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The Entry of Reovirus into L Cells Is Dependent on Vacuolar Proton-ATPase Activity

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Inhibitors of vacuolar proton-ATPase activity (5 μM bafilomycin A1 or 50 nM concanamycin A) prevented infection by reovirus particles but not by infectious subviral particles (ISVPs). Neither compound affected virus attachment or internalization. However, both compounds potently blocked cleavage of the viral protein μ1C. Finally, both reovirus particles and ISVPs efficiently translocated the toxin alpha-sarcin to the cytosol during virus entry. Bafilomycin A1 blocked translocation of alpha-sarcin by reovirus particles but not by ISVPs.

Mammalian reovirus, a member of the *Reoviridae* family, contains a segmented double-stranded RNA genome enclosed in two protein capsid layers with no lipid envelope (22). σ1 is the protein that interacts with cellular receptors, which comprise multiple sialoglycoproteins on the cell surface (15). The interaction of reovirus with the receptor leads to reversible conformational modifications not only of σ1 itself but also of the whole virus particle (2, 10, 24). Upon this interaction, reovirions appear to be internalized in endosomes, following the classic route of receptor-mediated endocytosis (23). Acidification of endosomes by vacuolar proton-ATPases, or even fusion of endosomes with lysosomes, activates peptidases that modify several virion proteins. Thus, protein μ1C is cleaved to δ and φ (1, 3, 14, 19) while σ3 is removed (26), giving rise to the so-called infectious subviral particles (ISVPs) (22). These particles are now activated to trespass the endosomal membrane to deliver the genome plus the core proteins to the cytoplasm where virus gene expression is initiated (5, 16, 17).

Several lines of evidence suggest that reovirions do indeed enter cells by the endosomal route. Thus, morphological evidence indicates that virions are present inside endosomes of infected cells (2, 23). In addition, infection by reovirus particles is sensitive to NH_4Cl (17, 24), a compound that increases endosomal pH levels. Interestingly, infection by ISVPs is insensitive to NH_4Cl (24). Despite this, it has been suggested that both reovirions and ISVPs enter cells by the same pathway (24). These findings were, however, rationalized by supposing that the low-pH step, a step not required by ISVPs, is required to activate the proteolytic attack of virus particles. Other authors, however, have indicated that ISVPs enter cells directly via the plasma membrane (2). This suggestion is based on the finding that ISVPs, unlike reovirus particles, are able to permeabilize the cell membrane (2, 16). The permeabilizing activity of ISVPs may be related to their ability to open multisized pores in artificial membranes (25).

Both bafilomycin A1 (BFLA1) and concanamycin A are potent inhibitors of the vacuolar proton-ATPase; in particular, concanamycin A is very selective and at nanomolar concentrations blocks this enzyme (4, 9). The recent discovery that these inhibitors of the vacuolar proton-ATPase potently block the entry of animal viruses that require a low-pH step to infect cells (11–13, 20, 21) prompted us to investigate the dependence of reovirus and ISVP entry on proton-ATPase activity. To this end, mouse L cells were infected with 1 PFU of either reovirus or ISVP per cell and treated with 5 μM BFLA1 for 2 h at 37°C. Then, the inhibitor and unadsorbed virus were washed and further incubated for 24 h at 37°C in the absence of BFLA1. Cells were lysed by three cycles of freezing and thawing and the titer of the virus obtained was determined. The results obtained indicated that BFLA1 inhibited the production of reovirus from 1.6×10^7 in the control to 3.5×10^5 in the treated samples, whereas the antibiotic had no effect on virus production when infected with ISVPs (2.1×10^8 in the control versus 2.0×10^8 in the presence of BFLA1). To investigate the step of the reovirus growth cycle affected by BFLA1, L cells grown in 24-well plates were infected with reovirus type 3 (Dearing strain) at a multiplicity of infection of 100 PFU per cell in the presence or absence of the macrolide inhibitors. After virus adsorption (2 h at 37°C), the cells were incubated in Dulbecco modified Eagle's medium. Protein labeling was performed with 20 μCi of [^{35}S]methionine (1.45 Ci/mmol; Amersham International, Amersham, United Kingdom) per ml added to methionine-free medium for the indicated period of time at 37°C. After the incubation time, the radiolabeled cell monolayers were dissolved in sample buffer (62.5 mM Tris-HCl [pH 6.8], 2% sodium dodecyl sulfate (SDS), 0.1 M dithiothreitol, 17% glycerol, 0.024% bromophenol blue). Samples were heated at 90°C for 5 min and electrophoresed on a 15% acrylamide gel overnight at 80 V. Fluorography was carried out in 1 M sodium salicylate. Finally, gels were dried and exposed to Agfa X-ray film. The results shown in Fig. 1A indicate that 5 μM BFLA1, when present from the beginning of infection, is a potent inhibitor of infection by reovirus particles. Similar results were obtained with concanamycin A, at concentrations as low as 50 nM, and with reovirus type 2 Jones (results not shown). Neither compound had adverse effects on cellular translation. Moreover, BFLA1 and concanamycin A did not prevent infection by ISVPs (Fig. 1A), suggesting that these particles, unlike intact reovirions, do not require vacuolar proton-ATPase activity for efficient infection of L cells. In order to determine when BFLA1 exerts its inhibitory effect on reovirus replication, the compound was added at different times after the reovirus inoculum. Figure 1B shows that BFLA1 added

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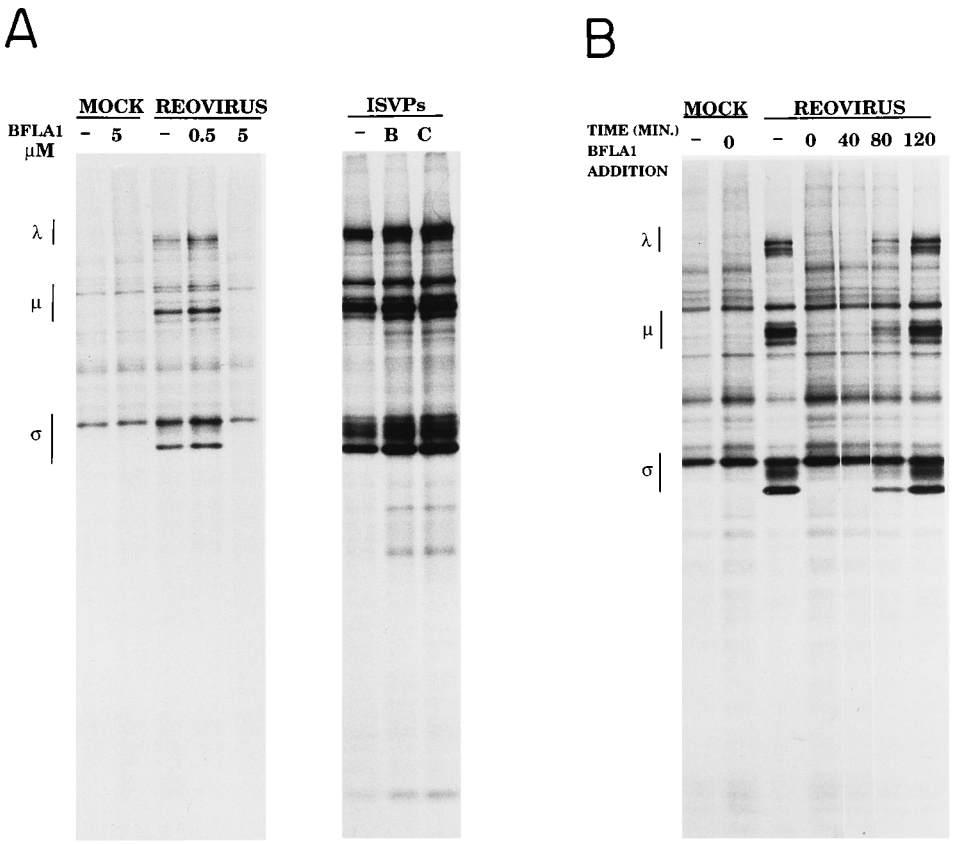

FIG. 1. (A) Effects of proton-ATPase inhibitors on infection by reovirus particles and ISVPs. L cells were mock infected or infected with reovirus type 3 or ISVPs. At the time of virus addition, the indicated concentrations of BFLA1 were added, and cells were then incubated in Dulbecco modified Eagle's medium at 4°C for 2 h (the adsorption period). The end of the adsorption period was considered time zero of infection. Cells were then changed at 37°C, incubated until 7 h p.i. and labeled for 1 h. Proteins were analyzed as described in the text. Experiments in lanes B and C were carried out with 5 μM BFLA1 and 50 nM concanamycin A, respectively. (B) Effects of addition of BFLA-1 at different times after reovirus infection. BFLA1 (5 μM) was added at the end of the adsorption period or at the times indicated. Incubation in the presence of the inhibitor lasted until protein labeling with [^{35}S]methionine. Proteins were analyzed as indicated above. —, lane without BFLA1.

either with the virus or 40 min later prevented infection but that the antibiotic had no effect when added 120 min later. This suggests that BFLA1 acts on steps of the reovirus life cycle that take place during the first 2 h of infection. In addition, these results indicate that the presence of BFLA1 beyond the second hour of infection does not interfere with the synthesis of reovirus proteins.

To investigate the effects of BFLA1 and concanamycin A on reovirus attachment and internalization, two different techniques were employed. The first approach involved the use of [^{35}S]methionine-labeled reovirions. In the absence of inhibitors, the radiolabeled virus bound to cells at 4°C, with the binding showing almost linear kinetics for 90 min; similar kinetics were observed in the presence of BFLA1 or concanamycin A (results not shown). This suggests that these compounds do not interfere with the recognition of cellular receptors and the attachment of reovirions to them. The second approach was based on electron microscopy. Cell surface-bound reovirus particles were observed both in the absence and the presence of BFLA1. As early as 15 min after the addition of reovirus, vesicles containing viral particles were observed. On the other hand, ISVPs were observed within cells in close proximity to the plasma membrane, both in the presence and absence of the inhibitors, supporting the idea of direct entry for ISVPs (results not shown). These findings indicate that neither the attachment of reovirus to the cell surface nor the internalization of reovirus particles in endosomes is the step blocked by the proton-ATPase inhibitors.

We next investigated the effect of BFLA1 on virus uncoating. Internalization of reovirus is followed by proteolytic degradation of several virion proteins, including μ1C, which is cleaved to generate two products known as δ and ϕ (1, 14, 19). Cell monolayers grown in 24-well plates were washed twice with cold phosphate-buffered saline and precooled at 4°C for 10 to 15 min. [^{35}S]methionine-labeled reovirus was added to the cells and allowed to adsorb for 2 h at 4°C. Unbound virus was then washed off, and cells were overlaid with Dulbecco modified Eagle's medium containing 0.5 or 5 μM BFLA1. Cells were harvested at different times postinfection (p.i.), and samples were prepared for SDS-polyacrylamide gel electrophoresis (PAGE). Figure 2 shows that δ can be detected 80 min after the addition of reovirus to L cells and that the amount present increases up to 240 min p.i. The presence of 5 μM BFLA1 blocks the generation of δ, which suggests that endosomal acidification is required for this proteolytic cleavage to occur. This result agrees with the finding that high concentrations of NH_4Cl inhibit reovirus uncoating (5, 17).

The majority of animal viruses investigated to date promote the coentry of protein toxins into the cytoplasm during the entry process (6, 7). For viruses that enter cells through endosomes, this phenomenon requires the activity of the vacuolar proton-ATPase, while viruses that fuse directly with the plasma

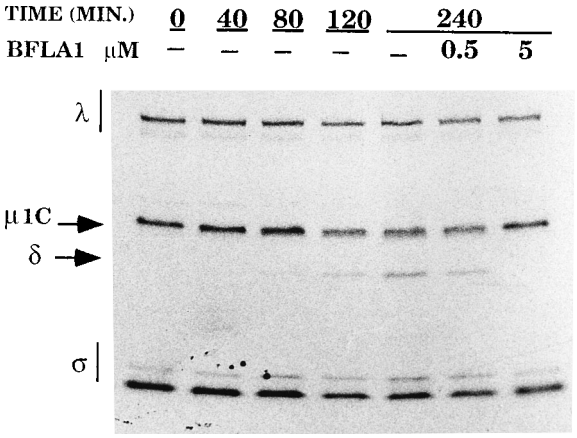

FIG. 2. Kinetics of digestion of reovirus outer-capsid proteins. [^{35}S]methionine-labeled reovirus type 3 was added to L cell cultures and allowed to adsorb for 2 h at 4°C. Unbound virus was then washed off, and cells were overlaid with Dulbecco modified Eagle's medium containing 0.5 or 5 μM BFLA1. Cells were harvested at different times p.i., and samples were prepared for SDS-PAGE. Numbers at the tops of the lanes indicate the times p.i. at which cells were harvested. —, lane without BFLA1.

membrane, such as Sendai virus, are able to enter and permeabilize cells to toxins such as alpha-sarcin, even in the presence of BFLA1 (7, 21). Moreover, poliovirus, which enters into cells through endosomes in a pH-independent manner, still requires vacuolar proton-ATPase activity to induce the coentry of alpha-sarcin (8, 21). Little is known as to how reovirus induces early membrane permeabilization, apart from the fact that ISVPs but not reovirions induce ^{51}Cr release from preloaded cells (2). Therefore, we decided to investigate the coentry of alpha-sarcin as mediated by reovirions and ISVPs. Figure 3 shows that both reovirions and ISVPs efficiently promote the entry of the toxin into the cytoplasm, as judged by the profound inhibition of translation observed. The concentration of alpha-sarcin used was extremely low, indicating that both reovirions and ISVPs potently permeabilize L cells to alpha-sarcin. Strikingly, BFLA1 totally blocked the permeabilizing effect of reovirions but had no effect whatsoever on ISVP-mediated toxin entry. These findings indicate that the vacuolar proton-ATPase activity is required for reovirus particles to permeabilize L cells to alpha-sarcin, while the coentry of alpha-sarcin with ISVPs is independent of this enzyme. Reovirus ISVPs thus behave in a way similar to Sendai virus (21).

The mechanisms for gaining access to the cytoplasm that are used by animal virus particles lacking lipid envelopes are much less well understood than those used by enveloped viruses. Most animal virus particles, whether naked or enveloped, enter cells by receptor-mediated endocytosis (6, 18). The blockade of the vacuolar proton-ATPase by BFLA1 does not inhibit the infection of cells by certain other animal viruses with naked particles, such as poliovirus or adenovirus (21), indicating that it is not necessary for the particles of these viruses to undergo pH-triggered conformational changes in order to cross the endosomal membrane. The infection of susceptible cells by reovirus, on the other hand, requires a low intraendosomal pH (5, 17), which is not the case for infection by poliovirus or adenoviruses (21). This step is necessary for activating the

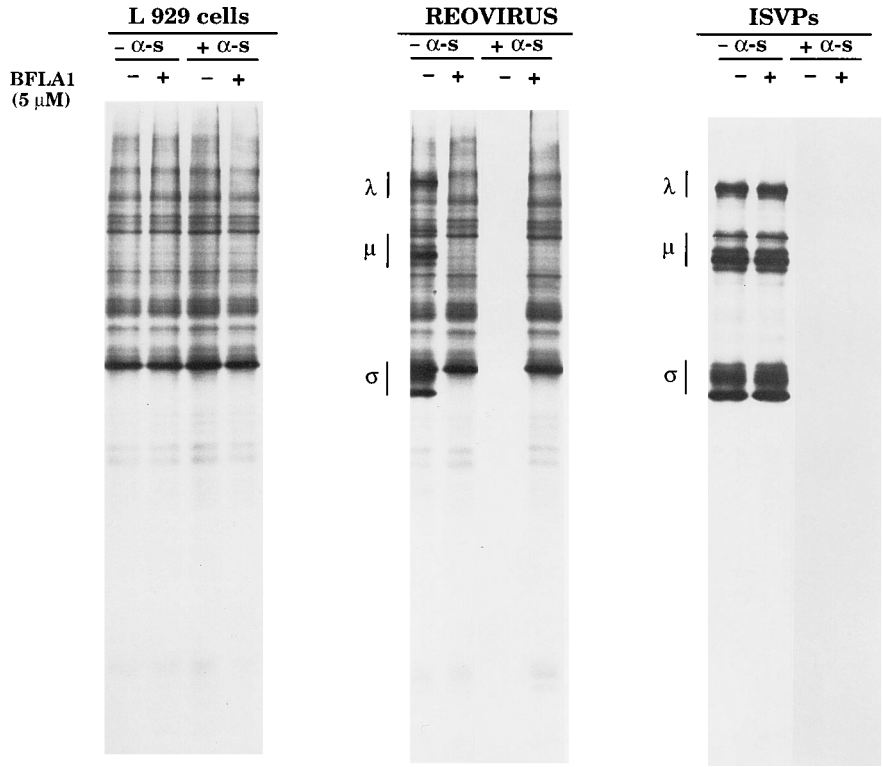

FIG. 3. Effect of BFLA1 on the entry of alpha-sarcin in the presence of reovirus and ISVPs. L cells were preincubated in the presence (+) or absence (−) of 1 μg of alpha-sarcin (α-s) per ml and 5 μM BFLA1 during 15 min at 37°C. After this time, cells were infected with reovirus type 3 or ISVPs at a multiplicity of infection of 100 PFU per cell in the presence of 5 μM BFLA1. Then, the cells were incubated at 37°C until protein labeling was performed from 7 to 8 h p.i. The proteins were analyzed by PAGE as described in the text.

proteolysis of certain reovirion proteins (5). Apart from this requirement, however, a low pH is not needed per se to induce the other conformational changes in reovirion components, which are presumably required for the crossing of the endosomal membrane, as indicated by our finding that ISVPs efficiently infect cells even in the presence of vacuolar proton-ATPase inhibitors.

Our present findings show that both reovirions and ISVPs efficiently translocate the alpha-sarcin to the cytoplasm. Coentry with ISVPs is not blocked by BFLA1, suggesting that neither an intraendosomal acidic pH nor a pH gradient is required for alpha-sarcin to cross the membrane under these conditions. It is conceivable that ISVPs and alpha-sarcin enter endosomes and that the endosomal membrane is then permeabilized to macromolecules by ISVPs in a pH-independent fashion. However, other evidence supports the notion that ISVPs enter cells directly across the plasma membrane. Specifically, ISVPs permeabilize the plasma membrane to ^{51}Cr, suggesting that macromolecules leak out of the cytoplasm during the process of ISVP entry into cells.

In conclusion, our present results indicate that reovirus particles but not ISVPs require vacuolar proton-ATPase activity to enter cells. In addition, our data on the coentry of the toxin alpha-sarcin provide support for the notion that ISVPs may infect cells by crossing the plasma membrane directly.

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