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Infectious Subvirion Particles of Reovirus Type 3 Dearing Exhibit a Loss in Infectivity and Contain a Cleaved σ1 Protein

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Mammalian reoviruses exhibit differences in the capacity to grow in intestinal tissue: reovirus type 1 Lang (T1L), but not type 3 Dearing (T3D), can be recovered in high titer from intestinal tissue of newborn mice after oral inoculation. We investigated whether in vitro protease treatment of virions of T1L and T3D, using conditions to generate infectious subvirion particles (ISVPs) as occurs in the intestinal lumen of mice (D. K. Bodkin, M. L. Nibert, and B. N. Fields, J. Virol. 63:4676–4681, 1989), affects viral infectivity. Chymotrypsin treatment of T1L was associated with a 2-fold increase in viral infectivity, whereas identical treatment of T3D resulted in a 10-fold decrease in infectivity. Using sodium dodecyl sulfate-polyacrylamide gel electrophoresis, we found that loss of T3D infectivity was correlated with cleavage of its σ1 protein. We used reassortant viruses to identify viral determinants of infectivity loss and σ1 cleavage and found that both phenotypes segregate with the σ1-encoding S1 gene. Comparable results were obtained when trypsin treatment of virions of T1L and T3D was used. In experiments to determine the fate of σ1 fragments following cleavage, the capacity of anti-σ1 monoclonal antibody G5 to neutralize infectivity of T3D ISVPs was significantly decreased in comparison with its capacity to neutralize infectivity of virions, suggesting that a σ1 domain bound by G5 is lost from viral particles after proteolytic digestion. In contrast to the decrease in infectivity, chymotrypsin treatment of T3D virions leading to generation of ISVPs resulted in a 10-fold increase in their capacity to produce hemagglutination, indicating that a domain of σ1 important for binding to sialic acid remains associated with viral particles after σ1 cleavage. Neuraminidase treatment of L cells substantially decreased the yield of T3D ISVPs in comparison with the yield of virions, indicating that a σ1 domain important for binding sialic acid also can mediate attachment of T3D ISVPs to L cells and lead to productive infection. These results suggest that cleavage of T3D σ1 protein following oral inoculation of newborn mice is at least partly responsible for the decreased growth of T3D in the intestine and provide additional evidence that T3D σ1 contains more than a single receptor-binding domain.

The mammalian reoviruses are important models for investigation of molecular determinants of viral pathogenesis (reviewed in reference 59). After oral inoculation into newborn mice, some reoviruses replicate in intestinal epithelium (49, 50, 70, 71) and spread to the central nervous system (31, 32, 39, 60), where they produce serotype-specific patterns of disease (64, 66). During entry of reovirus into the gastrointestinal tract, intact virions are subject to proteolysis by intestinal proteases, yielding infectious subvirion particles (ISVPs) (5, 9). Conversion of virions to ISVPs is required for adherence of reovirus to intestinal M cells (1) prior to transcytosis of virus and delivery to cells in the Peyer's patch mucosa, where primary replication of reovirus is thought to occur (39, 43, 71).

Some reovirus strains differ in the capacity to grow in intestinal tissue after oral inoculation of newborn mice. Type 1 Lang (T1L) can be recovered in high titer from intestinal tissue as long as 8 days after oral inoculation; in contrast, type 3 Dearing (T3D) cannot be recovered 4 days after inoculation under identical conditions (8, 33, 49). By using T1L × T3D reassortant viruses, these differences in viral infectivity were mapped genetically to the S1 and L2 gene segments (8). The S1 gene encodes viral attachment protein σ1 (35, 63), and L2 encodes core-spike protein λ2 (48, 68), which interacts with σ1 in the outer capsid (22). These observations raise the possibility that loss of T3D infectivity after oral inoculation is due to proteolytic digestion of T3D structural proteins (particularly σ1 or λ2) in the intestinal lumen (8).

Proteolytic processing of infecting virions also plays an important role in entry of reovirus into host cells. Steps in reovirus entry are initiated by attachment of virions to cellular receptors via the σ1 protein (35, 63) and virion uptake into cells by receptor-mediated endocytosis (11, 12, 57). Following endocytosis, viral outer capsid proteins σ3 and μ1/μ1C are proteolytically processed by cellular proteases within a vacuolar, endocytic compartment, yielding ISVPs (12, 15, 53, 57) which are probably identical to those generated either in vivo or in vitro by treatment of virions with intestinal proteases (reviewed in reference 43).

The fate of viral attachment protein σ1 during protease treatment of reovirus virions has not always been apparent from previous studies (10, 21, 30, 52); however, since ISVPs are infectious (52, 57), it seems likely that they contain σ1. Treatment of purified σ1 protein of T3D, but not T1L, with either chymotrypsin or trypsin results in cleavage of σ1 (25, 72). These studies suggest that differences in the capacity of T1L and T3D to grow in intestinal tissue of newborn mice might be due to differences in susceptibility of their σ1 proteins to proteolytic cleavage during generation of ISVPs.

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To provide a mechanistic explanation for differences in T1L and T3D infectivity in intestinal tissue, we investigated whether ISVPs generated from virions of these strains are fully infectious. Additionally, we conducted experiments to examine the fate of viral structural proteins during protease treatment. Our results indicate that treatment of T1L and T3D with the intestinal proteases chymotrypsin and trypsin, using conditions to generate ISVPs, is associated with strain-specific alterations in viral infectivity that correlate with sensitivity of σ1 protein to proteolytic cleavage. We used T1L × T3D reassortant viruses to determine the genetic basis of infectivity loss and σ1 cleavage, and we investigated the effect of σ1 cleavage on the capacity of T1L and T3D to bind L cells and erythrocytes. These studies establish a model to explain differences in reovirus infectivity at the site of entry into the host and contribute new information about cell attachment domains of the reovirus σ1 protein.

MATERIALS AND METHODS

Cells and viruses. Spinner-adapted L cells were grown in either suspension or monolayer cultures in Joklik's modified Eagle's minimal essential medium (Irvine Scientific, Santa Ana, Calif.) that was supplemented to contain 5% fetal bovine serum (HyClone Laboratories, Logan, Utah), 2 mM L-glutamine, and 100 U of penicillin, 100 μg of streptomycin, and 0.25 μg of amphotericin per ml (Irvine). Reovirus strains T1L and T3D are laboratory stocks. We also used T1L × T3D reassortant viruses 1.HA-3 and 3.HA-1 (66), EB1, EB121, G2, H9, and H41 (13), and KC19 (19). Purified virion preparations were made by using second- and third-passage L-cell lysate stocks of twice plaque-purified reovirus as previously described (27). To obtain purified virions containing ^{35}S-labeled proteins, Tran^{35}S-label (~12.5 μCi/ml; ICN Biochemicals, Costa Mesa, Calif.) was added to cell suspensions at the initiation of infection. Concentrations of virions in purified preparations were determined from the equivalence 1 unit of optical density at 260 nm = 2.1×10^{12} virions per ml (54). The particle-to-PFU ratio in these preparations was approximately 100 to 1.

Digestion of reovirus virions with chymotrypsin and trypsin. Purified virions of reovirus at a concentration of 2×10^{12} particles per ml in virion storage buffer (150 mM NaCl, 10 mM $MgCl_2$, 10 mM Tris [pH 7.5]) were digested for defined intervals with either 200 μg of *N*α-*p*-tosyl-L-lysine chloromethyl ketone (TLCK)-treated bovine α-chymotrypsin (Sigma Chemical Co., St. Louis, Mo.) per ml at 32 or 37°C or 100 μg of *N*α-*p*-tosyl-L-sulfonyl phenylalanyl chloromethyl ketone (TPCK)-treated bovine trypsin (Sigma) per ml at 32°C. Chymotrypsin digestion was stopped by adding 2 to 5 mM phenylmethylsulfonyl fluoride (Sigma) to the treatment mixtures and cooling at 0°C; trypsin digestion was stopped by adding 0.3 to 0.5 mg of soybean trypsin inhibitor (Sigma) per ml to the treatment mixtures and cooling at 0°C.

Determination of virus titer after protease treatment of reovirus virions. Virus titer after protease treatment of virions was determined by plaque assay (61). Samples were diluted serially 10-fold and used to infect L-cell monolayers in duplicate in six-well plates (Costar, Cambridge, Mass.). L cells then were overlaid with medium 199 (Irvine Scientific) supplemented to contain 2.5% fetal calf serum, 2 mM L-glutamine, 100 U of penicillin, 100 μg of streptomycin, 0.25 μg of amphotericin per ml, and 1% agar (Difco, Detroit, Mich.). Plaques were counted on day 7 after staining cells with neutral red (Fisher Scientific, Pittsburgh, Pa.).

SDS-PAGE of reovirus structural proteins. Discontinuous sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) was performed as previously described (42). Briefly, in preparation for electrophoresis, ^{35}S-labeled viral particles in virion storage buffer were mixed 1:1 with 2× sample buffer (250 mM Tris [pH 8.0], 4% 2-mercaptoethanol, 2% SDS, 20% sucrose, 0.02% bromophenol blue) and incubated at 95°C for 1 min. Samples then were loaded into wells of 5 to 20% (2.6% bis) polyacrylamide gradient gels and electrophoresed at 20- to 40-mA constant current until the dye front reached the bottom of the gel. Gels were treated with Enlightning (New England Nuclear Corp., Boston, Mass.), dried onto filter paper under vacuum, and exposed to RX film (Fuji Photo Film, Tokyo, Japan) at −70°C.

Binding of reovirus to L cells. Virus binding was analyzed by fluorescence-activated flow cytometry as previously described (51), with slight modifications. Purified virions (5×10^{10} particles) were adsorbed to 10^6 cells, after which a 1:20 dilution of rabbit polyclonal antireovirus serum (Cocalico, Reamstown, Pa.) was added. The secondary antibody was a 1:50 dilution of fluorescein isothiocyanate-conjugated goat F(ab′)$_2$ directed against rabbit immunoglobulin G (Sigma). Fluorescence intensity was determined with a FACS IV flow cytometer (Beckton Dickinson Co., San Jose, Calif.). Cells considered positive for virus binding were those demonstrating fluorescence intensity exceeding that displayed by cells treated with primary and secondary antibodies alone.

Neutralization of virions and ISVPs by monoclonal antibodies G5 and 5C6. Purified virions were digested for various intervals with chymotrypsin as described above and diluted in gelatin saline (61) to a concentration of 2×10^5 particles per ml (all digestions of T1L and 0-, 5-, and 20-min digestions of T3D) or 2×10^6 particles per ml (60- and 180-min digestions of T3D). Monoclonal antibodies 5C6, which is specific for T1L σ1 (62), and G5, which is specific for T3D σ1 (14), were added to diluted virus at a final concentration of 25 μg/ml, and the mixtures were incubation at 37°C for 60 min. Infectivity of antibody-treated virus was determined by plaque assay (61). Specific reduction in virus infectivity was calculated as the percent plaque reduction in the presence of the homotypic antibody relative to the number of plaques in the presence of the heterotypic antibody.

HA assay. Purified virions were serially diluted twofold from 2.0×10^{11} to 1.0×10^8 particles in 0.05 ml of cold phosphate-buffered saline (PBS) into 96-well round-bottom microtiter plates (Costar). Type O human erythrocytes were washed twice in cold PBS and resuspended at a concentration of 0.8% (vol/vol). Erythrocytes (0.05 ml) were added to wells containing virus and incubated at 4°C for 2 h. The smallest number of viral particles sufficient to produce hemagglutination (HA) was designated to equal 1 HA unit. The HA titer was defined as the number of HA units per 2×10^{11} particles, the largest number of viral particles used in these assays.

Growth of virions and ISVPs in neuraminidase-treated L cells. L cells were desialylated by incubation of 10^6 cells with 40 mU of *Arthrobacter ureafasciens* neuraminidase per ml in a total volume of 0.2 ml gelatin saline (61). Neuraminidase treatment at 37°C for 1.5 h was followed by a wash in ice-cold medium. Control cells were treated identically except for exclusion of neuraminidase. Cells then were resuspended in 0.2 ml of gelatin saline and adsorbed at 4°C for 1 h with 10^7 particles of intact virions or ISVPs generated by treatment of virions with chymotrypsin for 180 min as described above. After virus adsorption, cells were resuspended in 1 ml of medium and incubated at 37°C for 18.5 h. Cells were frozen and thawed (−70 and 37°C) twice, and lysates were titrated by plaque assay (61).

RESULTS

T1L and T3D differ in infectivity of ISVPs and susceptibility of σ1 protein to cleavage after chymotrypsin treatment. To determine whether reovirus strains T1L and T3D differ in infectivity after protease digestions that generate ISVPs as end products, purified virions of each strain were exposed to chymotrypsin for different times, using previously defined conditions (42). Aliquots of the treatment mixtures then were used to determine infectious titer by plaque assay on L-cell monolayers (Fig. 1). With T1L, a small increase in infectivity occurred early in the course of chymotrypsin treatment and was maintained over a 3-h period of digestion, similar to previous findings (21). With T3D, however, an early increase in infectious titer was followed by a moderate decrease, to approximately 10% of starting levels at later times of treatment.

To investigate the effects of chymotrypsin treatment on viral structural proteins, aliquots of digestion mixtures were analyzed by SDS-PAGE (Fig. 2). As expected, findings consistent with the generation of ISVPs were observed (loss of σ3 protein and stability of the δ and ϕ fragments of μ1C protein). With T1L, the σ1 protein was resistant to chymotrypsin digestion, as judged by continued presence of a σ1 band with normal electrophoretic mobility after as much as 4 h of treatment (Fig. 2 and data not shown). Similar findings were reported previously for both T1L (9, 44, 57) and type 2 Jones (27). With T3D, however, σ1 was cleaved by chymotrypsin such that the σ1 band decreased in intensity at later times of treatment (Fig. 2). The half-life of intact T3D σ1 was approximately 20 min in these experiments. These results indicate that changes in viral infectivity after chymotrypsin treatment correlate with the state of σ1 protein observed by SDS-PAGE. Furthermore, loss of T3D infectivity and cleavage of σ1 protein occurred with similar kinetics during the time course of chymotrypsin treatment (compare Fig. 1 and 2), suggesting a causal relationship between these two phenomena.

Differences in ISVP infectivity and σ1 cleavage after treatment of T1L and T3D with chymotrypsin segregate with the S1 gene. To identify viral determinants of differences between T1L and T3D ISVPs in infectivity and σ1 cleavage after chy-

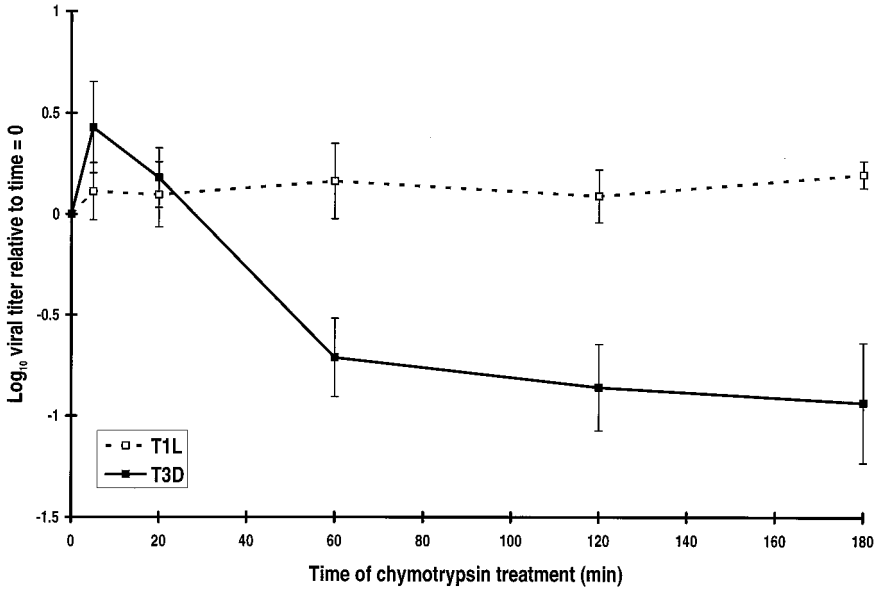

FIG. 1. Titers of reovirus virions during treatment with chymotrypsin to generate ISVPs. Purified virions of T1L and T3D at a concentration of 2×10^{12} particles per ml were treated with 200 μg of TLCK-treated bovine α-chymotrypsin per ml at 37°C for the specified intervals. Following chymotrypsin treatment, aliquots of the treatment mixtures were titrated on L-cell monolayers by plaque assay. Experiments were performed in triplicate, and the results are presented as the mean (±standard deviation) $\log_{10}$ titer relative to that at time zero.

motrypsin treatment, we used reovirus strains containing reassorted genomes derived from T1L and T3D (13, 19, 66). Purified virions of each reassortant virus were digested with chymotrypsin under identical conditions and subjected to plaque assay to determine effects on infectivity. The digests also were analyzed by SDS-PAGE to confirm that ISVPs had been generated and to determine the fate of σ1 (Table 1). We found that chymotrypsin treatment of reassortant viruses containing an S1 gene derived from T3D was associated with both a moderate decrease in infectivity and cleavage of σ1 protein, whereas treatment of reassortant viruses containing an S1 gene derived from T1L was associated with neither a loss in infectivity nor σ1 cleavage. No other reovirus gene was found to be associated with these phenotypes, indicating that they segregated exclusively with the σ1-encoding S1 gene.

Treatment of T1L and T3D with trypsin under conditions to generate ISVPs also links loss of infectivity to σ1 cleavage. ISVPs also can be generated by digestion of reovirus virions with trypsin under appropriate conditions (5, 9, 42). To determine whether treatment of T1L and T3D with trypsin is associated with changes in infectivity, virions of these strains were digested with trypsin for different times, and aliquots were used to determine infectious titer by plaque assay on L-cell monolayers (Fig. 3). Treatment of T1L with trypsin under

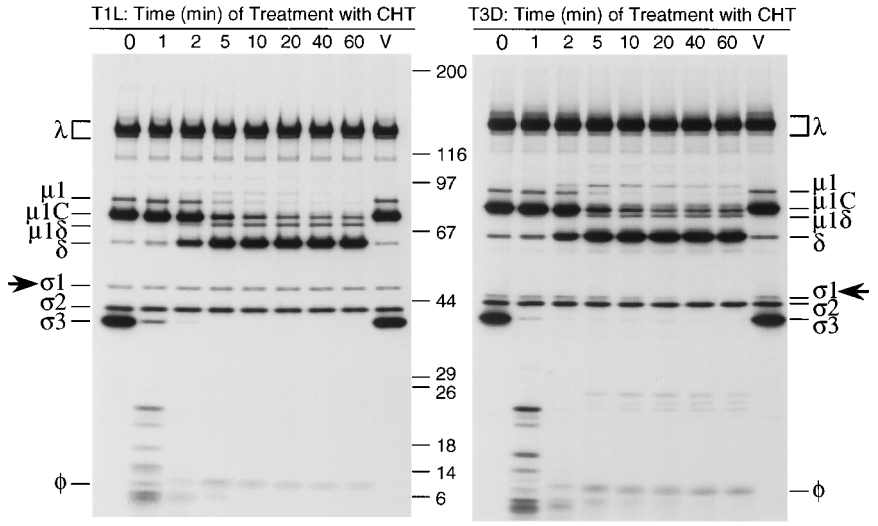

FIG. 2. Electrophoretic analysis of viral structural proteins of reovirus virions during treatment with chymotrypsin to generate ISVPs. Purified [^{35}S]methionine-labeled virions of T1L and T3D at a concentration of 2×10^{12} particles per ml were treated with chymotrypsin (CHT) at 32°C for the specified intervals. Equal volumes of samples (20 μl) were loaded into wells of 5 to 20% gradient SDS-polyacrylamide gels. After electrophoresis, gels were prepared for fluorography and exposed to film. Samples of untreated virions appear in the lanes labeled V. Viral proteins are labeled. The σ1 protein is indicated with arrows. Sizes are indicated in kilodaltons.

TABLE 1. Effects of chymotrypsin treatment on infectivity, σ1 cleavage, and HA capacity of T1L × T3D reassortant viruses

Virus strain	Genome segment[a]										Mean $\log_{10}$ titer (180 min)/titer (0 min) ± SD[b]	σ1 stability to chymotrypsin digestion[c]	Mean $\log_2$ HA titer (180 min)/HA titer (0 min) ± SD[d]
	L1	L2	L3	M1	M2	M3	S1	S2	S3	S4			
T1L	L	L	L	L	L	L	L	L	L	L	0.20 ± 0.07	Yes	0.00 ± 0.00
T3D	D	D	D	D	D	D	D	D	D	D	−0.94 ± 0.30	No	3.25 ± 0.50
EB121	D	D	L	D	L	D	L	D	D	D	1.02 ± 0.48	Yes	0.00 ± 0.00
EB1	L	D	L	L	D	L	L	L	D	L	0.85 ± 0.19	Yes	0.00 ± 0.00
3.HA-1	D	D	D	D	D	D	L	D	D	D	0.27 ± 0.09	Yes	0.00 ± 0.00
H41	D	D	L	L	L	D	L	D	D	L	0.26 ± 0.32	Yes	0.00 ± 0.00
1.HA-3	L	L	L	L	L	L	D	L	L	L	−0.55 ± 0.30	No	5.25 ± 0.96
KC19	L	L	L	L	D	L	D	L	D	L	−0.60 ± 0.11	No	4.33 ± 0.58
G2	L	D	L	L	L	L	D	L	L	L	−0.68 ± 0.11	No	4.67 ± 0.58
H9	D	D	L	D	L	L	D	D	D	D	−0.94 ± 0.37	No	4.33 ± 2.08

[a] Parental origin of each genome segment in the reassortant strains: D, genome segment derived from T3D; L, genome segment derived from T1L (13, 19, 66).
[b] Purified virions at a concentration of 2×10^{12} particles per ml were treated with 200 μg of TLCK-treated bovine α-chymotrypsin per ml at 37°C for either 0 or 180 min. Samples then were titrated on L-cell monolayers by plaque assay. Ratios of viral yields following chymotrypsin treatment are expressed as the means ± standard deviations of at least three independent experiments.
[c] Purified virions were treated with chymotrypsin for 180 min, using conditions to generate ISVPs as end products. Viral structural proteins were analyzed by SDS-PAGE for the presence of intact σ1.
[d] Purified virions at a concentration of 2×10^{12} particles per ml were treated with 200 μg of chymotrypsin per ml at 37°C for either 0 or 180 min. Samples then were tested for the capacity to agglutinate human type O erythrocytes. Ratios of HA titers are expressed as the means ± standard deviations of at least three independent experiments.

conditions to generate ISVPs was associated with a small increase in infectivity. Identical treatment of T3D, however, resulted in a moderate decrease in infectivity, to approximately 10% of starting levels within 2 min of trypsin treatment.

We used SDS-PAGE to examine the fate of σ1 protein during trypsin treatments to generate ISVPs from T1L and T3D virions. Similar to results for chymotrypsin digestion, the σ1 protein of T1L was resistant to trypsin digestion, whereas the T3D σ1 was cleaved by trypsin (Fig. 4). A difference between trypsin and chymotrypsin treatments was noted, however, in that the T3D σ1 protein was cleaved very rapidly by trypsin: in these experiments, a T3D σ1 band of normal mobility was lost after only 1 min of treatment (Fig. 4). Infectivity loss and σ1 cleavage occurred with similar kinetics during trypsin treatments of T3D (compare Fig. 3 and 4), as noted before with chymotrypsin (Fig. 1 and 2). Thus, results obtained from treatment of T3D virions with trypsin provide additional evidence that a moderate loss in infectivity and cleavage of σ1 protein are causally linked during generation of ISVPs.

To determine the genetic basis for differences in infectivity observed after trypsin treatment of T1L and T3D, purified virions of reassortant viruses 1.HA-3 and 3.HA-1 were treated with trypsin for different times, and aliquots were used to determine infectious titer (Fig. 3). These strains are single-

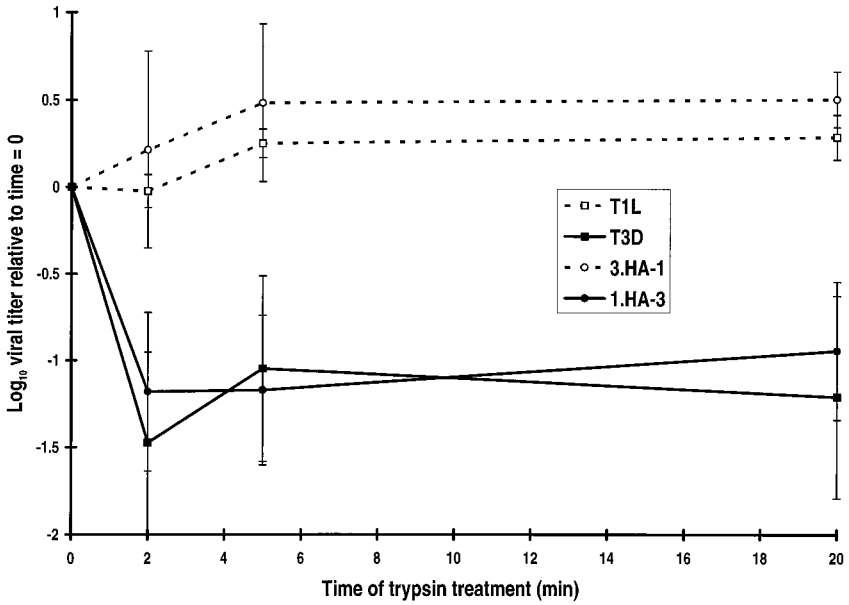

FIG. 3. Titers of reovirus virions during treatment with trypsin to generate ISVPs. Purified virions of parental strains, T1L and T3D, and reassortant strains, 3.HA-1 and 1.HA-3, at a concentration of 2×10^{12} particles per ml were treated with 100 μg of TPCK-treated bovine trypsin per ml at 32°C for the specified intervals. Following trypsin treatment, aliquots were titrated on L-cell monolayers by plaque assay. Experiments were performed in triplicate, and the results are presented as the mean (±standard deviation) $\log_{10}$ titer relative to that at time zero.

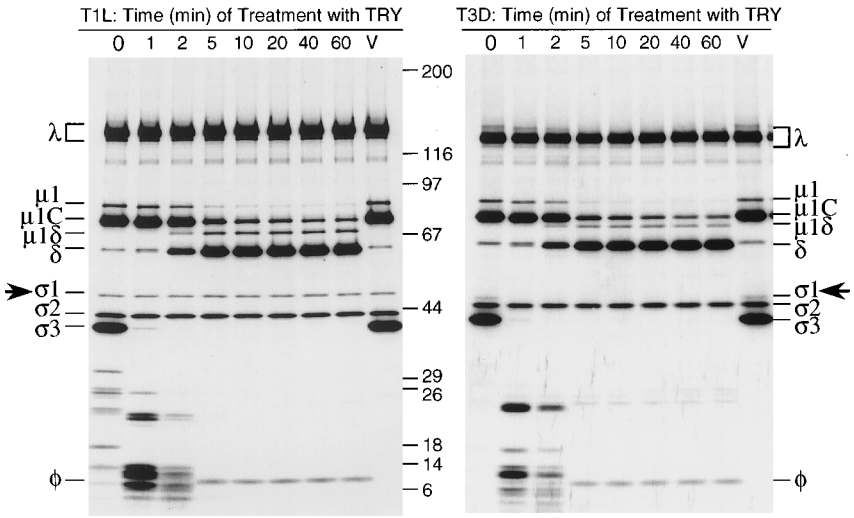

FIG. 4. Electrophoretic analysis of viral structural proteins of reovirus virions during treatment with trypsin to generate ISVPs. Purified [^{35}S]methionine-labeled virions of T1L and T3D at a concentration of 2×10^{12} particles per ml were treated with trypsin (TRY) at 32°C for the specified intervals. Equal volumes of samples (20 μl) were loaded into wells of 5 to 20% gradient SDS-polyacrylamide gels. After electrophoresis, gels were prepared for fluorography and exposed to film. A sample of untreated virions appears in the lanes labeled V. Viral proteins are labeled. The σ1 protein is indicated with arrows. Sizes are indicated in kilodaltons.

gene reassortants that contain either the S1 gene of T3D and the nine remaining segments of T1L (1.HA-3) or the S1 gene of T1L and the nine remaining segments of T3D (3.HA-1) (66). In these experiments, 3.HA-1 did not lose infectivity after trypsin digestion whereas infectivity of 1.HA-3 decreased to approximately 10% of starting levels. Therefore, similar to findings with chymotrypsin, differences in infectivity between T1L and T3D after treatment with trypsin segregated with the S1 gene in this limited analysis.

Chymotrypsin-treated virions of T1L and T3D differ in the capacity to bind L cells. Our findings next prompted us to examine the effects of chymotrypsin-mediated σ1 cleavage on other properties attributable to σ1. The σ1 protein is the viral attachment protein (35, 63), determinant of the type-specific neutralizing immune response (65), and the viral hemagglutinin (20, 47, 67). We reasoned that an examination of the capacity of chymotrypsin-generated ISVPs of T3D to mediate these σ1 functions might provide insight into the fate of σ1 fragments generated by chymotrypsin cleavage and the mechanism by which T3D ISVPs bind to cells.

We first determined the effect of chymotrypsin treatment on binding of T1L and T3D particles to L cells. Purified virions of each strain were treated with chymotrypsin for different intervals, and aliquots were tested for the capacity to bind L cells by using fluorescence-activated cytometric analysis (Fig. 5). With T1L, chymotrypsin treatment did not affect virus binding, con-

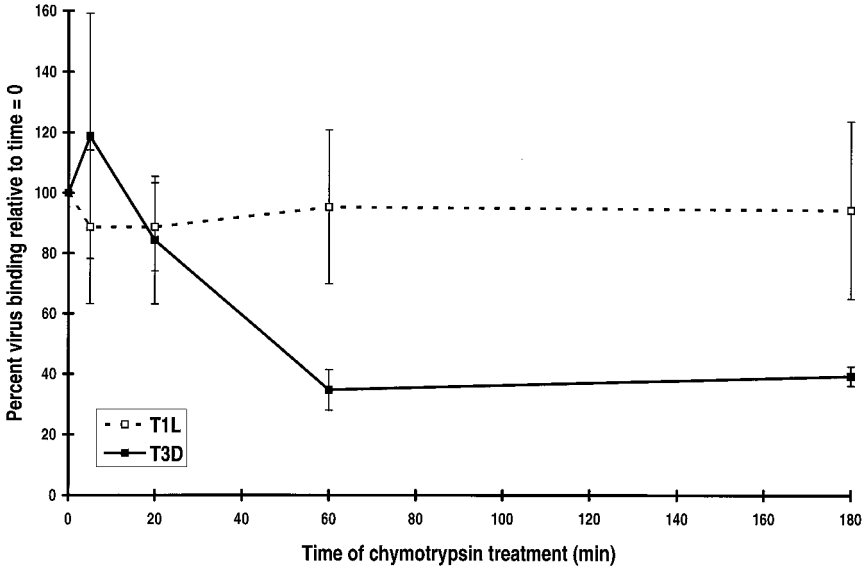

FIG. 5. Effect of chymotrypsin treatment on binding of reovirus virions to L cells. Purified virions of T1L and T3D were treated with chymotrypsin for the specified intervals, and 5×10^{10} particles were adsorbed to 10^6 L cells. Adsorbed virus was incubated with rabbit polyclonal antireovirus antiserum and then with fluorescein isothiocyanate-conjugated goat anti-rabbit antibody. Virus binding was quantitated by fluorescence-activated cytometric analysis. Experiments were performed in triplicate, and the results are presented as the mean (±standard deviation) virus binding relative to that at time zero.

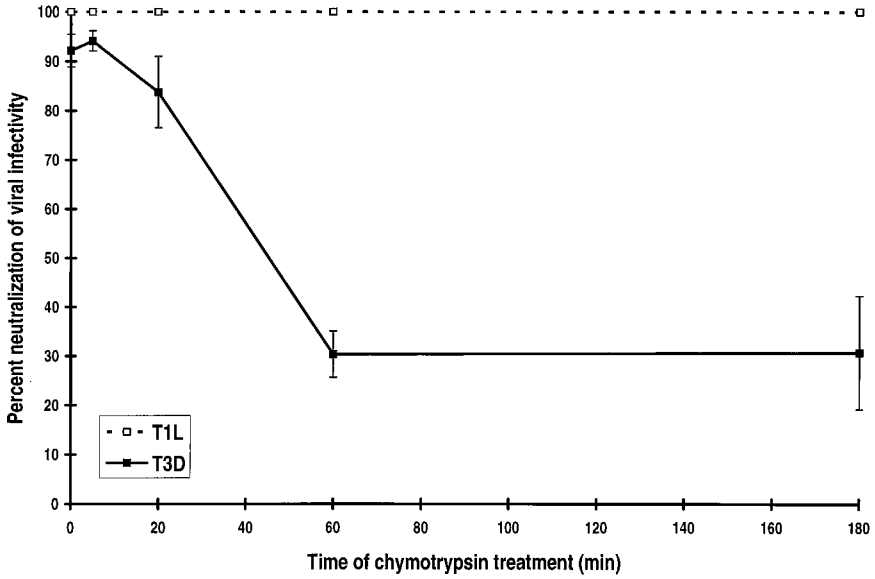

FIG. 6. Effect of chymotrypsin treatment of reovirus virions on the capacity of anti-σ1 monoclonal antibodies to neutralize viral infectivity. Purified virions of T1L and T3D were treated with chymotrypsin for the specified intervals, after which virus was diluted in gelatin saline to a concentration of 2×10^5 particles per ml (all digestions of T1L and 0-, 5-, and 20-min digestions of T3D) or 2×10^6 particles per ml (60- and 180-min digestions of T3D). Virus then was incubated with σ1-specific neutralizing monoclonal antibody 5C6 (T1L) or G5 (T3D) at a final concentration of 25 μg/ml. Infectivity of antibody-treated virus was determined by plaque assay. The capacity of antibody to specifically neutralize virus is expressed as the percent plaque reduction in the presence of the homotypic antibody relative to the number of plaques in the presence of the heterotypic antibody. Experiments were performed in triplicate, and the results are presented as the mean (±standard deviation) plaque reduction relative to that at time zero.

sistent with the observation that T1L does not lose infectivity following treatment with chymotrypsin. Binding of T3D, however, diminished significantly with continued chymotrypsin treatment, the kinetic profile of which approximated that of T3D infectivity (Fig. 1). Similar findings were obtained in a limited number of binding experiments performed with radiolabeled particles (data not shown). Thus, differences between T1L and T3D in infectivity and σ1 cleavage following chymotrypsin treatment correlate with differences in the capacity of these strains to bind L cells.

Binding of monoclonal antibody G5 to T3D is decreased after treatment of virions with chymotrypsin. Monoclonal antibody G5 is capable of neutralizing viral infectivity in assays using L cells (14), and it binds an epitope formed from sequences within the carboxy-terminal one-half of σ1 (7, 24, 40, 58, 69, 72). To determine whether the σ1 epitope bound by monoclonal antibody G5 remains associated with viral particles after chymotrypsin treatment, aliquots of digestion mixtures were used to determine infectious titer in the presence of G5 (Fig. 6). In these experiments, G5 antibody was capable of neutralizing infectivity of T3D particles generated by short times of chymotrypsin digestion, but its neutralization capacity was markedly decreased with particles generated by longer times of treatment. In control experiments, monoclonal antibody 5C6, which is directed against T1L σ1 (62), was capable of neutralizing the infectivity of T1L at all times of chymotrypsin digestion (Fig. 6).

Similar results were obtained in experiments to test more directly the binding of G5 antibody to T3D virions and ISVPs. Using an enzyme immunoassay (62) with particles of T3D or the reassortant 1.HA-3 and including ISVPs generated by either chymotrypsin or trypsin treatment, binding of G5 to ISVPs was found to be significantly decreased in comparison with its binding to virions (data not shown). These results suggest that the σ1 domain bound by monoclonal antibody G5 in T3D virions is either significantly altered or lost from T3D ISVPs.

Chymotrypsin treatment of T3D increases its capacity to produce HA. Previous studies suggested that a domain of the T3D σ1 protein important for both attachment to L cells and binding to monoclonal antibody G5 is distinct from a domain important for HA (14, 20, 40, 72). To investigate whether a σ1 domain important for HA remains associated with T3D ISVPs after chymotrypsin treatment, we digested purified virions of T1L and T3D with chymotrypsin for different times and then tested aliquots for the capacity to agglutinate human type O erythrocytes (Fig. 7). The HA titer of T1L was not affected by chymotrypsin treatment, whereas the HA titer of T3D increased approximately eightfold. These results are consistent with those from a previous study in which T3D, but not T1L, ISVPs were shown to have an increase in HA titer relative to virions (62).

In our experiments, most of the increase in HA titer by T3D particles was found to occur between 2 and 20 min of chymotrypsin treatment, and the increase was maintained over a 3-h period of digestion (Fig. 7 and data not shown). When trypsin was used to generate T3D ISVPs, an increase in HA titer was again observed, but mostly between 0 and 10 min of treatment (data not shown). The increased rate of HA enhancement upon trypsin versus chymotrypsin treatment correlates with an increased rate of σ1 cleavage by trypsin, suggesting that these two phenomena are causally linked. However, many other type 3 reovirus strains exhibit a similar increase in HA titer during generation of ISVPs but do not exhibit σ1 cleavage (17), indicating that an event other than σ1 cleavage during generation of ISVPs of type 3 reovirus must be responsible for their increases in HA titer.

In an effort to define how T3D ISVPs mediate HA, we performed experiments to determine the genetic basis for their increased HA capacity, using T1L × T3D reassortant viruses (Table 1). Purified virions of each reassortant virus were digested with chymotrypsin under identical conditions and assessed for capacity to produce HA. We found that chymotryp-

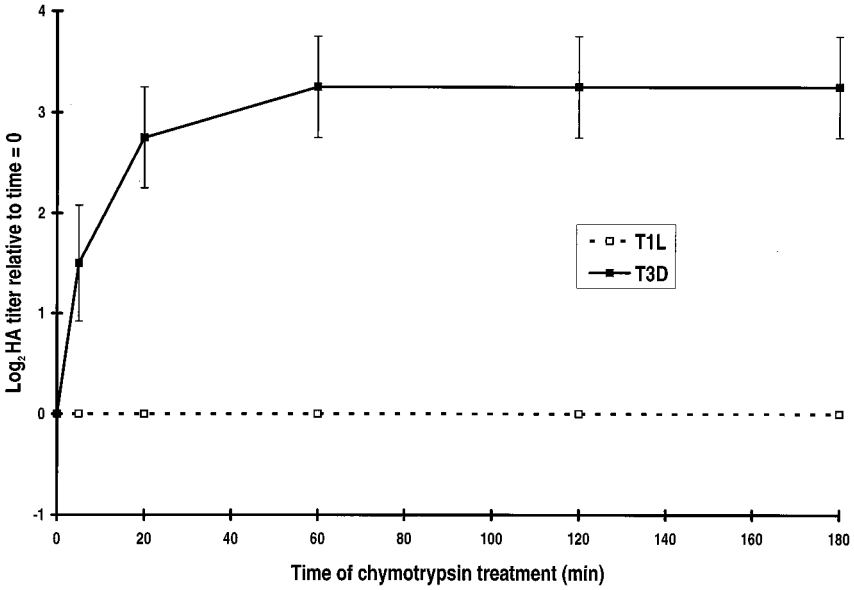

FIG. 7. HA titers of reovirus virions during treatment with chymotrypsin to generate ISVPs. Purified virions of T1L and T3D at a concentration of 2×10^{12} particles per ml were treated with 200 μg of chymotrypsin per ml at 37°C for the specified intervals. Following chymotrypsin treatment, aliquots of the treatment mixtures were tested for the capacity to agglutinate human type O erythrocytes. Experiments were performed in triplicate, and the results are presented as the mean (±standard deviation) $\log_2$ HA titer relative to that at time zero.

sin treatment of reassortants containing an S1 gene derived from T1L was associated with no increase in HA titer, whereas treatment of reassortants containing an S1 gene derived from T3D was associated with an increase in HA titer of slightly greater magnitude than that with T3D. No other reovirus genes were found to be associated with changes in HA titer after chymotrypsin treatment, indicating that this phenotype maps exclusively to the S1 gene. These findings strongly suggest that HA by T3D ISVPs continues to be mediated by σ1, despite its cleavage, and therefore further suggest that the portion of σ1 that contains the binding site for sialic acid on the erythrocyte surface (20) remains attached to T3D ISVPs.

ISVPs of T3D exhibit poor growth in neuraminidase-treated L cells. The capacity of type 3 reovirus to bind certain types of cells, including erythrocytes, depends on the presence of sialic acid on the cell surface (2, 20, 28, 29, 45–47). To determine whether a sialic acid-binding domain of σ1 protein mediates cell attachment by ISVPs, we treated L cells with *A. ureafasciens* neuraminidase to remove sialic acid (Fig. 8). Neuraminidase treatment of L cells resulted in a slight reduction in growth of T3D virions relative to untreated cells; however, neuraminidase treatment virtually abolished growth of T3D ISVPs. Growth of neither T1L virions nor ISVPs was affected by neuraminidase treatment of L cells (Fig. 8), consistent with previous studies suggesting that T1L does not use sialic acid as a component of its cellular receptor (16, 45). These results are consistent with a model in which T3D ISVPs containing cleaved σ1 proteins initiate infection by binding sialic acid residues on cells.

DISCUSSION

Loss of viral infectivity associated with cleavage of σ1 protein in T3D ISVPs. In this study, we investigated whether ISVPs of reovirus strains T1L and T3D exhibit differences in infectivity. The findings strongly suggest that T3D ISVPs have a reduced infectivity relative to T1L ISVPs because their attachment protein, σ1, has been cleaved. Moreover, the data suggest that σ1 cleavage results in decreased infectivity primarily by decreasing the capacity of viral particles to attach to cell surface receptors.

Proteolytic cleavage of viral attachment proteins is essential for infectivity of many viruses, including coronavirus (55), influenza virus (34), and rotavirus (4, 18). In those cases, cleavage is thought to be important for the additional function of the receptor-binding proteins as fusion proteins that mediate membrane penetration. In contrast, our data indicate that cleavage of the reovirus attachment protein σ1 is not essential for infection and instead is associated with a decrease in infectivity. There is no evidence that σ1 plays a direct role in membrane penetration by reovirus; on the contrary, another of the proteolytically processed outer capsid proteins of reovirus, μ1, is postulated to mediate this process (37, 42, 44). Thus, cleavage of different viral attachment proteins can have markedly different consequences for infection.

Cleavage of σ1 as a determinant of viral infectivity after oral inoculation. Our results suggest that proteolytic cleavage of σ1 may have a major effect on viral infectivity in animal hosts as well as in cell culture. In comparison with T1L, T3D grows poorly in the small intestine of newborn mice after oral inoculation (8, 33, 49), and this difference in growth maps genetically to the S1 and L2 gene segments (8). Whether or not σ1 protein is cleaved during generation of ISVPs in the intestinal lumen (5, 9) may be sufficient to explain the contribution of the σ1-encoding S1 gene to determining differences in viral growth exhibited by strains T1L and T3D in intestinal tissue. As with studies in cell culture, we predict that cleavage of T3D σ1 protein leads to reduced infectivity in intestinal tissue primarily through effects on cell attachment.

Despite this explanation, it is important to note that cleavage of reovirus proteins to generate ISVPs from virions appears to be essential for infection of the mouse intestine (1, 5). Thus, cleavage of viral proteins is not detrimental in itself; rather, it is the cleavage of only certain inappropriate proteins, such as σ1 in the case of T3D, that is detrimental. The suscep-

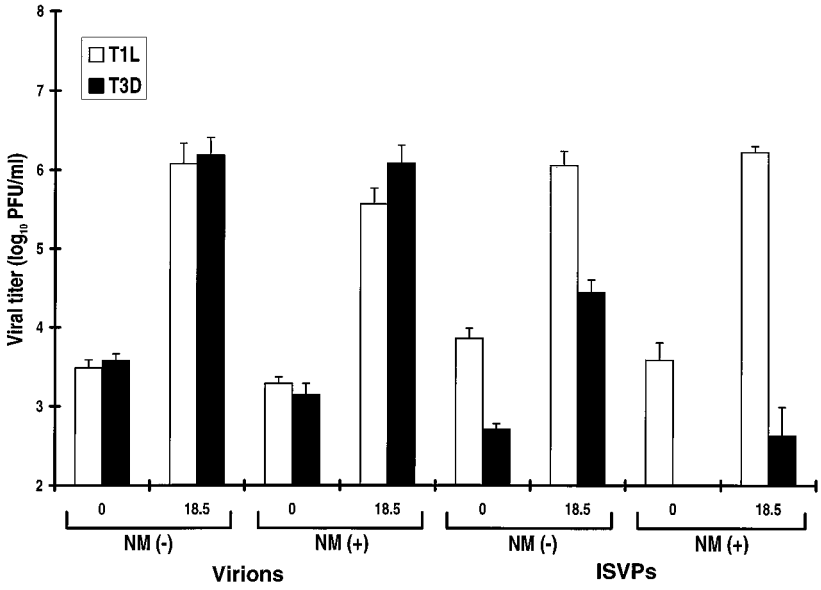

FIG. 8. Capacity of T1L and T3D virions and ISVPs to infect neuraminidase-treated L cells. L cells (10^6) cells) were either untreated [NM (−)] or treated [NM (+)] with 40 mU of *A. ureafasciens* neuraminidase per ml and infected with 10^7 virions or ISVPs generated by chymotrypsin treatment of virions. Following adsorption, cells were incubated at 37°C for either 0 or 18.5 h, after which time culture lysates were titrated by plaque assay. Experiments were performed in triplicate, and the results are presented as the log_{10} mean (±standard deviation) viral titer. Titers of T3D ISVPs in neuraminidase-treated L cells at time zero were undetectable (<50 PFU/ml).

tibility of T3D σ1 to protease cleavage may reflect that it is mutant in some sense.

Fate of the protease cleavage fragments of T3D σ1 protein. In at least some settings, the reovirus σ1 protein assumes the morphology of an extended, fibrous tail topped with a globular head (3, 26, 27). The σ1 tail is predicted to be formed from the amino-terminal two-thirds of σ1 sequence, and the σ1 head is predicted to be formed from the remainder (6, 23, 26, 41). Current understanding of how σ1 attaches to viral particles suggests that the amino-terminal tail is embedded in the λ2 spike (22, 27, 36, 38, 41), and we predict that it remains anchored after σ1 cleavage. In contrast, the carboxy-terminal fragment might be expected to float free from viral particles after cleavage. Consistent with this idea are previous reports which show that the amino- and carboxy-terminal trypsin fragments of purified T3D σ1 are physically separated after cleavage (24, 25, 56, 72). It remains possible, nonetheless, that the carboxy-terminal fragment of σ1 remains attached to viral particles via noncovalent interactions with another viral protein, such as λ2.

We tested these models by determining whether monoclonal antibody G5, which binds sequences within the σ1 head (7, 24, 40, 58, 69, 72), can bind or neutralize infectivity of T3D ISVPs relative to T3D virions. The results indicate that the capacity of G5 to bind and neutralize T3D ISVPs is markedly diminished, consistent with the notion that the σ1 head is liberated from T3D particles following protease treatment. Thus, if the carboxy-terminal portion of σ1 is either lost from T3D ISVPs or functionally inactivated after cleavage, it must be the remaining amino-terminal portion, or some other protein in the reovirus outer capsid, that mediates the residual attachment capacity of these particles.

T3D ISVPs bind to cellular receptors by a domain important for HA. Previous work suggests that sequences in both the head and tail regions of σ1 proteins from type 3 reoviruses are important for attachment to cells. Regions of sequence contained within the σ1 head are important for T3D binding to L cells (24, 40, 58, 69, 72) and also play a role in determining tropism of T3D for cells within the central nervous system of newborn mice (7, 32). Regions of sequence that form a predicted β-sheet structure within the σ1 tail are important for the capacity of T3D to produce HA (20) and to infect murine erythroleukemia cells (51).

We found that the capacity of T3D ISVPs to agglutinate human type O erythrocytes is not reduced by σ1 cleavage. On the contrary, agglutination of human erythrocytes by T3D ISVPs having cleaved σ1 proteins actually increases relative to virions, as noted previously (62). Thus, our results indicate that a σ1 receptor recognition region important for HA is not destroyed by σ1 cleavage and, in fact, is made more accessible for binding and agglutinating erythrocytes by the process of generating ISVPs.

Type 3 reovirus produces HA by binding to sialylated glycoproteins on the erythrocyte surface (2, 20, 28, 29, 46, 47), and previous reports suggest that sialic acid also is important for T3D binding to L cells (2, 28, 45). Using T3D ISVPs with cleaved σ1 proteins to infect neuraminidase-treated L cells, we tested whether a region of σ1 important for HA can facilitate infection of L cells by T3D ISVPs. The results show that in the absence of sialic acid, growth of T3D ISVPs is significantly decreased relative to growth of virions, indicating that infection of normal L cells by T3D ISVPs can be initiated by binding to sialylated receptors on the cell surface.

Results obtained in this study substantiate a model of type 3 reovirus cell attachment in which σ1 can engage the cell surface by two distinct receptor-binding domains (20), one within the σ1 head and another within the σ1 tail. Each domain may be independently capable of binding L cells; however, in the case of T3D ISVPs with cleaved σ1 proteins, it is probably the tail domain that mediates attachment. Such redundancy within a viral attachment protein might play an important role in the capacity of a virus to infect a wide variety of tissues in its host, as observed in newborn mice infected with mammalian reoviruses (reviewed in reference 59).

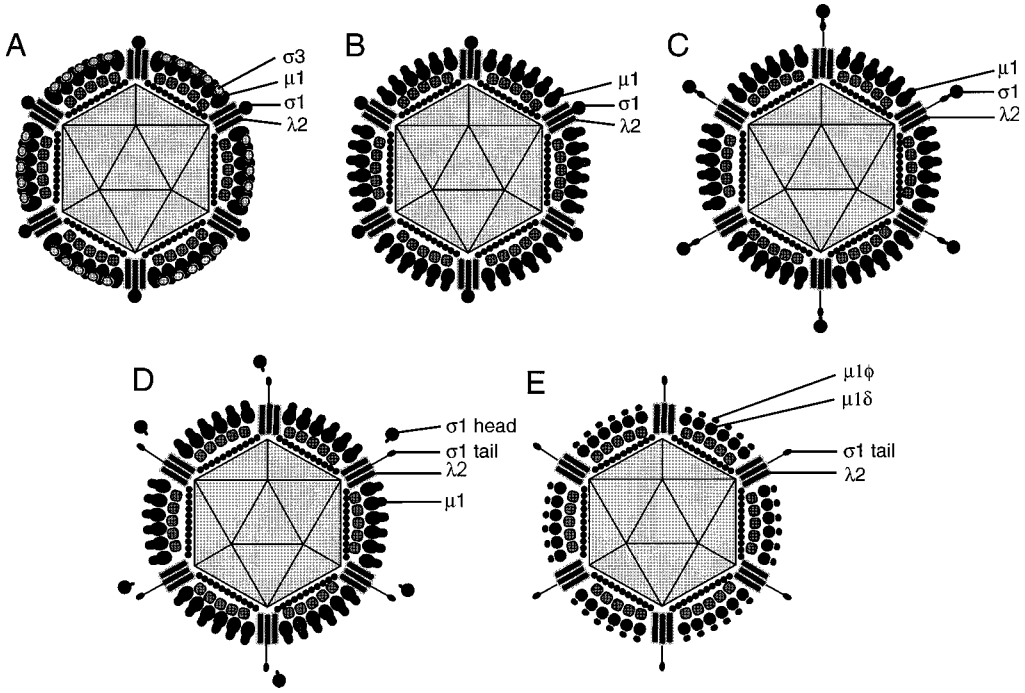

FIG. 9. Changes in outer capsid proteins of reovirus T3D during proteolytic processing of virions to generate ISVPs. (A) Intact virion; (B) cleavage of outer capsid protein σ3; (C) conformational change in viral attachment protein σ1; (D) cleavage of σ1 protein; (E) cleavage of outer capsid protein μ1/μ1C to μ1δ and φ. During proteolytic processing of reovirus virions in a variety of settings as described in the text, there is an ordered sequence of changes in the viral outer capsid, one possible description of which is shown here. In this model, outer capsid protein σ3 is cleaved first. Second, viral attachment protein σ1 undergoes a conformational change from a retracted structure to an extended, rod-like form. Third, outer capsid protein μ1/μ1C is cleaved to μ1δ/δ and φ, yielding the fully processed ISVP. According to our model, the head domain of T3D σ1 protein floats free after chymotrypsin or trypsin digestion, leaving the tail domain attached to the particle and capable of mediating attachment to sialic acid residues on the surface of cells.

Given the moderate decrease in infectivity for L cells observed with ISVPs that contain cleaved σ1, it seems likely that the receptor-binding domain in the tail of type 3 σ1 protein mediates attachment that is less efficiently productive of infection in L cells than that mediated by the head. Differences in efficiency of productive infection after binding by one of these two regions of σ1 may reflect a variety of characteristics, including receptor numbers, binding affinities, and capacity to induce receptor-mediated endocytosis after binding. Additional studies are required to define further the relative activities of these two regions of type 3 σ1.

Previous studies have explored the effects of treatment with various proteases on the structure and function of T3D σ1 protein. Trypsin cleaves purified or expressed T3D σ1 into a 26-kDa amino-terminal fragment and a 23-kDa carboxy-terminal fragment, which is capable of binding L cells and monoclonal antibody G5 (24, 25, 72). Amino-terminal sequencing of the 23-kDa fragment demonstrates that trypsin cleaves T3D σ1 after arginine 245 (24, 25). Chymotrypsin also cleaves expressed T3D σ1 protein. At chymotrypsin concentrations such as those used in this study, 21- and 23-kDa fragments are generated (25), and amino-terminal sequencing of these fragments indicates that chymotrypsin generates the 21-kDa fragment by cleaving T3D σ1 after leucine 261 and the 23-kDa fragment by cleaving after phenylalanine 239 (25).

Our data do not address the site at which T3D σ1 is cleaved by either chymotrypsin or trypsin; however, the site used by chymotrypsin to cleave T3D σ1 during generation of ISVPs is likely to be carboxy terminal to amino acids 198 to 204, sequences important for the capacity of type 3 reovirus to produce HA (20), and amino terminal to amino acids ~300 to 455, sequences important for T3D binding to G5 (7, 24, 40, 58, 69, 72). In concurrence with this expectation, a set of protein fragments, between approximately 20 and 26 kDa in size, was observed when we subjected T3D, but not T1L, to either chymotrypsin or trypsin digestion to generate ISVPs (Fig. 2 and 4); these fragments likely represent the cleavage products of T3D σ1. Thus, our current findings appear to concur with those obtained with expressed σ1 protein and suggest that a protease-sensitive region of T3D σ1 exists in approximately the middle of the protein.

Changes in viral outer capsid proteins during generation of ISVPs. Our study affirms that an ordered sequence of changes affects reovirus outer capsid proteins during proteolytic processing of virions to generate ISVPs (reviewed in reference 43). These changes are observed in several settings, including treatment of virions in vitro with either chymotrypsin (10, 21, 30, 42, 52, 57) or trypsin (5, 9, 42), in the endocytic compartment of reovirus-infected cells (15, 53, 57), or in the intestinal lumen (5, 9). As depicted in Fig. 9, the first step in processing of virions to ISVPs involves proteolytic cleavage of outer capsid protein σ3. This step is thought to be followed closely by a conformational change in viral attachment protein σ1, in which it assumes an extended, rod-like structure (22, 27). Finally, outer capsid protein μ1/μ1C is proteolytically cleaved to yield the stable, particle-bound fragments μ1δ/δ and φ, producing the fully processed ISVP. In the case of certain reovirus strains, including T3D in this study, the cleavage of protein σ1 is viewed to be unrelated to these more integral steps in the generation of ISVPs. The relationship between the cleavage of σ1 and its conformational change is not apparent from these experiments. However, the widely different rates of T3D σ1 cleavage mediated by chymotrypsin and trypsin suggest that cleavage and conformational change may be independent phe-

nomena. The early, mild increases in infectivity and attachment activities observed during protease treatments in this study may reflect the extension of σ1 in ISVPs; however, they may also reflect the degradation of portions of σ3 that provide steric hindrance to σ1-receptor interactions (62).

It is interesting that changes associated with the generation of T1L ISVPs do not affect the capacity of T1L to produce HA. These observations suggest that regions of T1L σ1 important for cell attachment have a topographically different arrangement within the protein or relative to other proteins in the reovirus outer capsid than those of T3D σ1. For example, receptor-binding regions of T1L σ1 might be equally accessible to receptor molecules in either a retracted or an extended conformation.

Studies of reovirus infectivity after treatment with proteases. The current results suggest that digestion with intestinal proteases is a mechanism by which reovirus strains T1L and T3D differ in tropism for intestinal tissue. Thus, the reovirus attachment protein appears to be a determinant of tissue tropism in host organisms by both its capacity to recognize different types of cellular receptors (64, 66) and its susceptibility to proteolytic digestion. Future experiments addressing σ1 cleavage and the mechanism of its effects on viral infectivity will provide additional information about the structure of σ1, how σ1 associates with other proteins in viral particles, and the role of σ1 in early events in infection of cells in culture and animal hosts.

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