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Trypsin Enhancement of Rotavirus Infectivity: Mechanism of Enhancement

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The infectivity of most rotaviruses is enhanced by treatment with trypsin. We studied the mechanism of enhancement by examining the effect of trypsin on rotavirus infectivity, aggregation, early interactions with host cells, and structure. The results indicated that trypsin does not increase levels of infectious virus by dispersion of aggregates or affect the efficiency or rate of attachment of virus to cells. A fraction of virus that was not infectious without trypsin treatment was found to attach to cells, but did not initiate antigen synthesis. When cells were infected with labeled, purified virus, increased levels of uncoated particles were found in cells infected with trypsin-treated virus. Infection of cells with trypsin-treated virus also led to greater levels of RNA synthesis early in the infection. The results suggest that trypsin converts a noninfectious fraction of virus into infectious virus by allowing this fraction to uncoat in the infected cell. Trypsin was found to cleave an 88,000-dalton structural polypeptide of bovine rotavirus generating 67,000- and 20,000-dalton cleavage products.

Rotaviruses, which are members of the family Reoviridae, are a major cause of gastroenteritis in a wide variety of animals (10), and as such they are currently under intense study, particularly cell culture-adapted strains.

A distinctive feature of rotavirus growth in cell culture is that virus infectivity can be increased by, and in some cases is dependent on, trypsin treatment. We have reported that treatment of stocks of bovine rotavirus (BRV) with as little as 10 ng of trypsin per ml increases virus infectivity (6). Trypsin treatment of virus was also shown to increase virus infectivity as much as 100-fold and growth in cells up to 10,000-fold. In that study, virus that was found to be infectious only when "trypsin treated" was defined as potentially infectious virus (PIV), whereas virus that was infectious without trypsin treatment was referred to as infectious virus (IV), following the nomenclature of Spendlove and Schaffer for reovirus (21). In another study, we reported that the infectivity of BRV was enhanced significantly by trypsin, marginally by bovine protease and galactosidase, and not at all by carboxypeptidase A or B, chymotrypsin, ficin, or lactase (3). Other reports dealing with the effect of trypsin on the propagation of rotaviruses in cell culture (1, 2, 6, 11, 22) and on rotavirus assay and detection (5, 14, 15, 17, 18) have appeared. Several aspects of the biological mechanism of trypsin-mediated enhancement of simian rotavirus (SRV) infectivity have been studied by Graham and Estes (11). Recently, Espejo et al. (9) reported modifications of SRV polypeptides with trypsin.

Since the infectivity of rotaviruses is increased by trypsin and the viruses replicate primarily in the epithelial lining of the upper intestinal tract (10), it is possible that trypsin present in the intestine of the host may contribute to the extent of the disease by increasing levels of IV. This underscores the need to investigate the mechanism of trypsin enhancement of rotavirus infectivity.

MATERIALS AND METHODS

Cells and virus. Madin-Darby bovine kidney (MDBK) and rhesus monkey kidney (MA-104) cells were maintained as previously described (6). BRV was grown in MDBK cells, and porcine rotavirus and SRV were grown in MA-104 cells. Stock virus preparations were stored at −80°C as Freon-extracted culture fluids. Cells were routinely infected with trypsin-treated virus (10 μg of trypsin per ml, 10 min at 37°C) at a multiplicity of infection of 10 to 20. Virus was grown in Eagle minimal essential medium (MEM) without fetal bovine serum.

Aggregation studies. Virus preparations with and without prior trypsin treatment were passed through 200-nm-pore-size polycarbonate filters (13 mm; Nuclepore Corp.). For sonication studies, virus preparations treated or not treated with trypsin were sonicated with

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a Biosonic III Sonifier for various times. Virus assays were performed by using an immunofluorescence assay (4), in which MDBK cells grown on circular glass cover slips were infected with portions of virus, incubated for 16 to 20 h, and stained with fluorescent antibovine rotavirus antibody. Infected cells were then counted under UV illumination.

Virus labeling, purification, and analysis. For polypeptide analysis, BRV was grown in MDBK cells with MEM containing 25 μCi of ^{3}H-amino acids per ml and one-fourth the normal amount of amino acids. Virus was Freon extracted (0.26 ml of Freon per ml of culture fluids) and purified with alternate sedimentation and isopycnic centrifugation in CsCl gradients. L virions (infectious, double shelled; $p = 1.36$; see reference 7) were collected and dialyzed against Dulbecco phosphate-buffered saline, treated or not treated with trypsin, and dialyzed against water. Lyophilized samples were then analyzed on 9% polyacrylamide gels as described by Laemmli (12), and the gels were fluorographed.

Attachment and uncoating studies. BRV was labeled with [^{3}H]uridine (10 μCi/ml) in MDBK cells, Freon extracted, and centrifuged into single CsCl gradients as described (7). The L-virion band was collected and dialyzed against phosphate-buffered saline. MDBK cells were then infected with the labeled virus that had been treated or not treated with trypsin. For attachment studies, portions from duplicate cultures infected with 0.02 ml of virus were removed at various times and counted in a liquid scintillation counter. For uncoating studies, after infection with the labeled L virions and absorption for 1 h, virus inocula were removed by washing and MEM was added to the cultures. At various times after infection, infected cells were harvested and extracted with Freon 113 (0.26 ml per ml culture fluids) by homogenization at 12,000 rpm in a Virtis 60K homogenizer and centrifuged at 1,000 × *g* for 5 min, and the supernatant was layered onto preformed CsCl gradients ($p = 1.34$ to 1.39 in 0.15 M NaCl–0.01 M Tris-chloride–1 mM CaCl, pH 7.5). The gradients were centrifuged for 90 min at 100,000 × *g* and fractionated by bottom displacement, and single-drop fractions were counted in a liquid scintillation counter.

RNA synthesis in infected cells. MDBK cells in 24-well plates were pretreated for 1 h with 5 μg of actinomycin D per ml in MEM. The cells were then infected, in the presence of actinomycin D, with BRV treated or not treated with trypsin. The inocula contained 2 μCi of [^{3}H]uridine per ml. Samples were solubilized at various times with 1% sodium dodecyl sulfate, 8 M urea, and 2% 2-mercaptoethanol. Samples were sonicated, and duplicate portions from duplicate cultures were precipitated with cold trichloroacetic acid and filtered onto nitrocellulose filters. Washed filters were dried and counted.

RESULTS

Effect of sonication and filtration on rotavirus preparations. There are several possible mechanisms by which trypsin may increase the infectivity of rotaviruses. To determine whether rotavirus preparations contain aggregates that are dispersed by trypsin, virus samples were sonicated or passed through membrane filters that would only allow the passage of aggregates of three or four virus particles. When untreated virus samples were filtered, the titer was approximately the same before and after filtration (Table 1). Trypsin treatment of the sample after filtration increased the titer approximately 50-fold. If the virus sample had contained aggregates, the aggregates would have been retained on the filter and the virus titer after filtration would have been less than the titer before filtration. The fact that the infectivity of the untreated sample was increased by trypsin treatment after filtration also indicates that the sample did not contain aggregates that were dispersed by trypsin. When virus preparations were sonicated, no increase in titer as a result of sonication was found (Table 2). If the virus in the preparation had been aggregated, we would have expected sonication to increase the virus titer by dispersing the aggregates. The possibility that sonication resulted in disruption of the virus and loss of infectivity may be ruled out since no loss in the infectivity of trypsin-treated samples was found during the period of sonication.

Attachment of rotavirus to cells. Another possibility is that trypsin may increase the efficiency of attachment of rotavirus to the cell surface. This was studied in two different ways. First, purified L virions labeled with [^{3}H]uridine were used to study attachment of the virus to cells. Neither the rate nor the efficiency of attachment of radiolabeled rotavirus was affected by trypsin treatment (Fig. 1). Second, since the fraction of labeled virus that was infectious was not known, attachment of infectious virus was studied by the immunofluorescence assay. Neither the rate nor the efficiency of attachment of infectious virus was affected by treating or not treating the virus before infection. It was also of interest to determine whether the noninfectious (PIV) fraction of virus attached to cells. When

TABLE 1. *Effect of filtration of BRV treated or not treated with trypsin*

Treatment	Titer before filtration[a]	Titer after filtration	
		Trypsin treated	Not trypsin treated
Trypsin treated	5.0×10^8	4.4×10^8	5.7×10^8
Not trypsin treated	8.0×10^6	4.7×10^8	5.3×10^6

[a] Titer obtained by immunofluorescent assay. For experimental details, see text. Titer is expressed as immunofluorescent cell-forming units per milliliter.

TABLE 2. *Effect of sonication on BRV treated or not treated with trypsin*

Treatment	Titer before sonication[a]	Titer at each time after sonication			Avg titer after sonication
		30 s	60 s	120 s	
Trypsin treated	1.4×10^9	9.8×10^8	9.9×10^8	1.7×10^9	1.2×10^9
Not trypsin treated	7.1×10^7	8.6×10^7	6.0×10^7	6.9×10^7	7.1×10^7

[a] Titer obtained by immunofluorescent assay. For experimental details, see text.

this was studied (see legend to Fig. 2 for experimental details), it was found that untreated PIV attached to cells at the same rate and efficiency as trypsin-treated and untreated virus (Fig. 2). The fact that the level of IV in the sample used to study attachment was 100-fold greater when treated with trypsin indicates that although untreated PIV attached to cells, no viral antigen was synthesized in cells as a result of PIV attachment. This suggests that the infectivity of PIV was aborted at some step after the virus has attached to the cell.

Uncoating of parental rotavirus. Since our results indicate that trypsin does not affect attachment of rotavirus to cells and that the infectivity of PIV is aborted at a stage after attachment, the effect of trypsin on uncoating of parental L virions in infected cells was studied. We have previously shown (7) that labeled L virions are rapidly and efficiently uncoated in infected cells to D particles, a particle that differs from the L virion in that it lacks infectivity and three outer structural polypeptides and has a buoyant density in CsCl that is 0.02 g/ml greater than that of L virions. D particles have also been shown to transcribe the viral genome with an endogenous viral RNA polymerase, an enzyme activity latent in L virions (8). In the present study, the intracellular uncoating of trypsin-treated and untreated, purified, labeled L virions was examined. Trypsin treatment did not affect the density in CsCl of L virions in the inocula (Fig. 3). When cells were infected with this treated and untreated labeled virus, various amounts of the L virions were converted to D particles as evidenced by the loss of radioactivity in the L-virion peak and the corresponding increase of radioactivity in the D-particle peak (Fig. 3). When the virus was trypsin treated before infection, approximately 44% of the input L virions were uncoated during 1 h of absorption, whereas only 15% of the untreated L virions were uncoated during this same time. Uncoating of treated and untreated virus continued during the times studied, with the final fraction of uncoated L virions becoming approximately 72% when the input virus was trypsin treated and 40% when the virus was not treated (Fig. 4). From 0 to 120 min postinfection, approximately twofold more of the input L virions were uncoated when the input virus was treated with trypsin before infection than when the input virus was not treated. In addition, when the virus was not treated with trypsin before infection, a greater fraction of L virions were not uncoated, which indicates that PIV not treated with trypsin probably did not uncoat in infected cells.

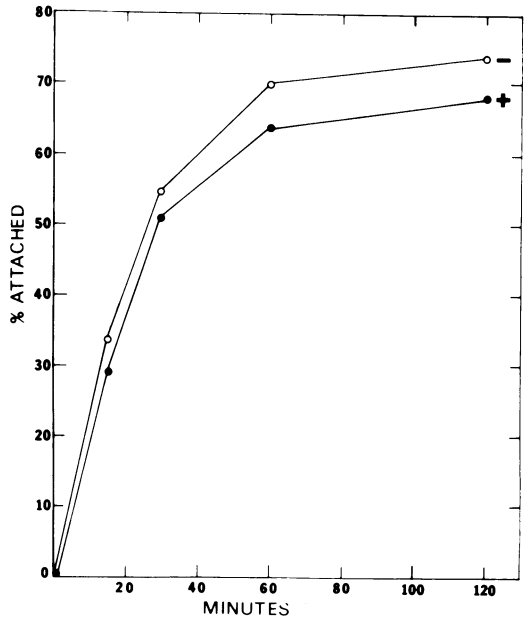

FIG. 1. *Rate of attachment of BRV treated (+) or not treated (−) with trypsin. BRV L virions labeled with [^{3}H]uridine and purified with one cycle of CsCl gradient centrifugation were treated or not treated with trypsin and used to infect MDBK cells. At various times, triplicate samples of inocula from duplicate cultures were removed and counted.*

RNA synthesis in infected cells. When the synthesis of [^{3}H]uridine-labeled RNA was studied in actinomycin D-treated cells infected with virus treated or not treated with trypsin, roughly twofold more acid-insoluble labeled RNA was found in cells infected with trypsin-treated virus throughout the times studies (Fig. 5). This result agrees well with the difference in the levels of uncoated particles found in cells infected with treated and untreated virus (Fig. 4).

Rotavirus structural polypeptides

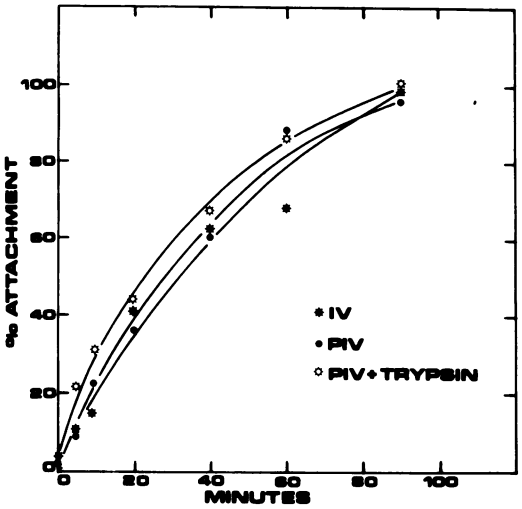

FIG. 2. *Effect of trypsin on attachment of infectious BRV to MDBK cells. MDBK cells on 15-mm circular cover slips were infected with BRV treated (PIV + trypsin) or not treated (IV, PIV) with trypsin before inoculation. At various times, triplicate cover slips were washed three times and incubated for 16 h with MEM without fetal bovine serum. The cover slips were then fixed with cold acetone and stained with fluorescein-conjugated rabbit antibovine rotavirus immunoglobulin G. Attachment of untreated PIV was studied by determining the amount of PIV removed at various times from the untreated inocula. The untreated inoculum was removed from the cells at various times, diluted 100-fold, treated with trypsin, and assayed. Percentage of attachment was calculated by comparing the number of infected cells at each time with the number at 90 min.*

treated with trypsin. The effect of trypsin on structural polypeptides of BRV was then studied. BRV L virions grown in our laboratory routinely consist of eight structural polypeptides ranging in estimated molecular weight from 24,000 to 132,000, although polypeptide 5 is not consistently observed (Fig. 6). When identical amounts of this purified virus were treated with various levels of trypsin, P3 was found to decrease in relative amount with an increase in labeled material that comigrated with P5. When BRV was grown and labeled in the presence of 2 μg of trypsin per ml and then purified, P3 could not be detected (Fig. 6, lane G), but the material that comigrated with P5 was easily observed. It thus appears that the material that comigrates with P5 is a cleavage product of P3, with an estimated molecular weight of 67,000. An additional cleavage product that migrated slightly faster than P8 was also observed and had an estimated molecular weight of 20,000. The sum of the molecular weights of both cleavage products is 87,000, which is identical to the estimated molecular weight of P3. These results are identical to those obtained by Espejo et al. (9) with SRV was treated with trypsin.

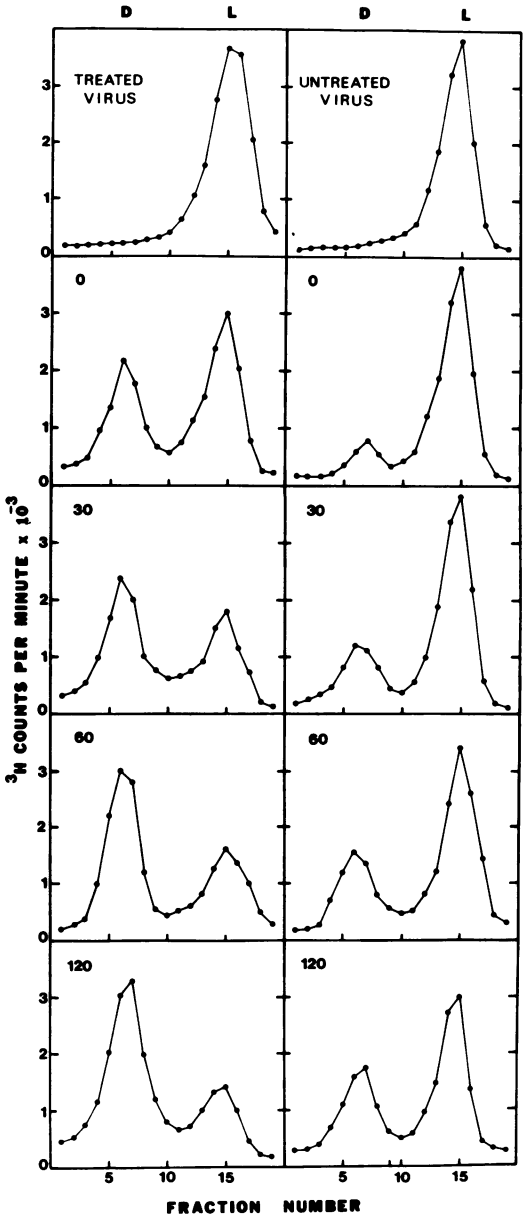

FIG. 3. *In vivo uncoating of BRV L virions treated or not treated with trypsin. BRV was labeled with [^{3}H]uridine in MDBK cells. L virions were collected from single CsCl gradients, dialyzed against phosphate-buffered saline, and used to infect MDBK cells with and without prior trypsin treatment. After 1 h of absorption, unattached virus was removed by washing and MEM was added. At various times, samples were harvested and analyzed with CsCl gradients as described in the text.*

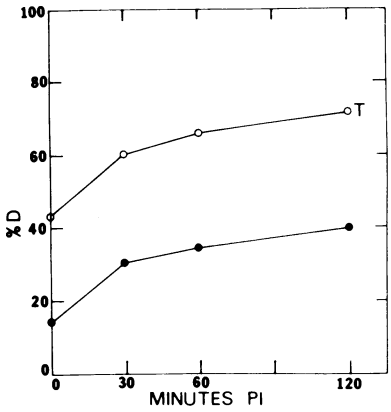

FIG. 4. *Percentage of conversion of L virions to D particles in vivo. The percentage of the counts in D particles compared with the total counts in L and D present in the CsCl gradients shown in Fig. 3 is illustrated.*

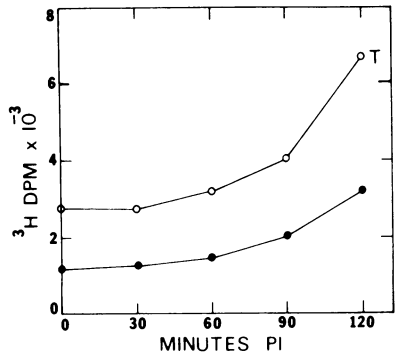

FIG. 5. *RNA synthesis in cells infected with BRV treated or not treated with trypsin. MDBK cells pretreated with actinomycin D were infected with treated or untreated virus. Total RNA was labeled with [^{3}H]uridine and processed at various times as described in the text. Average acid-insoluble disintegrations per minute in duplicate samples from duplicate cultures are shown.*

DISCUSSION

Proteolytic enhancement of the infectivity of viruses such as Sendai (16), influenza A and B viruses (13), reovirus (20, 21), and rotavirus (1, 2, 6, 11) has been demonstrated. Structural changes in viral polypeptides with enzyme treatment of various ortho- and paramyxoviruses and reovirus have been well documented, but mechanisms of infectivity enhancement remain, in all cases, obscure.

Possible mechanisms of trypsin-mediated enhancement of rotavirus infectivity may include effects on the cell surface that influence virus attachment or penetration, digestion of viral inhibitors such as interferon or antibody, dispersion of viral aggregates, or an effect that is expressed at some step during virus replication. We feel that the results of the present study, as well as those reported by others, allow us to rule out many of these possibilities and provide evidence for a proposed mechanism.

The possibility that trypsin exerts its effect on the cell has been examined by Barnett et al. (3), who studied the effect of trypsin treatment on cells and virus when the enzyme was inhibited with soybean trypsin inhibitor before infection. It was found that virus infectivity was increased only when the virus, not the cells, was treated with trypsin. Graham and Estes (11) found that treatment of MA-104 cells with trypsin and removal of the enzyme before infection did not result in infectivity enhancement of SRV. These results suggest that trypsin enhancement is due to an effect on the virus and not the cell.

Graham and Estes (11) and our laboratory (unpublished data) have found that the infectivity of purified SRV and BRV, respectively, is enhanced with trypsin. Since viral inhibitors in culture fluids would have been removed during purification, these results indicate that digestion of viral inhibitors by trypsin probably does not play a role in infectivity enhancement.

In the present study, we found that trypsin probably does not disperse viral aggregates or affect the attachment of virus to cells. The fact that trypsin treatment of rotaviruses has been shown in many laboratories to increase levels of IV indicates that the amount of PIV present in virus samples is greater than the amount of IV present. Our results indicate that trypsin enhances the infectivity of rotavirus by allowing a greater fraction of parental virus to uncoat in the infected cell. This conclusion is supported by the fact that without trypsin treatment of virus, lower levels of RNA synthesis and uncoated (D) particles were found in infected cells. In addition, untreated virus was found to not initiate antigen synthesis even though this fraction of virus was shown to attach to cells. These results may be explained by the lack of intracellular uncoating of the potentially infectious fraction of virus. When virus was treated with trypsin before infection, increased levels of uncoated particles and RNA synthesis were found in infected cells. Therefore, it appears that when trypsin increases the level of IV present in a viral inoculum, increased levels of uncoated particles in infected cells result. Since conversion of L virions to D particles in vitro has been shown to activate the endogenous viral RNA polymerase (8), the fact that fewer L virions were uncoated in cells infected with untreated virus may account for the lower levels of labeled RNA

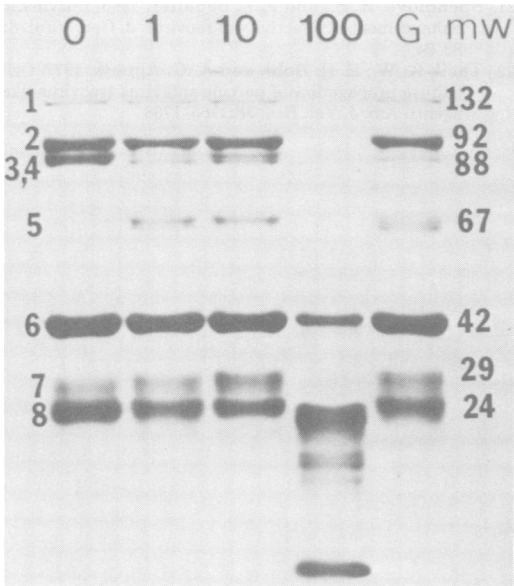

FIG. 6. *Effect of trypsin on structural polypeptides of BRV. BRV L virions labeled with ^{3}H-amino acids were purified, treated or not treated with trypsin, and analyzed on 9% polyacrylamide gels as described in the text. mw, Estimated molecular weights ($\times 10^3$) determined by comparison with the migration of SRV polypeptides. Molecular weight estimates of SRV polypeptides were from Smith et al. (19). ○, Not trypsin treated; 1, 10, and 100 indicate the amount of trypsin (in micrograms per milliliter) used to treat virus; G, virus grown in the presence of 2 μg of trypsin per ml.*

synthesis in cells infected with untreated virus, as shown, as well as the lack of viral antigen synthesis in cells infected with untreated virus.

The result of trypsin-mediated structural modifications of BRV polypeptides reported here is consistent with the results obtained by Espejo et al. (9). Although trypsin cleaves a rotaviral structural polypeptide, the manner in which this cleavage affects uncoating of virus is presently unknown.

In summary, we found that trypsin has no effect on rotavirus aggregation or attachment of virus to cells and that untreated virus (PIV) attaches to cells but does not induce viral antigen synthesis because it is probably not uncoated in infected cells. Results found when virus uncoating and RNA synthesis in infected cells were studied indicate that trypsin increases the fraction of parental virus uncoated and subsequent levels of RNA in the cells. Evidence that suggests that P5 in purified virus is not a primary gene product but is derived from P3 by trypsin cleavage is also discussed. We conclude that trypsin may enhance the infectivity of BRV by converting a noninfectious fraction of virus (PIV) into infectious virus, which then allows this fraction to uncoat in the infected cell.

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