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Intraluminal Proteolytic Activation Plays an Important Role in Replication of Type 1 Reovirus in the Intestines of Neonatal Mice

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Oral inoculation of suckling mice with reovirus serotype 1 (strain Lang) results in the conversion of intact virions to intermediate subviral particles (ISVPs) in the intestinal lumen. Digestion of virus in vitro with chymotrypsin or trypsin reveals two distinct forms of ISVPs, while the predominant species of ISVPs found in the small intestinal lumen appears to be identical to the chymotrypsin product. The in vivo conversion of virions to ISVPs was blocked by pretreatment of mice with protease inhibitors, resulting in inefficient replication of reovirus in intestinal tissue. The early inhibition of viral replication in suckling mice pretreated with protease inhibitors was not observed when suckling mice were inoculated with ISVPs generated by in vitro digestion with either chymotrypsin or trypsin. However, replication was decreased during secondary rounds of replication in mice receiving repeated doses of protease inhibitors, suggesting that luminal proteolytic digestion is important in rendering progeny virions infectious in the gut.

To initiate infection, reoviruses enter their mammalian hosts via the gastrointestinal tract, where they undergo primary replication before spreading in the host (12). Previous studies with mice have shown that reovirus type 1 penetrates the intestinal epithelium via membranous (M) cells, specialized epithelial cells overlying Peyer's patches, before infecting other intestinal epithelial cells, which they enter via their basolateral surfaces (3, 17, 28, 29). While in the intestinal lumen, reovirions are exposed to the luminal contents, including a variety of proteolytic enzymes which may have important effects on early events in the viral life cycle. The effect of such enzymes on reoviruses may be mediated in part by the M2 gene, which encodes the μ1c outer capsid protein of the virion. Genetic studies have indicated that the M2 gene determines the response of reoviruses to digestive proteases (8, 16). Treatment of purified reovirus with chymotrypsin results first in the loss of an outer capsid protein, σ3, followed by the cleavage of μ1c to δ, a lower-molecular-weight form (5, 10, 18, 21, 23). The resultant viral particle has been termed an intermediate subviral particle (ISVP).

Many viruses, such as rotavirus (2, 7), Sendai virus (19), astrovirus (14), coronavirus (22), and influenza virus (13), require specific proteolytic activation with trypsin for efficient propagation in tissue culture. For rotavirus, Sendai virus, and influenza virus, other proteases, such as chymotrypsin, have been shown to be incapable of activating the viruses in vitro. Type 1 reovirus replicates efficiently in murine L cells without the addition of exogenous proteases. Predigestion of the intact type 1 reovirions with chymotrypsin results in the formation of ISVPs which have a shorter eclipse phase than intact virions and can overcome the inhibitory effects of ammonium chloride and other lysosomotrophic agents on viral replication in L cells (20, 23).

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Little is known about the effect of the contents of the lumen of the gastrointestinal tract on primary replication of type 1 reovirus in small intestinal epithelial cells. Bodkin et al. showed that reovirus is converted to ISVPs in the intestinal lumen of neonatal mice (4). In this report, we demonstrate that proteolytic conversion of intact type 1 reovirions to ISVPs in the intestinal lumen is a major factor in its efficient replication in the intestine of the suckling mouse.

Initially, the effect of different digestive enzymes on the structure of intact reovirus type 1 was determined (Fig. 1). In vitro digestion with chymotrypsin and trypsin as previously described (23) resulted in formation of ISVPs (lanes 3 and 4), with loss of σ3 and cleavage of μ1c to its lower-molecular-weight form, δ. The δ observed after trypsin digestion had an apparent lower molecular weight by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (59 versus 60 kilodaltons) than the δ observed after chymotrypsin digestion. The δ observed in ISVPs recovered from the murine small intestine (lane 5) was identical by SDS-PAGE to that generated with chymotrypsin. Pepsin digestion at pH 3.0 (lane 1) appeared to remove only σ3 without affecting μ1c. Mock digestion at pH 3.0 and digestions with pepsin at pH 5.0 and 6.0 showed no loss of σ3 (data not shown). Preparations of trypsin- and chymotrypsin-treated virus consistently had particle-to-PFU ratios of 70 to 80, while those of mock-digested virus had ratios of 150 to 200. Pepsin-treated virus was not demonstrably infectious when titers were determined on L cells.

In order to determine whether ISVP formation in the intestinal lumen is important for viral replication in the intestine, we sought to inhibit luminal proteolysis of virions (Fig. 2). Ten-day-old BALB/c/ByJ mice from seronegative dams were given 1 mg of aprotinin and 0.025 mg of chymostatin intragastrically 1 h before administration of 5×10^5 cpm of purified ^{35}S-labeled reovirus type 1 (23). Four hours later, luminal virus was recovered by rinsing and concentrated by ultracentrifugation at $200{,}000 \times g$ before SDS-

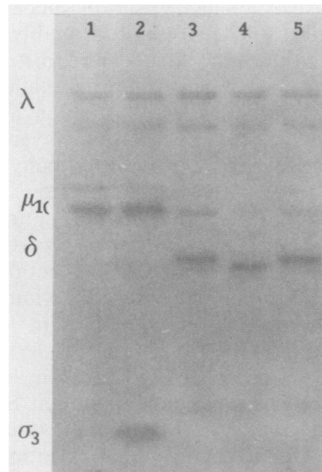

FIG. 1. Fluorogram showing the effect of in vitro digestion of virus by various gut proteases. ^{35}S-labeled virus was digested with 0.1 mg of protease per ml for 1 h at 37°C and then diluted in Laemmli sample buffer and subjected to SDS-PAGE. Lanes: 1, pepsin digest; 2, mock digest; 3, chymotrypsin digest; 4, trypsin digest; 5, virus recovered from the small intestine of a suckling mouse 2 h after peroral inoculation. Positions of the proteins λ, μ1c, δ, and σ3 are indicated.

PAGE and autoradiography. Virus recovered from control mice 4 h postinoculation had undergone digestion to ISVPs (Fig. 2, lane 3). Aprotinin (lane 4) or chymostatin (lanes 7 and 8) pretreatment of mice resulted in moderate inhibition of in vivo ISVP formation, while pretreatment of mice with both aprotinin and chymostatin (lanes 5 and 6) resulted in more effective inhibition of ISVP formation in vivo. There was some loss of σ3 from virions in animals pretreated with both aprotinin and chymostatin (30%), but there was little conversion of μ1c to δ (6%).

To determine the effects of protease inhibitors on in vivo replication of reovirus in the intestine, 10-day-old mice were pretreated with either protease inhibitors (aprotinin and chymostatin) or buffer 1 h before intragastric inoculation with either 10^5 PFU of intact type 1 reovirus or in vitro-generated ISVPs. Because there was an approximately 2.5-fold increase in infectious titer when ISVPs were compared with intact virions in L-cell plaque assays, the number of ISVPs or virus particles was adjusted appropriately. Animals were killed 12, 24, and 48 h after inoculation, and their small intestines were frozen in gel saline for subsequent plaque assay on L-cell monolayers as previously described (12). In control experiments protease inhibitors added in vitro to purified reovirus type 1 at concentrations up to 10 times that given to animals had no effect on plaque assay results. Viral titers in the small intestines of mice pretreated with inhibitors before inoculation with intact virions were significantly lower than in controls at 12 to 24 h (Fig. 3). Furthermore, the reduction in viral titers in intestinal tissues associated with the administration of protease inhibitors was not observed when animals were inoculated with ISVPs. Since the protease inhibitors were presumably effective within the intestinal lumen for only a limited time, we then repeated these experiments with administration of the inhibitors every 12 h (Fig. 4). Viral replication in the intestine was even further reduced in the mice inoculated with intact virions and treated with serial doses of protease inhibitors. Mice that were treated with serial doses of inhibitors and

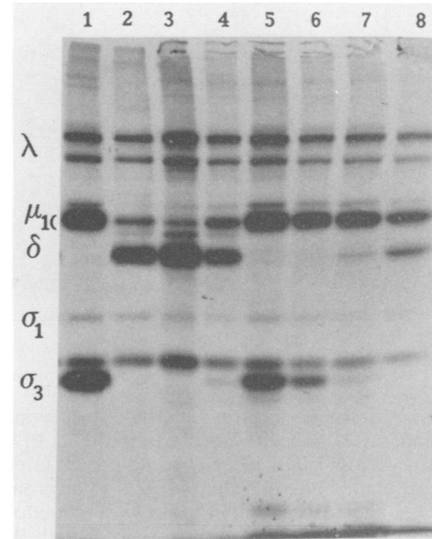

FIG. 2. Fluorogram showing the effect of protease inhibitors on in vivo virus digestion in the intestinal lumen. Radiolabeled virus was administered to 10-day-old suckling mice which had been pretreated with phosphate-buffered saline or protease inhibitors. Mice were killed 4 h postinoculation, and virus was recovered from the intestinal lumen by rinsing and was then subjected to SDS-PAGE and fluorography. Lanes: 1, ^{35}S-labeled purified type 1 reovirus; 2, virus digested with 0.1 mg of chymotrypsin per ml in vitro; 3, virus recovered from a mouse pretreated with phosphate-buffered saline; 4, virus recovered from a mouse pretreated with aprotinin alone; 5 and 6, virus recovered from two mice pretreated with aprotinin and chymostatin; 7 and 8, virus recovered from two mice pretreated with chymostatin alone. Positions of the proteins λ, μ1c, δ, σ1, and σ3 are indicated.

inoculated with ISVPs had titers comparable with those of control animals at 12 h, but these reached a plateau thereafter, suggesting that after the initial round of replication of ISVPs subsequent replication required luminal proteolysis.

In other experiments, the growth of trypsin- and chymotrypsin-generated ISVPs in intestines of mice pretreated with

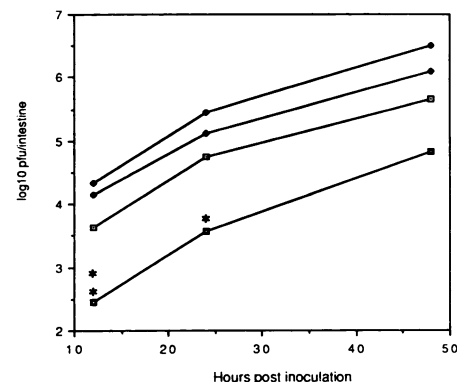

FIG. 3. Viral titers in intestinal tissue after a single dose of protease inhibitors. Mice were inoculated with 10^5 PFU of type 1 reovirus (⊡ and □) or type 1 reovirus ISVPs (◆ and ◇) 1 h after pretreatment with either phosphate-buffered saline (⊡ and ◆) or protease inhibitors (□ and ◇). Datum points represent the mean of three to five animals. Symbols: * and **, $P < 0.05$ and $P < 0.02$ compared with phosphate-buffered saline-pretreated type 1 reovirus, respectively.

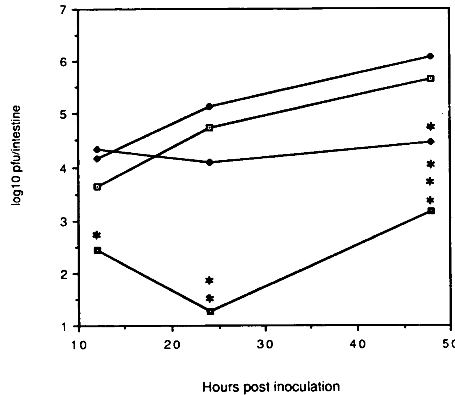

FIG. 4. Viral titers in intestinal tissue after serial doses of protease inhibitors. Mice were inoculated with 10^5 PFU of either type 1 reovirus (⊡ and □) or ISVPs (◆ and ◇) 1 h after treatment with either phosphate-buffered saline (⊡ and ◆) or protease inhibitors (□ and ◇). The administration of protease inhibitors or phosphate-buffered saline was continued every 12 h until the intestine was harvested. Datum points represent the mean of three to five animals. Symbols: *, **, and ***, $P < 0.05$, $P < 0.02$, and $P < 0.001$ compared with phosphate-buffered saline-treated type 1 reovirus, respectively.

protease inhibitors was compared (Fig. 5). Both species of ISVPs were able to successfully replicate in the presence of the protease inhibitors. Pepsin-digested ISVPs that had lost σ3 but retained μ1c had a >1,000,000-fold reduction in titer from the original stock of purified intact virions when assayed on L cells. These were not demonstrably infectious in vivo (data not shown).

These data indicate that proteolytic digestion of reovirus type 1 plays an important role in efficient primary viral replication in the intestine of suckling mice. Many enteric viruses, such as rotavirus and astrovirus, are dependent on

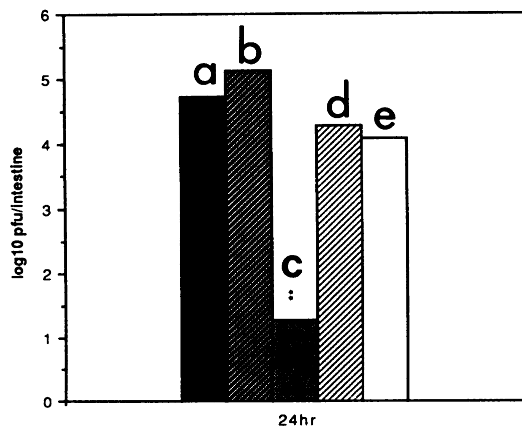

FIG. 5. Comparison of growth at 24 h postinoculation of trypsin-digested ISVPs and chymotrypsin-digested ISVPs in the intestines of suckling mice treated with protease inhibitors every 12 h. Mice were inoculated with 10^5 PFU of type 1 reovirus (a and c), chymotrypsin-generated ISVPs (b and e), or trypsin-generated ISVPs (d) 1 h after pretreatment with either protease inhibitors (c through e) or phosphate-buffered saline (a and b). The pretreatment was repeated 12 h postinoculation, and the intestines were harvested at 24 h. Values are expressed as means of three to five animals. *, $P < 0.02$ compared with phosphate-buffered saline-treated type 1 reovirus (a).

exogenous proteases such as trypsin for successful propagation in tissue culture (7, 14) and probably exploit host intestinal proteases in vivo. A recent report describes protection of suckling mice from rotavirus challenge by treatment with protease inhibitors (27). Influenza virus, which requires exogenous protease activation for growth in some tissue culture systems, can be proteolytically activated for replication in the presence of nasal secretions from children (1) and by staphylococcal proteases (24). Further evidence for the importance of proteases for the growth of influenza virus in vivo comes from reports of protection of chickens and mice from lethal influenza virus infection by treatment with protease inhibitors such as leupeptin, aprotinin, and aminocaproic acid (26, 30, 31). The necessity of host proteases for secondary rounds of replication has been demonstrated for chymotrypsin-dependent mutant Sendai viruses in the mouse lung (25). Type 1 reovirus is unusual in that it requires proteases for in vivo replication in intestinal tissue yet grows readily, although slower and to slightly lower yields, in tissue culture without proteases and in the presence of potent protease inhibitors in fetal calf serum. Many of the protease-dependent viruses require specific proteases for growth (2, 19). Reovirus type 1 grows in the murine gut and in tissue culture after either trypsin or chymotrypsin digestion, although in our suckling mouse model most of the intraluminal digestion appeared to be due to chymotrypsin-like activity. Further studies will be needed to elucidate the molecular basis and biologic significance of the two distinct forms of δ, which result from digestion with trypsin or with chymotrypsin.

Early events in target cell-virus interactions are likely involved in the mechanism by which digestion of type 1 reovirus to ISVPs enhances replication of reovirus in intestinal tissue. Removal of σ3 and cleavage of the μ1c peptide by chymotrypsin digestion of reoviruses have been shown to result in extension of the σ1 peptide, perhaps making it more accessible to cellular receptors (9; M. L. Nibert, D. B. Furlong, and B. N. Fields, unpublished data). Increased efficiency of binding of ISVPs to target cells might be particularly important, since the site of type 1 reovirus penetration of the intestinal epithelium is known to be the M cell, which is found exclusively in the epithelium overlying domes of intestinal lymphoid follicles (28, 29) and is present in very small numbers compared with other intestinal epithelial cell types.

Another possible mechanism of proteolytic enhancement might lie in the facilitation of virus penetration through the target cell plasma membrane. In many protease-dependent viruses, cleavage of a surface fusion protein results in activation of hydrophobic regions which facilitate interaction between the protein and the target cell plasma membrane, ultimately resulting in entry of the viral genome into the cytoplasm so that it may replicate. Recent studies of rotavirus have revealed sequence homology between Vp3, the rotavirus outer capsid protein, which is cleaved by trypsin in proteolytic activation, and fusion proteins of several alphaviruses (15). Although such a region in the μ1c is not present, the work of Sturzenbecker et al. (23) demonstrated that cleavage of reoviruses to ISVPs is important for viral entry into the cytoplasmic compartment. They demonstrated that predigestion with chymotrypsin allows infecting virions to bypass endosomal blockade by ammonium chloride. That intestinal infection, unlike L-cell infection, is facilitated by conversion of intact virus to ISVPs may reflect differences in the endosomal or lysosomal compartment in intestinal epithelial cells compared with L cells.

Viral entry could also occur via direct membrane penetration without a need for the endocytic pathway. Some investigators have described evidence for direct penetration of host cell plasma membranes by protease-activated reovirus serotype 3 (6) and rotavirus (11). Others were unable to detect direct penetration of L cells by reovirus serotype 1 ISVPs (23). Despite specific efforts (D. M. Bass, unpublished results), we have not been able to observe evidence by transmission electron microscopy for such a mode of entry for reovirus type 1 ISVPs into intestinal cells.

Whatever the mechanism of reovirus type 1 proteolytic activation may be, such activation might be seen as fulfilling an important function in the viral life cycle. Fully encapsulated viruses might offer far greater environmental stability than ISVPs, which are generated only in response to the correct milieu for primary replication.

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