 1995 Academic Press, Inc.

INTRODUCTION

The replication of mouse hepatitis virus strain A59 (MHV-A59) is initiated by translation of replicase proteins from the 31-kb plus-sense genome RNA, followed by transcription of a full-length minus-strand intermediate which acts as a template to direct the synthesis of full-length plus-strand genome RNA as well as six subgenomic RNA species. The proteins responsible for these RNA-dependent RNA polymerase activities are predicted to be encoded in gene 1, the 5′-most gene of the MHV genome (Denison and Perlman, 1986, 1987; Gorbalenya *et al.*, 1989; Lee *et al.*, 1991). Gene 1 of MHV-A59 comprises 22 kb of the genome and contains two overlapping open reading frames, ORF 1a and ORF 1b, which possess a ribosomal frameshift in the overlap region (Fig. 1) (Breedenbeek *et al.*, 1990; Lee *et al.*, 1991). Together these two ORFs potentially encode up to 800 kDa of polyprotein products. Protein products representing approximately 30% of the gene 1 coding capacity have been detected during *in vitro* translation of MHV virion RNA, as well as in MHV-infected cells (Leibowitz *et al.*, 1982; Denison and Perlman, 1986; Denison and Perlman, 1987; Denison *et al.*, 1991; Denison *et al.*, 1992). A protein pattern of N-p28-65-50-240-C represents the mature protein products beginning at the amino-terminus of the gene 1 polyprotein (Hughes *et al.*, 1993; Denison *et al.*, 1995).

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Virus-encoded proteinases are involved in processing the gene 1 polyprotein. Three proteinase domains have been identified in ORF 1a of MHV-A59 gene 1 (Fig. 1). The two domains encoded in the 5′ 6 kb of ORF 1a are thought to be papain-like proteinases. The third domain, encoded in the region between 10 and 11 kb from the 5′ end of the genome, is predicted to encode a proteinase with catalytic domains and general structure similar to the 3C proteinases of the picornaviruses (Boursnell *et al.*, 1987; Gorbalenya *et al.*, 1991; Lee *et al.*, 1991). Only the first papain-like proteinase (PLP-1) has been experimentally demonstrated to be involved in gene 1 polyprotein processing; it cleaves the amino-terminal gene 1 protein p28 (Baker, 1993).

Cysteine and serine proteinase inhibitors have been used to study the expression and processing of the gene 1 polyprotein both in cells and *in vitro*. These studies have demonstrated that leupeptin and zinc chloride inhibit viral polyprotein processing (Denison and Perlman, 1986; Denison *et al.*, 1992). We have shown that leupeptin also inhibits replication of MHV-A59 (Denison *et al.*, 1992). However, because of the variable cell penetrability of leupeptin and the cellular toxicity of zinc chloride, they are not optimal agents to investigate interference with virus replication. In contrast, E64d is a specific cysteine proteinase inhibitor which readily penetrates cells, has very limited cellular toxicity, and has been shown to inhibit polyprotein processing and virus replication of the picornavirus foot-and-mouth disease virus (FMDV) (Kleina and Grubman, 1992). The parent compound, E64 (L-*trans*-epoxysuccinyl-leucylamido(4-guanidino)butane), was developed as an inhibitor of cellular calpain but also

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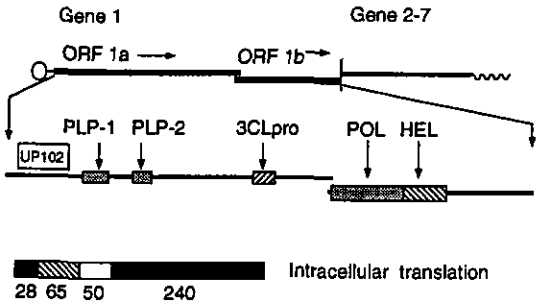

FIG. 1. MHV-A59 gene 1 structure, putative functional domains, and translation products. Gene 1 is enlarged to show the putative functional domains and antisera used in this study. Hatched boxes are predicted functional domains including two papain-like proteinases (PLP-1 and PLP-2), 3C-like proteinase (3CLpro), RNA polymerase(POL), and RNA helicase(HEL). The white box represents the cDNA clone used to express the fusion protein for induction of polyclonal antiserum UP102. Our current model of gene 1 products in DBT cells is also shown. The mature protein pattern is shown, but not precursors or processing pattern. Amino- and carboxy-termini have been identified only for p28.

irreversibly inhibits other cysteine proteinases such as cathepsin B and papain (Mehdi, 1991). It binds covalently via the C-2 carbon of the inhibitor oxirane ring to the thiol group of the catalytic cysteine residue of the proteinase, resulting in a stable thioester bond which inactivates the proteinase molecule. E64 is very active against papain and papain-like proteinases; however, it is a highly charged molecule with poor membrane permeability. In contrast, E64d is an uncharged diethyl ester derivative of E64 which maintains the activity of E64 but is highly membrane permeable. E64d has not previously been utilized in studies of coronavirus replication or polyprotein expression and processing.

In this study we have analyzed the effects of E64d on MHV-A59 virus replication and protein processing. We demonstrate that E64d can effectively inhibit MHV virus replication. In addition, we demonstrate that inhibition of gene 1 protein processing is associated with complete inhibition of MHV RNA synthesis even when given at late times of infection, long after polyprotein expression and RNA synthesis have been initiated in the virus-infected cell. These data indicate that processing of polyprotein molecules is required at all times of infection in order to sustain new RNA synthesis and virus replication.

MATERIALS AND METHODS

Viruses and cells

MHV-A59 is a laboratory strain which was used throughout this study. The virus was plaque purified three times and passaged twice prior to use. DBT cells were used for virus growth, isolation, infected cell lysates, and plaque assay (Hirano *et al.*, 1976). Cells were maintained in DMEM, 10% fetal calf serum (FCS) and infections were performed in DMEM, 2% FCS.

Proteinase inhibitors

E64d was originally obtained from Phil Sonnett at the USDA. Subsequently E64d was obtained from Matreya, Inc. E64d was dissolved in DMSO at a stock concentration of 100 mg/ml and used at concentrations of 50–500 μg/ml in DMEM, 2% FCS.

Infection of DBT cells with MHV-A59 and labeling of viral proteins

Confluent monolayers of DBT cells in 60-mm petri dishes were infected with MHV-A59 at an m.o.i. of 20 PFU/cell in DMEM, 2% FCS for 30 min at 37°. Excess virus and medium were removed and the cells were incubated in DMEM without methionine (Gibco) with 2% FCS for the remainder of the experiment. Actinomycin D (Sigma) was added to the medium at 4 hr postinfection (p.i.), to a final concentration of 10 μg/ml. [^{35}S]Methionine (Translabel; ICN) was added to the medium at a concentration of 200 μCi/ml for periods as indicated in individual experiments. Label was added when cells were approximately 20% involved in syncytia (6.5 hr p.i.) and cells were labeled for 1.5 hr.

Immunoprecipitation of gene 1 polyprotein products

Antiserum UP102 was induced against a fusion polypeptide expressed in *Escherichia coli* from a 1.8-kb cDNA which is predicted to span amino acids 1 to 600 in ORF 1a, as previously described (Hughes *et al.*, 1993; Weiss *et al.*, 1994). UP102 has been shown to precipitate two major proteins, p28 and p65. Proteins were immunoprecipitated as previously described (Denison *et al.*, 1992). Briefly, whole-cell lysates of MHV-A59-infected DBT cells were prepared by the addition of RIPA lysis buffer (10 m*M* Tris, pH 7.4, 150 m*M* NaCl, 1% NP-40, 1% sodium deoxycholate, 1% SDS, 1 m*M* PMSF). After lysis, aliquots of lysate equivalent to one-half plate were diluted in 9 vol of RIPA buffer without SDS to achieve a final concentration of SDS of 0.1%. Protein A–Sepharose beads (Sigma) which were prearmed with specific polyclonal antibodies or preimmune antibodies were added to the diluted lysates (30 μl per 1 ml of diluted lysate), rocked for 2 hr at 4°, and washed four times with alternating high (1 *M* NaCl) and low (100 m*M* NaCl) RIPA buffer. The beads were boiled in 50 μl of 2× SDS loading buffer (Laemmli, 1970) for 5 min and electrophoresed on a 5–18% SDS gradient polyacrylamide gel. Radiolabeled proteins were visualized by fluorography and quantitated by densitometry using the NIH program Image (Rasband, 1994) on a Macintosh IIci computer.

Plaque assays

Plaque assays were performed on confluent monolayers of DBT cells in six-well plates. Cells were infected for 30 min at 37° with virus from medium supernatants

diluted in gel saline, followed by medium/agar overlay for 24–30 hr and subsequent addition of agar containing 0.02% neutral red for 6–12 hr with counting of clear plaques. During plaque reduction inhibition assays, stock virus of known titer (2×10^8 PFU/ml) was diluted to a concentration of 100 PFU/200 μl and 200 μl was added to each well for 1 hr in the absence of E64d. Duplicate plates were overlayed with agar containing E64d at concentrations of 50 to 500 μg/ml and incubated for 30 hr. Agar/medium containing neutral red was added and clear plaques were counted at 36 hr p.i. All plaque assay and plaque reduction inhibition experiments were performed in duplicate and the experiments were repeated twice.

RNA synthesis and characterization

RNA synthesis was analyzed by two methods. First, cell monolayers were infected with MHV-A59 at an m.o.i. of 20 PFU/cell. At 4 hr p.i., actinomycin D was added at a concentration of 20 μg/ml. E64d (400 μg/ml) was added to the medium at various times from −1 to 7 hr p.i. to determine the effect on RNA synthesis. Because E64d was dissolved in DMSO (stock 100 μg/μl), separate aliquots of medium containing actinomycin D were set up to which the E64d was added, followed by rapid vortexing. The medium was used to replace that over the cell monolayer. [^{3}H]Uridine (ICN) was added to 100 μCi/ml final concentration from 8 to 9 hr p.i. Whole-cell lysates were prepared from all plates at 9 hr p.i. as with immunoprecipitation experiments, using RIPA lysis buffer. Aliquots equivalent to 10^5 cells were TCA precipitated, vacuum filtered onto Whatman glass filters, dried, and counted in a Beckman scintillation counter.

To analyze the effects of E64d on the synthesis of individual RNA species, four 60-mm plates were infected at an m.o.i. of 20 PFU/ml. Actinomycin D was added at 3 hr p.i. and E64d (400 μg/ml) was added at 5 hr p.i. [^{3}H]Uridine (100 μCi/ml) was added at 5 or 6 hr p.i. to one untreated and one E64d-treated plate. The cells were labeled for 1 hr and then lysed and total mRNA was purified using 1 ml of RNA-STAT (Tel-Test "B," Inc.) according to the manufacturer's instructions. Purified RNA was resuspended in 200 μl of 100% formamide (Chomczynski, 1992), and samples equivalent to 10^5 cells (10 μl) were electrophoresed on 0.8% formaldehyde–agarose gels. The gel was treated with 1% diphenyloxazole in 100% methanol for 2 hr, followed by precipitation in water, drying at room temperature, and exposure to Kodak X-omat film. Quantification of RNA was performed by scanning autoradiograms using an Apple OneScanner and Ofoto software (Apple) and densitometry with the program NIH Image (Rasband, 1994). Results were confirmed by excision of bands and scintillation counting.

RESULTS

Inhibition of gene 1 protein processing

To investigate E64d-specific proteinase inhibition, we utilized antiserum UP102, which has been previously shown to detect products processed from the amino-terminal portions of the gene 1 polyprotein, specifically, p28 and p65 (Fig. 1) (Denison *et al.*, 1995). Viral proteins were labeled with [^{35}S]methionine from 6 to 7 hr p.i. in the presence or absence of E64d, and whole-cell lysates were immunoprecipitated with UP102. In the absence of E64d, both p28 and p65 were detected. The addition of E64d prior to the addition of radiolabel resulted in greater than 90% inhibition of p65 processing (Fig. 2). p65 processing was not affected by 2 m*M* leupeptin or 2 m*M* PMSF (data not shown). In contrast, E64d had very little effect on the cleavage of p28, with only an 8% reduction in p28 processing compared with the control infection. Thus E64d clearly inhibits at least one well-defined protein processing event in the gene 1 polyprotein.

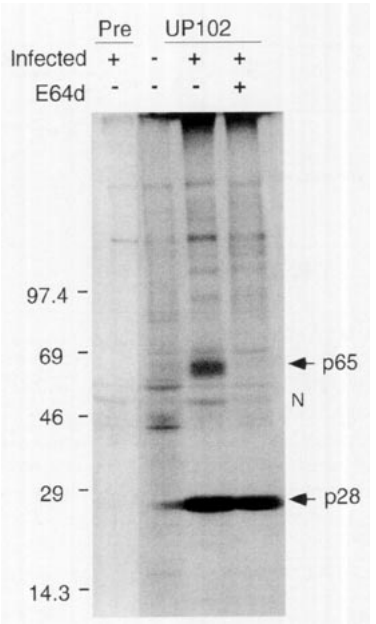

FIG. 2. Inhibition of intracellular processing of gene 1 proteins. Gene 1 proteins were labeled with [^{35}S]methionine from 7.5 to 9 hr p.i. in the presence of actinomycin D (20 μg/ml) and in the presence or absence of E64d (400 μg/ml). Whole-cell lysates were immunoprecipitated with preimmune rabbit sera or antiserum UP102. Mock-infected cells were used as controls. Molecular mass markers are to the left of the gel. The band labeled N indicates a nonspecifically precipitated protein, which was also detected by other antibodies and preimmune serum and migrates with nucleocapsid. The locations of specific gene 1 proteins are to the right of the gel. Pre, preimmune serum.

Inhibition of MHV replication in single-cycle growth experiments

While performing experiments to determine the effect of E64d on polyprotein processing, we observed that E64d also inhibited MHV-A59-induced syncytia formation in DBT cells. We therefore sought to determine the effect of E64d on MHV replication in a single-cycle growth experiment of MHV-A59 in murine DBT cells. Cell monolayers were infected at an m.o.i. of 20 PFU/cell for 1 hr at 37°, followed by removal of unbound virus and replace-

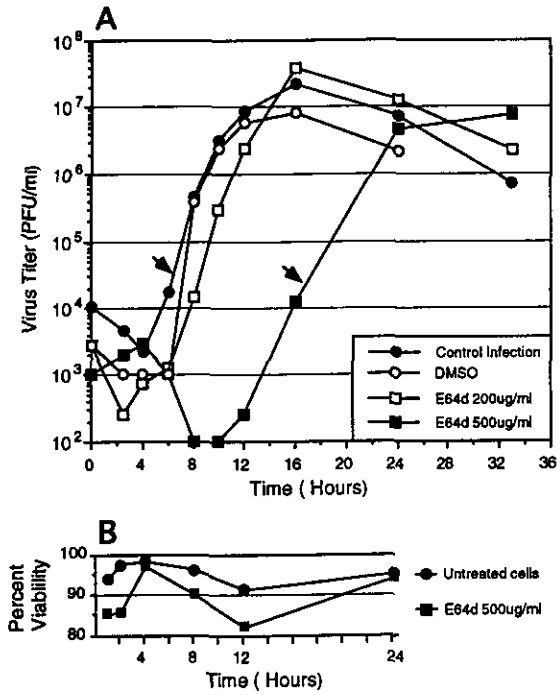

FIG. 3. *E64d inhibition of MHV-A59 replication.* (A) *DBT cell monolayers* were infected at an m.o.i. of 20 PFU/cell for 1 hr at 37°, followed by removal of unbound virus and replacement with medium containing 200 or 500 μg/ml of E64d, 1.5% DMSO, or with unsupplemented DMEM 2% FCS. Supernatant virus titers were measured at intervals from 0 to 32 hr p.i. Arrowheads indicate the time of first detection virus-induced syncytiae. (B) Viability of uninfected DBT cells in the presence of E64d. *DBT cell monolayers were incubated in the presence or absence of* 500 μg/ml of E64d for 24 hr and checked for viability by trypan blue exclusion at the time points indicated.

ment with medium containing 400 μg/ml of E64d. Plates were not supplemented with E64d; single dosing was utilized in order to assess the duration of inhibition. The titer of virus in the medium supernatants was measured from 0 to 50 hr p.i. (Fig. 3). In addition, the monolayers were observed for the appearance of alterations in cell morphology as well as for the characteristic syncytia of A59 infection. The single-cycle growth curve was typical of A59 infection in the absence of E64d; an increase in virus titer occurred at approximately 5 to 6 hr p.i., followed by an exponential increase in the virus titer which peaked by 10 to 12 hr p.i. and gradually declined thereafter for as long as it was measured. This growth curve was associated with initial detection of viral-induced syncytia at 6 hr p.i., involvement of 80–100% of the monolayer in syncytia at the peak of virus titer, and complete destruction of the monolayer by 18 hr p.i. In contrast, the plate containing medium with E64d showed a slow, gradual decline in virus titer for the first 16 hr after infection, resulting in a greater than 5 log reduction in titer compared to the control infection at 8 hr p.i. Interestingly, at 14 hr p.i. syncytia were observed in the plates containing medium with E64d. This observation was associated with an increase in virus titer, and subsequent virus titers and visible virus-induced cytopathic effect paralleled that of the control infection lacking E64d. This entire experiment was repeated with identical results; complete inhibition of virus replication and CPE for 16 hr followed by initiation of what appeared to be a normal virus growth cycle (Fig. 3). Neither E64d nor its solvent, DMSO, caused cellular toxicity at the concentrations used. E64d did result in alteration of the cell morphology; cellular dendrites were retracted and the cells appeared more rounded in comparison with their normal polygonal shape. Despite this alteration of DBT morphology, the cells were viable as determined by trypan blue exclusion and their capacity to support MHV replication at 16 to 24 hr after addition of E64d (Fig. 2). Similar results have been reported during studies of E64d inhibition of FMDV in LF-BK bovine kidney cells (Kleina and Grubman, 1992).

E64d plaque reduction inhibition

To determine more precisely the degree of inhibition of replication of a population of viruses, we performed a plaque reduction assay in the presence of E64d (Fig. 4). E64d inhibited replication of MHV in a dose-dependent manner, with only one visible plaque observed in the presence of 400 μg/ml of E64d in the overlying agar. The ID_{50} was less than 50 μg/ml, with 400 μg/ml resulting in a 99% inhibition of plaque formation. The appearance of visible plaques was delayed for up to 12 hr in all E64d-containing plates when compared to the control. Virus titering was also performed in the presence or absence of 400 μg/ml of E64d, from dilutions of 10^{-1} to 10^{-8} (data not shown). Compared to the control plates, where complete destruction of the monolayer was observed at 24 hr in the 10^{-1} to 10^{-5} dilutions, the plates containing E64d showed innumerable microscopic syncytia (two to six nuclei) with an intact monolayer and large unaffected areas of the monolayer. At higher dilutions, limited numbers of syncytia developed into plaques, with the remain-

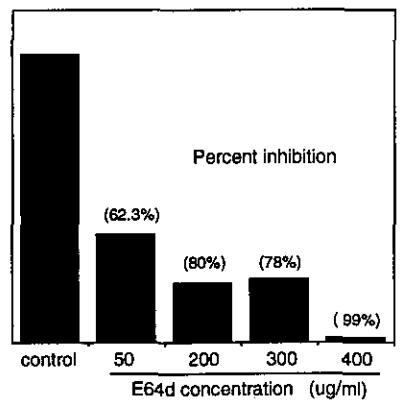

FIG. 4. Plaque reduction inhibition by E64d. DBT cell monolayers in 60-mm dishes were infected with 100 PFU of MHV A59 and allowed to adsorb for 1 hr at 37°. The monolayers were overlayed with agar/medium containing no proteinase inhibitor or E64d in the concentrations shown. Plaques were counted at 36 hr p.i.

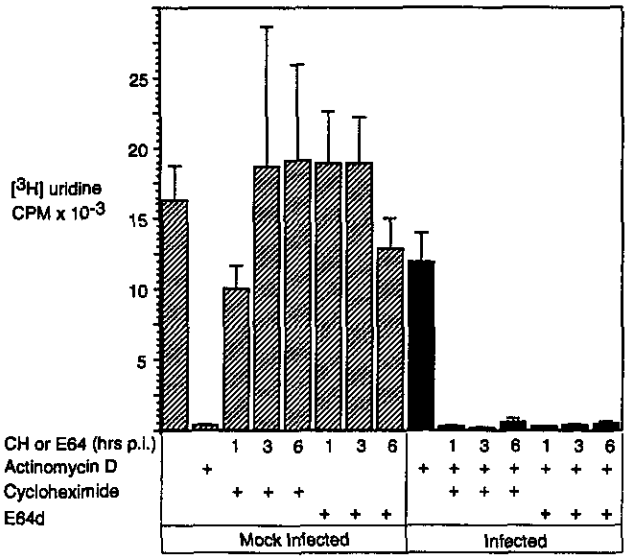

FIG. 5. Inhibition of MHV RNA synthesis by E64d. DBT cell monolayers in 60-mm dishes were mock infected or infected with MHV-A59 at an m.o.i. of 20 PFU/cell for 1 hr followed by incubation at 37° for 8 hr. Various combinations of actinomycin D (20 μg/ml), cycloheximide (20 μg/ml), and E64d (400 μg/ml) were added to the overlying medium. Actinomycin was added at 6 hr p.i. Cycloheximide and E64d were added at the times in hours indicated. [3H]Uridine (100 μCi/ml) was added at 8 hr p.i. and the cells were lysed at 9 hr p.i. for measurement of TCA-insoluble radioactivity.

der visible throughout the 36 hr only as microscopic syncytia.

Inhibition of new RNA synthesis at early and late times p.i.

The single cycle growth curve results indicated that E64d-mediated inhibition of MHV replication occurred at a point well after initial infection and virus entry. The point of interference of E64d with MHV replication was assessed by determining whether E64d resulted in diminished viral RNA synthesis and when E64d needed to be added to inhibit RNA synthesis. Cell monolayers were infected at an m.o.i. of 20 PFU/cell. Cycloheximide, E64d, or no inhibitor was added to the overlying medium at 1, 3, or 6 hr p.i. Actinomycin D (20 μg/ml) was added at 7 hr p.i. and [3H]uridine was added from 8 to 9 hr p.i., followed by assay of TCA-insoluble radioactivity from whole-cell lysates. Cycloheximide was chosen as a positive control since it has been shown to inhibit MHV RNA synthesis even when added at late times of infection (Sawicki and Sawicki, 1986).

The addition of E64d to the medium resulted in complete inhibition of MHV-specific RNA synthesis at 8 to 9 hr p.i. (Fig. 5). The shutoff of MHV RNA synthesis at 8–9 hr occurred when E64d was added as late as 6 hr p.i., even though viral syncytia were present, indicating that virus replication and therefore RNA synthesis was well underway. The pattern of E64d-associated inhibition of new RNA synthesis measured at 8 to 9 hr p.i. paralleled that seen with cycloheximide. This was clearly attributable to E64d inhibition of viral RNA synthesis rather than cellular toxicity since neither cycloheximide nor E64d inhibited cellular RNA synthesis in the absence of actinomycin D during these experiments.

Rate of reduction of MHV RNA synthesis by E64d

To determine the kinetics of E64d-associated inhibition of RNA synthesis, E64d was added to sets of plates at various time points during infection and the rate of virus RNA synthesis was determined at several time points thereafter (Fig. 6). The shutoff of viral RNA synthesis was very rapid, with a $t_{1/2}$ of less than 30 min. When E64d was added at early time points (1 to 3 hr p.i.), no MHV RNA synthesis was ever detected. When E64d was

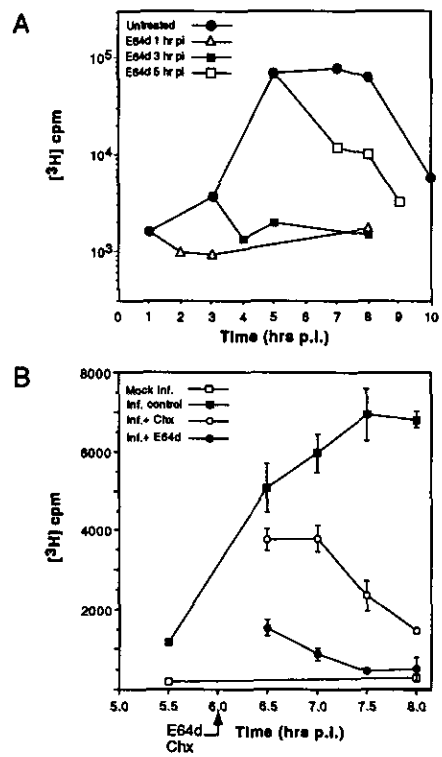

FIG. 6. Half-life of E64d inhibition of MHV RNA synthesis. (A) Monolayers of DBT cells in 60-mm dishes were infected at 20 PFU/cell for 1 hr and overlayed with medium. E64d (400 μg/ml) was added to sets of three plates each at 1, 3, and 6 hr p.i. [3H]Uridine (100 μCi/ml) was added for 1 hr beginning at the times p.i. indicated on the graph and cells were lysed and assayed for TCA-insoluble counts. Actinomycin D was added 1 hr prior to [3H]uridine labeling in all cases. (B) Comparison of E64d and cycloheximide. DBT cells were infected at an m.o.i. of 100 PFU/cell for 30 min at 37°. Actinomycin D (20 μg/ml) was added to all plates $\frac{1}{2}$-hr before addition of [3H]uridine (50 μCi/ml). Cycloheximide (100 μg/ml) or E64d (400 μg/ml) was added to four plates each at 6 hr p.i., and the plates were labeled with [3H]uridine for $\frac{1}{2}$-hr intervals thereafter, from 6 to 8 hr p.i. Time points indicate TCA-insoluble counts of cell lysates obtained at the end of the labeling periods. Error bars are standard deviation of two replicate counts. Uptake of [3H]uridine in mock-infected cells was measured in the presence of actinomycin D. The entire experiment was repeated with identical results.

added to the medium at the peak of viral RNA synthesis (5 to 7 hr p.i.), shutoff of RNA synthesis was rapid and essentially complete within 2 hr after its addition.

The rate of shutoff of new viral RNA synthesis was more specifically assessed in a direct comparison of cycloheximide and E64d (Fig. 6). The agents were added to replicate plates at 6 hr p.i., and the uptake of [^{3}H]-uridine into viral RNA was determined at serial 30-min intervals until 8 hr p.i. In the control untreated infection, the rate of new RNA synthesis increased in each interval measured, concurrent with increasing involvement of the monolayer in viral syncytia. In contrast, both cycloheximide and E64d caused a significant reduction in viral RNA synthesis during the first 30 min after addition, with increasing inhibition in every $\frac{1}{2}$-hr interval thereafter. Compared with cycloheximide, E64d caused a much more rapid decline in the rate of RNA synthesis, and the degree of reduction was greater than cycloheximide in all time periods. Within 30 min after addition, cycloheximide caused a 26% inhibition of [^{3}H]uridine uptake compared with the untreated control; E64d resulted in a 63% reduction in the same time period. By 1 hr after addition E64d caused a 93% inhibition of new viral RNA synthesis compared with a 66% inhibition in the presence of cycloheximide.

MHV RNA species inhibited by E64d

The ability of E64d to rapidly shut down viral RNA synthesis even at late times of infection indicated that positive-strand RNA synthesis was inhibited. We therefore investigated which MHV RNA species were inhibited by E64d at this time of maximal RNA synthesis. E64d was added to virus-infected cells at 5 hr p.i., followed by addition of [^{3}H]uridine either from 5 to 6 hr p.i. or from 6 to 7 hr p.i. The cells were lysed and total MHV RNA was analyzed on denaturing formaldehyde–agarose gels (Fig. 7). Analysis of genomic and subgenomic RNA species by densitometry demonstrated that compared with the infection in the absence of E64d, total viral RNA synthesis was inhibited 55% within the first hour after addition and greater than 99% by 2 hr after addition of E64d. RNA species 1 through 7 were equivalently inhibited, as demonstrated by the data showing that molar ratios of RNA species 1 through 7 were the same in treated and untreated cells.

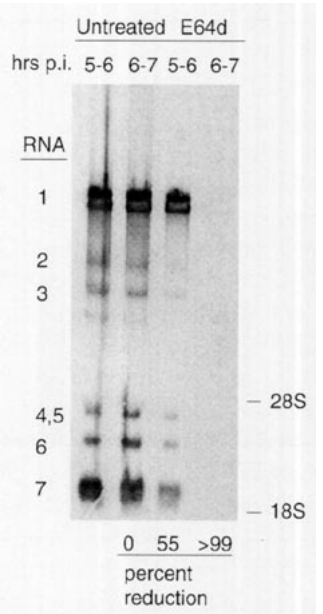

FIG. 7. Reduction of specific MHV RNA species by E64d. Monolayers of MHV-infected DBT cells were infected with MHV-A59 at an m.o.i. of 20 PFU/ml. At 3 hr p.i., actinomycin D (20 μg/ml) was added to the medium. E64d (400 μg/ml) was added to two plates at 5 hr p.i. and two plates remained untreated. One of the treated and one of the untreated plates were labeled with [^{3}H]uridine (100 μCi/ml) from 5 to 6 hr p.i. and the other two plates were labeled from 6 to 7 hr p.i. At the end of the labeling periods total RNA was isolated from cells and separated on formaldehyde–0.8% agarose gels. MHV RNA species are indicated at the left of the gel and the locations of unlabeled 28 S and 18 S RNA are to the right. The percentage reduction in RNA synthesis compared to the untreated 5 to 6 hr labeled cells is shown below the gel.

DISCUSSION

We have demonstrated that E64d blocks at least one step in the proteolytic processing of the gene 1 polyprotein in virus-infected cells. Associated with inhibition of processing was a decrease in virus-induced CPE and a reduction in virus titer. Doses of E64d in excess of 400 μg/ml resulted in greater than 99% inhibition of replication in plaque reduction assays and 4 log reductions in virus titers at 8 hr p.i. during single-cycle growth experiments. The diminished virus titer and viral cytopathic effect were associated with a rapid, specific inhibition of new viral RNA synthesis.

The one proven and two additional predicted proteinases in gene 1 are thought to possess cysteine catalytic residues. Two of these proteinases are papain-like proteinases, a type against which E64d has been shown to be particularly effective (Mehdi, 1991); therefore it was not surprising that E64d was an effective inhibitor of processing. It was unexpected that only the carboxy-terminal cleavage of p65 was inhibited by E64d, whereas the amino-terminal cleavage, which is presumably shared with the carboxy-terminus of p28, was not inhibited. It has been demonstrated that p28 is cleaved *in vitro* by the first papain-like proteinase (PLP-1) in a *cis*-autoproteolytic manner (Baker, 1993). The fact that p28 cleavage was minimally inhibited by E64d suggests that another proteinase activity may be responsible for the carboxy-terminal cleavage event which liberates p65. An alternate explanation is that PLP-1 is responsible for both cleavages, but prior to p28 cleavage is in a conformation which excludes E64d. E64d does not inhibit p28 cleavage during *in vitro* translation of genome RNA (data not shown), but since the pattern of translation of ORF 1a is different

than that seen during infection, it has not yet been possible to assess the cleavage of p65 in this system. Specifically, p65 has not been detected during *in vitro* translation of either purified virion RNA or of synthetic ORF 1a RNA transcripts. Thus, although E64d clearly inhibits gene 1 polyprotein processing, not all proteolytic events are blocked. Because of this differential inhibition, E64d may provide a means of investigating the specificity of individual proteolytic events, and notably those important for maturation of proteins involved in viral RNA synthesis. More specifically, it appears that inhibition of p28 cleavage is not responsible for inhibition of RNA synthesis in E64d-treated cells.

We do not yet know which proteinase is inhibited by E64d. The role of E64d in inhibition of FMDV replication has been well defined. E64d specifically and irreversibly inhibits the FMDV papain-like L proteinase, but not the serine-like 2A or 3C proteinases (Kleina and Grubman, 1992). Analogy to the FMDV results suggest that it is more likely that one of the two papain-like proteinases is inhibited by E64d, rather than the 3C-like proteinase. There is as yet no experimental evidence to support this hypothesis, and it is therefore still possible that the 3C-like proteinase may be involved.

Polyprotein processing is required at all times of coronavirus infection for new RNA synthesis and virus replication

Our study demonstrates that the inhibition of proteinase activity, whether specifically viral or viral and cellular, results in inhibition of MHV replication, which in turn results from shutoff of viral RNA synthesis. The pattern of inhibition of new MHV RNA synthesis in the presence of E64d is similar to that seen following the addition of cycloheximide to MHV-infected cells, as first demonstrated by Sawicki and Sawicki (Sawicki and Sawicki, 1986). They showed that the addition of cycloheximide at late times of MHV infection in DBT cells resulted in a rapid decline in the rate of MHV RNA synthesis and concluded that continued translation of MHV replicase proteins is necessary for ongoing viral RNA synthesis. We have confirmed these results and compared them with rates of RNA synthesis in the presence of E64d. E64d addition also results in rapid shutoff of viral RNA synthesis when added as late as 6 hr after infection, and the rapidity and degree of inhibition of new MHV RNA synthesis in the presence of E64d are significantly greater than those occurring with cycloheximide. This would be expected if maturation of replicase or accessory proteins is the rate-limiting step in transcription of new RNA molecules. Our data suggest that E64d may be irreversibly inactivating all available molecules of proteinase, thereby effectively eliminating the pool of replicase proteins at one time. Cycloheximide blocks the translation of new gene 1 polyprotein molecules, but processing of preexisting molecules could ostensibly continue until the pool is exhausted, manifesting as a gradual decrease in the rate of synthesis of MHV RNA. Our results also support the conclusion that the viral protein(s) involved in MHV RNA synthesis act in a stoichiometric fashion, and further suggest that polymerase activity requires proteolytic activity and therefore cannot occur when the protein(s) is in its uncleaved precursor state. These conclusions are supported by the observation that MHV replication can resume at 16 hr p.i. if E64d is added at only 1 hr p.i. and is not supplemented. Since E64d is a specific cysteine proteinase inhibitor which irreversibly binds and permanently inactivates a molecule of proteinase, degradation of E64d cannot alone account for escape of the virus from inhibition. Rather our data suggest that translation of the polyprotein from gene 1 continues in the presence of the inhibitor and that the consumption of E64d by binding to continuously synthesized proteinase molecules, along with degradation, finally allows the concentration of proteinase to exceed that of inhibitor, at which time a full round of virus replication is initiated.

There are several ways that E64d could cause shutoff of MHV RNA synthesis. E64d may be inhibiting the maturation of one or more of the proteinases encoded by ORF 1a by blocking the *cis*-autoproteolytic cleavage of the proteinase from the polyprotein. This in turn might result in inhibition of *cis* and/or *trans* cleavage of the RNA-dependent RNA polymerase(s) from the ORF 1b polyprotein. Failure of maturation of the polymerase protein would then result in loss of RNA synthetic activity. Alternatively, inhibition of processing could occur at the point where mature proteinase is available to act *in cis* or *in trans* at other substrate sites. This could limit cleavage of polymerase itself or of other proteins, such as p65, which might be involved in RNA synthesis. Finally, since E64d is known to inhibit calpain, it could be inhibiting processing of cellular proteins involved in MHV RNA binding and replicase complex formation. It has been determined for several RNA viruses that cellular proteins are involved in regulation of viral RNA transcription and translation, and cellular proteins which bind to the 5′ end of the plus-sense and the 3′ end of the minus-sense MHV-JHM genome RNA have recently been described (Furuya and Lai, 1993). Thus, it is possible that E64d could also be inhibiting maturation of cellular proteins involved in coronavirus RNA synthesis.

E64d is a potent inhibitor of MHV replication, which will be useful in studying the processing and functions of the gene 1-encoded proteins. It may provide a means of determining the sensitivity and specificity of the coronavirus-encoded proteinases. We are investigating the activities of the predicted MHV 3C-like proteinase, using E64d to assess its processing activities and specificity. We are also using this agent to look at the processing kinetics of p65 from the ORF 1a polyprotein. Finally, E64d

may also be a useful tool in the investigation of the pathogenesis of MHV infections, due to its potent inhibitory effect and demonstrated low toxicity in mice.

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