Proteolytic Processing of Reovirus Is Required for Adherence to Intestinal M Cells

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Reovirus adheres specifically to apical membranes of mouse intestinal M cells and exploits M-cell transepithelial transport activity to enter Peyer's patch mucosa, where replication occurs. Proteolytic conversion of native reovirus to intermediate subviral particles (ISVPs) occurs in the intestine, but it is not known whether conversion is essential for interaction of virus with M cells. We tested the capacity of native virions, ISVPs, and cores (that lack outer capsid proteins) to bind to intestinal epithelial cells *in vivo* and found that only ISVPs adhered to M cells. Thus, intraluminal conversion of native reovirus to ISVPs is a prerequisite for M-cell adherence, and outer capsid proteins unique to ISVPs (either $\sigma 1$ or products of $\mu 1$) mediate interaction of virus with M-cell apical membranes.

Many viruses gain entry to their hosts by crossing an epithelial barrier. In mammalian intestines, a large number of pathogens enter regions of the intestinal mucosa containing organized mucosal lymphoid tissues, which are composed of lymphoid follicles covered by a specialized follicle-associated epithelium (4, 8, 17). The follicle-associated epithelium contains M cells, a unique epithelial cell type whose function is to transport samples of luminal material, including antigens and microorganisms, to the underlying mucosa for the purpose of inducing a protective mucosal immune response (12). In mice, reovirus types 1 and 3 ingested orally exploit the vesicular transepithelial pathway of M cells to invade the mucosa, proliferate locally, and spread systemically (15, 24). By adhering specifically to the apical membranes of M cells, reoviruses ensure their own transepithelial transport and efficient delivery to mononuclear cells (most likely macrophages) within the Peyer's patch mucosa, where replication takes place. Thus, M-cell adherence is the first direct encounter of reovirus with the cells of the host and an essential first step in viral pathogenesis.

The biochemical features that allow reoviruses to recognize and adhere to M cells are not known. Interaction with M cells is a general strategy used by a variety of microorganisms whose life cycle involves crossing mucosal barriers (11). For example, poliovirus (19), human immunodeficiency virus (1), and several gram-negative bacteria (11) also selectively bind to M cells and invade via this route. Neither the M-cell receptors nor the microbial ligands involved for any infectious agent have been identified. The molecular structure of reovirus and the sequence of biochemical alterations that occur in reovirus capsid proteins during the early stages of host cell infection have been defined in detail (5, 15). Thus, reovirus provides a valuable

model system with which to explore the basis of selective microbe–M-cell interactions.

The reovirus outer capsid is composed of 600 copies of each of two major proteins: $\mu 1$ ($\mu 1C/\mu 1N$), which forms the basic shell of the external capsid, and $\sigma 3$, which decorates the native virus surface (5). In an intact virion, 5% of the $\mu 1$ protein is present in an uncleaved form while 95% has been cleaved to generate a small myristoylated $\mu 1N$ fragment and a large $\mu 1C$ protein; both fragments are found in the outer capsid (16). In addition, about 36 to 48 copies of viral hemagglutinin protein $\sigma 1$ are located (probably as trimers or tetramers) at the icosahedral vertices along with an additional protein, $\lambda 2$, that spans the capsid (9, 13). Treatment of virus with trypsin or chymotrypsin under controlled conditions *in vitro* results in an intermediate subviral particle (ISVP) generated by proteolytic removal of $\sigma 3$ and cleavage of $\mu 1C$ into a large, amino-terminal subfragment called δ and a smaller carboxy-terminal subfragment termed θ . δ , θ , and $\mu 1N$ all remain associated with the ISVP. In addition, there is a transition of $\sigma 1$ from a folded to an extended conformation (6). ISVPs are stable and highly infectious. Further protease treatment removes the remaining outer capsid proteins, yielding stable viral cores that cannot infect cells *in vitro* but are transcriptionally active (7).

In cultured cells, proteolytic cleavage to ISVPs occurs in endosomes or lysosomes (20), but in mice, native virions first encounter proteases in the intestinal lumen. Although ISVPs are generated in the intestine (3) and inhibitors of luminal serine proteases reduce viral infectivity in mice (2), it is not known at what step the conversion of virus to ISVPs is essential for infectivity, i.e., for interaction of reovirus with M cells, or for the subsequent infection of mucosal macrophages. In this study, we directly tested the capacity of three forms of reovirus—native virions, ISVPs, and cores—to bind to the apical membranes of mouse intestinal epithelial cells.

We first tested whether ISVPs generated by protease treatment *in vitro* are able to selectively recognize and adhere to M-cell apical membranes *in vivo*. Reovirus serotype 1 (Lang) from a standard laboratory stock was grown in mouse L cells in suspension culture, purified, and concentrated as described previously (14, 22). Numbers of viral particles were determined spectrophotometrically, and infectivity was measured by

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FIG. 1. Adherence of reovirus ISVPs to M cells in mouse intestines. ISVPs were generated by chymotrypsin digestion *in vitro* and inoculated into ligated loops of mouse ileum containing a Peyer's patch. Epithelial cells of tissues collected after 30 min were examined by electron microscopy. ISVPs were consistently observed on apical membranes and in endocytic vesicles of M cells but were generally absent from neighboring absorptive cells. Thus, ISVPs can selectively bind to M cells. Bar, 1 μ m.

plaque assay using L-cell monolayers. ISVPs were prepared by chymotrypsin digestion of purified reovirus (10) and injected into ligated loops of distal mouse ileum containing a Peyer's patch. Two 6-week-old female BALB/c mice (Charles River Laboratories, Wilmington, Mass.) were anesthetized with 2,2,2-tribromoethanol (Avertin; Aldrich Chemical Co., Milwaukee, Wis.). About 5 cm of the distal ileum was exposed through a midline incision and flushed with phosphate-buffered saline. A short segment (approximately 1 cm long) was ligated and inoculated with 10^{12} ISVPs, and the intestine was returned to the abdominal cavity. After 30 min, mice were killed by cervical dislocation and the loops were excised, rinsed with cold phosphate-buffered saline, and immersed in a fixative solution containing 2.5% glutaraldehyde, 2% formaldehyde, 4 mM CaCl_2 , and 2 mM MgCl_2 in 0.1 M Na cacodylate buffer. Mucosal samples were postfixated in 1% OsO_4 and 0.5% uranyl acetate and processed for electron microscopy as previously described (23).

Binding and endocytosis of ISVPs were evaluated by direct examination of the epithelium by electron microscopy. ISVPs were consistently observed on M-cell apical membranes (Fig. 1) and within endocytic vesicles in the apical cytoplasm of M cells, whereas few viral particles were associated with neighboring absorptive cells. Occasional absorptive cells both on the follicle-associated epithelium and on adjacent villi showed adherence of large numbers of ISVPs to the glycocalyx covering the apical microvillous border, but there was no evidence of endocytosis by these cells.

Having confirmed that ISVPs can selectively bind to M cells, we then sought to determine whether conversion of native virions to ISVPs is a prerequisite for M-cell binding. M-cell binding and endocytosis of native virions were evaluated in the presence and absence of protease inhibitors administered intraluminally at concentrations previously shown to inhibit viral infectivity *in vivo* (2, 3). It was important first to confirm that native virions were indeed converted to ISVPs in the intestinal lumens of our untreated mice and that conversion was effectively blocked by the intraluminal infusion of protease

inhibitors that we used *in vivo*. This was done by inoculating intestinal segments with 10^{12} virus particles that had been metabolically labeled with [^{35}S]methionine (20), recovering luminal contents after 30 min, and analyzing viral proteins by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and fluorography (3). Reovirus ISVPs are readily distinguished from native virions by this method because in ISVPs, the $\sigma 3$ band, prominent in lysates of native virus, is absent or reduced and the native viral 72-kDa band representing the intact $\mu 1\text{C}$ protein is shifted downward to a 59-kDa band representing delta (16). It should be noted that the ISVPs also contain $\mu 1\text{N}$ and θ , but these smaller fragments are not detected under the PAGE conditions of this experiment (14, 16). Luminal material recovered from control mice 30 min after inoculation with native virions showed a viral protein profile typical of ISVPs, indicating that endogenous proteases in the normal mouse intestine had effectively converted virus to ISVPs (Fig. 2).

Pilot experiments designed to confirm the ability of protease inhibitors to block reovirus conversion showed that when 7 μg of aprotinin per ml alone was added to the viral suspension just before infusion into the intestinal lumen, conversion to ISVPs was substantially but not completely inhibited (data not shown). A mixture of 7 μg of aprotinin per ml and 10% fetal bovine serum, however, completely prevented the conversion of native virus to ISVPs (Fig. 2), and thus, both aprotinin and serum were used in subsequent experiments. To rule out a possible direct effect of aprotinin or serum on virus binding unrelated to protease inhibition, ISVPs were generated by chymotrypsin treatment of virus *in vitro* and inoculated into ligated intestinal loops of two mice, with or without aprotinin and serum. Adherence of reovirus particles to M cells is usually followed by endocytosis, and *in vivo*, both events are occurring at any one time; therefore, estimates of total viral binding should take into account virus particles already taken up in endocytic vesicles, as well as those on apical cell surfaces. In this and subsequent experiments, viral adherence to M cells was visualized by electron microscopy and analyzed quantita-

FIG. 2. Protease inhibitors prevent conversion of native reovirus to ISVPs in the intestinal lumen in vivo. Intestinal segments were inoculated with 10^{12} native reovirus particles (type 1/Lang) that had been metabolically labeled with [35 S]methionine. Luminal contents were recovered after 30 min and analyzed by SDS-PAGE and fluorography, along with control preparations of native reovirus and ISVPs generated in vitro. In lane 1, native reovirus shows three prominent bands representing viral proteins λ , $\mu 1c$, and $\sigma 3$. In lane 2, ISVPs differ from native virions in that the $\sigma 3$ band is lost and the $\mu 1c$ protein band is shifted downward to a band representing the $\mu 1c$ product, δ . Smaller fragments of $\mu 1c$ ($\mu 1N$ and θ) were not retained in this gel. In lane 3, viral proteins recovered from mice inoculated with native virus along with aprotinin and serum show a protein profile typical of native virus. Thus, protease inhibitors effectively prevented the conversion of native virus to ISVPs. In lane 4, viral proteins recovered from the intestines of control mice show a profile typical of ISVPs, indicating that endogenous intestinal proteases have converted virus to ISVPs.

tively by counting the viral particles associated with the surface or apical endocytic vesicles of each M cell observed in ultrathin sections cut perpendicular to the surface of the follicle-associated epithelium (Fig. 3). The counts obtained indicated not the total number of virus particles associated with individual M-cell surfaces but rather a representative fraction thereof which could be used to compare M cells of one animal to those of another. The statistical significance of differences in these counts was evaluated by a two-tailed *t* test using Statview software on an Apple Macintosh computer. This analysis showed that binding of ISVPs to M cells was unaffected by aprotinin or serum (Fig. 3A).

To directly assess the role of proteolytic processing on viral adherence to M cells, 10^{12} native virions, either with or without aprotinin and serum, were inoculated into ligated ileal loops of six mice in three separate experiments. Peyer's patch tissues were collected after 30 min and processed and analyzed as described above. Native reovirus administered without inhibitors adhered efficiently to M cells (Fig. 3B and 4A), as previously observed (24). When endogenous luminal protease activity was inhibited, however, M-cell binding and endocytosis of virus were significantly decreased (Fig. 3B and 4B). Thus, intraluminal conversion of native reovirus to ISVPs is a prerequisite for M-cell adherence.

We then sought to determine which viral proteins might be involved in interaction of ISVPs with M cells. Prolonged protease treatment of ISVPs in vitro completely removes the $\sigma 1$ protein, as well as $\mu 1N$, δ , and θ (the three subfragments derived from $\mu 1$ and $\mu 1c$). The resultant viral cores are devoid

FIG. 3. Effect of proteolytic processing on the binding of native reovirus and ISVPs to M cells in vivo. Results of three separate experiments are shown. In all cases, viral particles were inoculated into ligated ileal loops of mice, Peyer's patch tissues were collected after 30 min, and adherence to M cells was analyzed by electron microscopy as described in the text. (A) Reovirus ISVPs were produced in vitro and inoculated either with or without protease inhibitors (PI). Binding of ISVPs to M cells was unaffected by inhibitors, indicating that ISVPs can adhere to M cells without further protease processing. (B) Native reovirus particles were inoculated either with or without protease inhibitors. Virus administered without inhibitors adhered efficiently to M cells. In the presence of inhibitors, however, M-cell binding and endocytosis of virus were significantly decreased ($P = 0.0052$). Thus, intraluminal conversion of native reovirus to ISVPs is a prerequisite for M-cell adherence. (C) ISVPs and viral cores were produced by controlled digestion of reovirus in vitro, and conversion of ISVPs to cores was confirmed by SDS-PAGE (data not shown). Equal numbers of cores or ISVPs (10^{12} particles) were injected into ligated loops of mouse ileum. Electron microscopic analysis showed that ISVPs were consistently present on M-cell surfaces but cores were not ($P = 0.0002$).

of outer capsid components, with the exception of the $\lambda 2$ spikes at the vertices of the particle (5, 15). If $\lambda 2$ is involved in ISVP binding to M cells, then both ISVPs and cores would be expected to bind. If binding is mediated by any of the subfragments of $\mu 1/\mu 1c$ or the extended $\sigma 1$ protein, however, cores should be unable to bind.

Cores were produced by digestion of native reovirus particles in vitro with chymotrypsin (14). SDS-PAGE of the digested particles showed loss of the $\sigma 1$ and delta bands (data not shown), confirming that conversion to cores was complete. In three separate experiments, equal numbers of cores or ISVPs (10^{12} particles) were injected into ligated loops of mouse ileum (two mice per experiment and one or two ligated loops per mouse) and association of viral particles with M cells was assessed by electron microscopy as described before. ISVPs were present on M-cell surfaces and in M-cell endosomes, as expected, but adherence or endocytosis of cores was not observed (Fig. 3C), although clusters of viral cores were seen associated with luminal debris. To test the possibility that such aggregation reduced the numbers of viral cores that were free to diffuse to the epithelial surface, we inoculated a large number of cores (10^{13} particles) but still did not observe M-cell binding or endocytosis. Thus, removal of the outer capsid protein $\sigma 1$ and $\mu 1/\mu 1c$ fragments abolished M-cell adherence.

These observations provide an important clue as to the nature of this virus-cell interaction. The fact that ISVPs, the active, infectious form of reovirus, adhere selectively to M cells of Peyer's patches whereas native virions and viral cores do not indicates that outer capsid proteins unique to ISVPs mediate interaction of virus with apical membrane components of M cells. The intact viral particle is a compact, highly stable

FIG. 4. Adherence of reovirus to M cells in the absence and presence of protease inhibitors. Native reovirus was inoculated into ligated intestinal loops as described in the legend to Fig. 3B. (A) In the absence of inhibitors, viral particles were observed adhering to apical surfaces and in endocytic compartments of M cells. (B) In the presence of protease inhibitors, little or no virus adhered to M-cell surfaces. Bar, 1 μ m.

structure whose function is thought to be related to survival outside the host and passage through the acidic environment of the stomach (15). The ISVP is generated in the small intestine and is dramatically altered: the $\sigma 3$ protein is removed, the $\mu 1$ protein and its product $\mu 1C$ are further cleaved, and attachment protein $\sigma 1$ is extended (5, 15). In this activated form, the virus is primed to interact with cells. The two most prominent surface components of ISVPs, $\sigma 1$ and $\mu 1$ products (including fragments $\mu 1N$, δ and θ), are thought to have different functions during infection of target mononuclear cells (5). The $\sigma 1$ protein binds to host cell surface receptors that appear to be sialylated glycoproteins or possibly glycolipids (8). The $\mu 1$ protein and its products have recently been shown to interact

with cell membranes, as evidenced by the capacity of ISVPs to cause release of ^{51}Cr from prelabeled cells (10) and to generate ion channels in planar lipid bilayers (21). It is thus possible that either $\sigma 1$ or products of $\mu 1$ are involved in M-cell recognition and entry. The $\lambda 2$ pentamers, proteins that form the spike to which the $\sigma 1$ protein is attached, are also found at the surface of the ISVP, but the presence of $\lambda 2$ pentamers on cores (which do not bind to M cells) argues against a direct role in M-cell recognition. Further studies are required to determine which of these proteins is critical for M-cell attachment.

We have established that proteolytic processing of native reovirus by enzymes in the intestinal lumen of a host is a prerequisite for M-cell transport of virus into Peyer's patch mucosa. These studies confirm that digestive proteases are the key signal that triggers conversion of intact viral particles to infectious ISVPs (2, 3). Reovirus thus serves as a striking example of how viruses use specific internal environments of the host as part of their own life cycle. The fact that other viruses and microorganisms use M cells of the gastrointestinal tract as a portal of entry (12) raises the possibility that other pathogens also exploit this enzyme-rich environment to change from an inactive, nonadherent form to an active form that can bind to M cells. Although there is no evidence for this, there is abundant evidence that gut proteases convert viruses from one form to another (18). It is possible that intraluminal alterations enable certain bacterial, as well as viral, pathogens to bind to M cells.

The M-cell transepithelial transport pathway used by reovirus leads directly to mucosal sites that are specialized for generation of mucosal immune responses (8). This suggests that information about the mechanism whereby reovirus interacts with M cells could be exploited to deliver other antigens to mucosal inductive sites. Although engineering of foreign proteins into reovirus is not feasible, reovirus outer capsid proteins might serve as targeting molecules to enhance the effectiveness of oral vaccines.

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