INHIBITION OF CORONAVIRUS MHV-A59 REPLICATION BY PROTEINASE INHIBITORS

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INTRODUCTION

Replication of mouse hepatitis virus is initiated by translation and processing of the gene 1 polypeptide. The increasing complexity of this pattern of replicase protein expression is becoming apparent. MHV-A59 encodes approximately 800 kDa of polypeptide within the two overlapping open reading frames of gene 1. In addition, at least one proteinase activity encoded by gene 1 has been identified^{1,2} and two more are predicted, along with putative polymerase, helicase, NTP binding and possibly growth factor like proteins³⁻⁵. Only the first 380 kDa of protein products of MHV gene 1 ORF 1a have been described and characterized as to the pattern of expression and processing in virus infected cells⁶. Specifically, proteins of N-p28-65-290-C constitute the initial translation products of this region. The cleavage between p28 and p65 has been best described, both in vitro and in virus-infected cells⁷. As of yet no specific functions have been ascribed to any of these proteins. Thus a great deal remains to be learned about the processing pattern and functions of MHV gene 1 encoded proteins.

Based on sequence analysis and demonstrated activity of an ORF 1a encoded proteinase, it is assumed that most or all of the processing of the gene 1 polypeptide is effected by viral proteinases. Thus, one of the tools which has been extensively used in the investigation of gene 1 expression is the addition of proteinase inhibitors to in vitro or intracellular translation reactions, in order to determine the effect on polypeptide expression and to determine the specificity of proteolytic events involved in maturation of gene 1 products. The processing of the N-terminal protein p28 was defined based on the inhibition of appearance in the presence of leupeptin or ZnCl_2 ⁸. Subsequently, the cleavage of the p290 protein into p50 and p240 in cells was also defined in part by its inhibition in the presence of leupeptin⁷.

Leupeptin is an amino acid aldehyde (N-acetyl-leu-leu-arg-aldehyde), which inhibits most cysteine and trypsin-like serine proteinases⁹. Leupeptin acts by forming a stable,

noncleavable tetrahedral intermediate in the substrate binding region of the proteinase. However leupeptin is a reversible inhibitor, and is also degradable, so we have sought more stable, irreversible inhibitors of specific serine and cysteine proteinase inhibitors to allow more careful dissection of the proteolytic pathway of the gene 1 polyprotein. One such agent which will be described in this paper is e64 (L-trans epoxysuccinyl-leucylamido(4-guanidino)butane), a specific inhibitor of cysteine (thiol) proteinases such as calpain, cathepsin B and papain¹⁰. E64 irreversibly binds the thiol group of cysteine catalytic residues, thereby inactivating the proteinase molecule. Thus e64 is considered an excellent active site titrant for studies of cysteine proteinases. We have utilized e64d, the uncharged diethyl ester derivative of e64, because of its excellent cell penetrability. This agent has previously been used to describe inhibition of L proteinase of the picornavirus, foot and mouth disease virus (FMDV)¹¹. Leupeptin and e64d both can penetrate cells, and both are very non toxic to cell monolayers and to mice, and thus make excellent agents to study effects on virus protein processing in virus infected cells or intact animals.

During studies of polyprotein processing in MHV-A59 infected DBT cells¹², we observed that addition of either leupeptin or e64d to media at early times of infection resulted in significantly decreased viral syncytia formation. If the inhibitor is added early enough, the yield of viral nonstructural proteins at 8-10 hours is markedly decreased (Denison, unpublished data). The present studies are predicated on that observation and the hypothesis that the presence of proteinase inhibitors diminishes virus replication by acting at the level of inhibition of gene 1 polyprotein processing, an essential step in virus replication. We here show that both leupeptin and e64d inhibit MHV-A59 virus replication in DBT cells. Leupeptin and e64d both result in diminished viral RNA synthesis and infectious virus, even when added at relatively late times post infection. Thus polyprotein processing appears to be required throughout infection in order for ongoing RNA synthesis to occur.

MATERIALS AND METHODS

Virus stocks, cells, and proteinase inhibitors. Stocks of MHV-A59 were plaque purified times three and passaged at low MOI prior to use. DBT cells were used for virus growth, isolation, infected cell lysates and plaque assay¹². Cells were maintained in DMEM 10% fetal calf serum (FCS) and infections were performed in DMEM 2% FCS. Leupeptin was obtained from Sigma, and was dissolved in water at stock concentrations of 200mM and used at final concentrations of 2mM. 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 100mg/ml and used at concentrations of 500 µg/ml in DMEM 2% FCS.

Plaque assays. Plaque assays were performed on confluent monolayers of DBT cells in six-well plates. Cells were infected for 30 minutes at 37°C with virus from media supernatants diluted in gel saline, followed by media/agar overlay for 24-30 hours and subsequent addition of agar containing 0.02% neutral red for 6-12 hours with counting of clear plaques. All plaque assays were performed in duplicate and the experiments were repeated twice.

Determination of infectious virus and cytopathic effect. Confluent monolayers of DBT cells in 60mm petri dishes were infected with MHV-A59 at an MOI of 20 PFU/cell in DMEM 2% FCS for 30 min. at 37° C. Plates were rinsed with Tris-saline pH 7.4 three times prior to addition of overlay media. For assays of infectious virus, monolayers were overlaid with 2ml of media and 100µl aliquots were removed at the time points indicated in the individual experiments. For assay of proteinase inhibitors, leupeptin (2mM) or e64d (500µg/ml) was added to the media 1 hour p.i. Monolayers were carefully observed by two

independent observers who were blinded to each other, and estimates were made of the percent involvement of each monolayer by virus-induced syncytiae prior to determination of supernatant virus titers.

Determination of viral RNA synthesis. Infection was initiated exactly as described above. Actinomycin D was added to the media at a final concentration of 10 µg/ml at 4 hours p.i. when determinations of RNA synthesis were to be made at 8-9 hrs p.i. [³H] uridine (NEN) was added at a final concentration of 200 µCi/ml at times indicated in the presence of leupeptin (2mM) or e64d (500 µg/ml) added as shown in individual experiments. At the end of the labeling period, plates were washed on ice with PBS times three, lysed in hypotonic solution containing 1% SDS, and lysates were passed through a 23 gauge needle. Aliquots were taken for determination of TCA insoluble radioactivity, and were always performed in triplicate.

RESULTS AND DISCUSSION

Leupeptin Inhibition of Virus Titer and RNA Synthesis

We first investigated the effect of leupeptin on MHV-A59 virus replication in single cycle growth experiments in DBT cells by adding leupeptin at 1 hour p.i. to the overlying media, with or without supplementation every three hours throughout the infectious cycle (Figure 1).

Visually, we observed a delay in the appearance of viral syncytia in the leupeptin-treated cells. When virus titer was determined at the various time points, the eclipse phase of virus replication was markedly affected at all concentrations of leupeptin, with a delay of 4-6 hours, compared with the control infection, before increases in supernatant virus titers were observed. Virus titers in leupeptin-treated cells were diminished at all times points compared with controls, with the most marked reduction measured in those cells treated with 2mM leupeptin and supplemented every 3 hours, where 2.8 log reductions in virus titer were noted at 12 hours post infection.

We next investigated the importance of time of addition of leupeptin on replication inhibition. In the single-cycle growth experiments, leupeptin was added 1 hour after infection, and the results were therefore consistent with inhibition of an event occurring after binding and entry (Figure 2). Therefore we sought to determine whether leupeptin resulted in inhibition of viral RNA synthesis as a marker of inhibition of maturation of proteins expressed from gene 1. DBT cells were infected with MHV-A59 and 2mM leupeptin was added at various times from -1 hr to 8 hrs after infection. In parallel experiments we measured either virus titer at 8 hours after infection or uptake of [³H]uridine into actinomycin D resistant RNA at 8 to 9 hours after infection. When leupeptin was added at or before 2 hrs p.i., there was a greater than 90% reduction in supernatant virus titer measured at 9 hrs p.i. The titer was substantially decreased when leupeptin was added as late as 7 hrs p.i., suggesting interference with an ongoing intracellular process, such as protein translation, processing or RNA synthesis.

DBT cell morphology, cell replication, and protein and RNA synthesis were unaffected by the presence of up to 10mM leupeptin, indicating that the effect on MHV-A59 replication was not due to cellular cytotoxicity (data not shown). There did not appear to be any direct antiviral effect of leupeptin on the virus, since incubation of stock virus with leupeptin did not reduce measurable virus titer. Leupeptin also did not interfere with maturation cleavage of the spike (S) surface glycoprotein. No increase in infectious virus particles was observed when MHV grown in the presence of leupeptin was incubated with trypsin at 37°C for up to two hours. These data strongly suggest that early proteolytic events,

Figure 1. Inhibition of MHV-A59 replication by leupeptin. Infections were performed as described in Materials and Methods. Monolayers of DBT cells were infected with MHV-A59 at an MOI of 10 PFU/ml. Leupeptin was initially added at 1 hr p.i. to all plates except the control (no leu). For those plates which were supplemented, leupeptin was added at 3 hour intervals directly to the media. Samples were obtained at the times indicated and titer was determined by plaque assay on DBT cell monolayers.

possibly those important for ORF 1a processing, may be the site of leupeptin-mediated inhibition of MHV replication.

The results of the parallel experiment, in which viral RNA was labeled with [³H] uridine in the presence of actinomycin D, are shown in Figure 2. The results are surprisingly similar to those found when measuring infectious virus titer. When leupeptin was added before 2 hrs p.i., viral RNA synthesis at late times was at or below the level of mock-infected control cells in the presence of actinomycin, or essentially absent. Also similar to the virus titer experiment, addition of leupeptin up to 7 hrs p.i. resulted in a significant reduction in viral RNA synthesis at 8 to 9 hours. Together, the results of these experiments indicate that an ongoing intracellular viral replication event is inhibited by leupeptin, and that the inhibition manifests as diminished RNA synthesis with concomitant reduction in virus titer.

E64d Inhibition of MHV-A59 RNA Synthesis and Replication

Because leupeptin is a reversible inhibitor, and because of its broad specificity for both cysteine and serine proteinases, it is not possible to state whether the need for supplementation and the decreasing inhibition when added at late times of infection is due

Figure 2. Leupeptin inhibition of MHV-A59 replication and RNA synthesis. A) Virus titer at 9 hours p.i. Leupeptin was added at the times p.i. indicated, except for the control, to which no leupeptin was added. Supernatant virus was harvested at 9 hours post infection and titered on DBT cells. B) Incorporation of [3 H]uridine into viral RNA. Leupeptin was added at the times p.i. indicated. Actinomycin D was added to virus infected monolayers at 4 hrs p.i. and [3 H]uridine was added from 8-9 hrs p.i., followed by cell lysis and measurement of TCA insoluble radioactivity. "Uninf." indicates uninfected cells in the presence of actinomycin D and "no leu" indicates infected cells in the presence of actinomycin D but in the absence of leupeptin.

to reversibility of inhibition or synthesis of new proteinase molecules. Thus we investigated the inhibition of replication with e64d, the specific, irreversible cysteine proteinase inhibitor. This was potentially a good agent because all of the known or predicted gene 1-encoded proteinases are thought to possess cysteine catalytic residues. In addition, its irreversible inhibition is such that binding to a molecule of proteinase eliminates that molecule. Therefore any late resumption of replication can be attributed the activity of newly available proteinase. Finally, it has been shown that e64d inhibits at least one cleavage in the gene 1 polypeptide, at the carboxy terminus of p65¹³. We first repeated the single cycle growth experiments with 500 μ g/ml of e64d during MHV-A59 infection of DBT cells, and visually estimated percent involvement in syncytia prior to titrating supernatant virus at various time points from 0 to 24 hours (Figure 3).

E64d added at 1 hour p.i. completely blocked virus replication as measured both by titer and visible cytopathic effect when compared to control. At 10 hours post infection, there were no syncytia visible in the e64d treated plates, and virus titer was diminished greater than 6 logs compared to the control. E64d was added at only one time, 1 hr p.i.; thus it was interesting that replication as measured by both titer and syncytia estimates recommenced after a 12-16 hour lag. This indicated both that the cell monolayer was not adversely affected by the e64d and that the inhibitor was exhausted, possibly by a combination of degradation and binding to proteinase. These results were entirely consonant with those observed with leupeptin, but the inhibition was much more complete.

We next examined the effect of e64d on viral RNA synthesis, in order to assess the degree of inhibition and to determine the role of time of addition (Figure 4). Similar to leupeptin, e64d had no effect on cellular RNA synthesis in the absence of actinomycin D at 8 hours after addition, which was consistent with experience with other cell types¹¹.

In contrast, the addition of e64d (500 μ g/ml) at any time from -1 to 7 hours p.i. resulted in complete ablation of viral RNA synthesis as measured by actinomycin D resistant [3 H]uridine incorporation at 8-9 hrs p.i. These results show that e64d can inhibit new RNA synthesis for more than 9 hours when added at the beginning of infection, and also demonstrate that the half-life of that inhibition is short, likely less than 1 hour. This was

Figure 3. Inhibition of MHV-A59 virus replication by e64d. Single cycle growth of MHV-A59 in DBT cells was determined. The monolayer was infected at an MOI of 20 in absence of proteinase inhibitor (Control), or e64d (500 µg/ml in DMSO). The percent involvement of the monolayer in viral syncytia was determined as described in the Materials and Methods. Aliquots of overlying media were taken at the time points indicated and titer determined. The log titer is to the left of the figure and the %syncytia to the right.

consistent with the results obtained with leupeptin, but was more rapid acting and complete when added at late times of infection.

Requirement for Continuous Proteolytic Processing in MHV Replication

Both leupeptin and e64d have been used to inhibit processing of the gene 1 polyprotein. We here demonstrate that the addition of either of these agents to virus infected cells

Figure 4. Time of addition of e64d and inhibition of RNA synthesis. 60mm plates of confluent DBT cells were infected at an MOI of 20. Actinomycin D (10 µg/ml) was added to all infected cells and one uninfected plate at 4 hrs p.i. E64d was added to the media of infected cells at the times indicated from -1 hr to 7 hrs p.i., it was added to the uninfected cell control at 1 hr after mock infection. No e64d was added to the infected control. [³H]uridine was added at 200 µCi/ml at 8 hrs p.i. and cells were lysed at 9 hrs p.i. after a one hour labeling period. TCA insoluble incorporation into RNA was determined.

inhibits new viral RNA synthesis, and therefore output virus titer. The effect on titer more impressive at early times of infection, whereas even at late times of addition, RNA synthesis measured shortly after addition is already diminished. E64d inhibited virus replication to a greater extent than leupeptin (5.0 vs 2.8 log reduction at 8-10 hrs), and also demonstrated a longer duration of inhibition (up to 16 hrs). E64d also more completely inhibited new viral RNA synthesis (>90%) at late times of infection (7 hrs p.i.) than leupeptin.

Together the results obtained with leupeptin and e64d suggest a model in which continued protein expression and processing, is required for viral RNA synthesis to occur, and that interference with processing allows rapid depletion of the proteins involved in the RNA synthesis activities. It has been previously demonstrated that ongoing protein synthesis is necessary for coronavirus RNA synthesis¹⁴, and our data show that processing is required as well. Early inhibition of proteolysis may result in markedly diminished RNA templates for transcription, protein synthesis, and packaging into progeny virions. Our results further suggest that some of the proteins involved in RNA synthesis, whether polymerases directly or proteinases more indirectly, have a short half-life of activity during replication, rather than being continuously available throughout infection once translated and processed. As the entire scheme of gene 1 polyprotein processing becomes apparent, these proteinase inhibitors will allow us to assess specifically which proteolytic events are required for ongoing RNA synthesis.

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