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Proteolytic Enhancement of Rotavirus Infectivity: Molecular Mechanisms

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The polypeptide compositions of single-shelled and double-shelled simian rotavirus particles were modified by exposure to proteolytic enzymes. Specificially, a major outer capsid polypeptide (VP3) having a molecular weight of 88,000 in double-shelled particles was cleaved by trypsin to yield two polypeptides, VP5* and VP8* (molecular weights, 60,000 and 28,000, respectively). The cleavage of VP3 by enzymes that enhanced infectivity (trypsin, elastase, and pancreatin) yielded different products compared to those detected when VP3 was cleaved by chymotrypsin, which did not enhance infectivity. The appearance of VP5* was correlated with an enhancement of infectivity. Cleavages of the major internal capsid polypeptide VP2 were also observed. The VP2 cleavage products had molecular weights similar to those of known structural and nonstructural rotavirus polypeptides. We confirmed the precursor-product relationships by comparing the peptide maps of the polypeptides generated by digestions with V-8 protease and chymotrypsin. The remaining rotavirus structural polypeptides, including the outer capsid glycoproteins (VP7 and 7a), were not altered by exposure to pancreatic enzymes. Cleavage of VP3 was not required for virus assembly, and specific cleavage of the polypeptides occurred only on assembled particles. We also discuss the role of cleavage activation in other virus-specific biological functions (e.g., hemagglutination and virulence).

Proteolytic enzymes enhance rotavirus infectivity and are important for rotavirus propagation and detection (1, 2, 10, 26). However, the mechanism(s) by which proteolytic enzymes enhance rotavirus infectivity remains unknown. Recent studies have suggested that these enzymes (i) act directly on the virus particles in the extracellular environment (3, 10), (ii) convert noninfectious particles to infectious particles (6, 10), and (iii) allow multiple cycles of replication to occur in normally restrictive cells (10). We undertook this study to determine whether trypsin treatment of virus particles cleaves a specific virus polypeptide and whether this cleavage is directly correlated with activation of infectivity.

This study showed that the polypeptide compositions of homogeneous preparations of both double- and single-shelled particles are altered by exposure to proteolytic enzymes. We found that a major outer capsid protein (VP3; molecular weight, 88,000) is cleaved and yields two polypeptides, VP5* and VP8* (molecular weights, 60,000 and 28,000, respectively), confirming the existence of VP3 as a major outer capsid polypeptide and its susceptibility to cleavage by trypsin (9). We also show the biological significance of this cleavage by its correlation with enhancement of viral infectivity.

This study confirms that processing of rotavirus primary gene products occurs and supports our hypothesis that polypeptide modifications represent important regulatory steps for rotavirus replication or virus infectivity or both (10, 15). Finally, this study demonstrates that many of the previously described differences in the numbers and molecular weights of the polypeptides of purified rotavirus particles (4, 8, 13, 15-22, 27, 28) represent differences caused by the exposure of the virus to proteolytic enzymes before analysis.

MATERIALS AND METHODS

Cells and virus. Fetal rhesus monkey MA104 cells were grown in medium 199 supplemented with 5% fetal bovine serum, 0.03% glutamine, 0.075% sodium bicarbonate, 100 U of penicillin per ml, and 100 μg of streptomycin per ml. SA11 virus stocks were prepared at 37°C in MA104 cells at a low multiplicity of infection in the absence of serum or exogenously added trypsin. Virus infectivity was quantitated by a plaque assay (24).

Isotopic labeling and purification of virus. Vi-

rus was grown in roller bottles (850 cm^2) at a high multiplicity of infection (>10 PFU/cell). Radiolabeled virus was prepared by adding 10 μCi of L-[^{35}S]methionine (1,075 Ci/mmol; Amersham Corp., Arlington Heights, Ill.) per ml to serum-free medium containing 1/10 the normal concentration of methionine and 5 μg of actinomycin D (P.L. Biochemicals, Milwaukee, Wis.) per ml. In some cases, the virus inoculum was preactivated by adding trypsin (2× crystallized; Worthington Diagnostics, Freehold, N. J.) to the virus-containing medium (10 μg/ml; 4 U/ml), and the virus-trypsin mixture was incubated at 37°C for 30 min before inoculation of cells. After virus adsorption, the monolayers were washed extensively to remove residual trypsin, and either medium lacking trypsin and serum was added or medium lacking serum but containing 0.4 to 1 U of trypsin per ml was added for the remainder of the infection. Double-shelled and single-shelled particles were purified from infected cell lysates (15). Virus concentrations were determined by optical density readings at 260 nm and by particle counts, using the kinetic attachment method (23); these concentrations averaged 3.1×10^{11} particles per unit of optical density at 260 nm for purified double-shelled preparations.

Enzymes and chemicals. All enzyme preparations were filter sterilized, frozen at −20°C, and thawed once immediately before use. Trypsin (2× crystallized) and tolylsulfonyl phenylalanyl chloromethyl ketone-treated trypsin (Worthington) were prepared in a solution containing 0.001 N HCl, tolylsulfonyl lysyl chloromethyl ketone-treated chymotrypsin, and porcine elastase (Sigma Chemical Co., St. Louis, Mo.). V8 protease (Miles Laboratories, Inc., Elkhart, Ind.) and alpha-chymotrypsin (Sigma) were prepared in distilled water. All enzyme preparations were monitored for enzyme purity as previously described (10).

Polyacrylamide gel electrophoresis. Polyacrylamide gel electrophoresis was performed by using 12% polyacrylamide slab gels (thickness, 0.75 mm; acrylamide/bisacrylamide ratio, 100:1) and a modified Laemmli discontinuous buffer system which contained 0.5 M urea (14, 15). Electrophoresis and fluorography were performed as previously described (15).

Enzyme treatments. Purified virus preparations were mixed with buffer or enzymes and incubated in a 37°C water bath for 30 min. Some samples were pelleted by centrifugation in an Airfuge rotor (Beckman Instruments, Inc., Fullerton, Calif.) at 100,000 × *g* for 30 min, and the pellets were suspended in electrophoresis sample buffer (5 mM Tris-hydrochloride, pH 6.8, 8% glycerol, 10% sodium dodecyl sulfate, 0.5 M urea, 5% 2-mercaptoethanol, 0.003% phenol red) and boiled for 2 min before they were loaded onto gels. Other samples were aliquoted and diluted directly in 0.05 M Tris-hydrochloride–0.15 M NaCl (pH 7.4) for immediate infectivity assays and mixed directly with electrophoresis buffer for polypeptide analyses on gels.

Peptide mapping. The precursor-product relationships of the polypeptides were analyzed by using staphylococcal V8 protease or chymotrypsin or both and the peptide mapping procedure of Cleveland et al. (7). The partial peptide patterns were compared on fluorograms after 3- to 6-week exposures at −70°C.

RESULTS

Trypsin alteration of purified double-shelled and single-shelled SA11 virus particles. The polypeptide compositions of purified double-shelled and single-shelled rotavirus particles were evaluated in 12% polyacrylamide gels. We found striking differences in the gel patterns; the simplest pattern was that of single-shelled particles from virus grown in the absence of trypsin (Fig. 1, lane A). These particles contained three polypeptides (VP1 [125,000 daltons], VP2 [94,000 daltons], and VP6 [41,000 daltons]). Single-shelled particles obtained from virus grown in the presence of trypsin contained two additional polypeptides, which had molecular weights of 88,000 and 84,000 (Fig. 1, lane B). The lack of a significant decrease in VP1 associated with the appearance of the 88,000- and 84,000-dalton polypeptides suggested that these additional polypeptides arose from proteolytic degradation of VP2. This was confirmed subsequently by peptide mapping (see below). Because these polypeptides were structural components of the virus, we refer to them as viral polypeptides (VP) but indicate their origins as cleavage products by an asterisk (i.e., VP3* and VP4*).

Double-shelled virus particles obtained from virus grown in the absence of trypsin revealed a

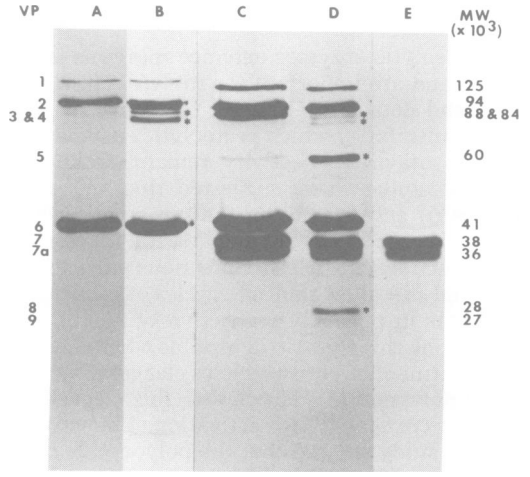

FIG. 1. *Polypeptide patterns of single-shelled (lanes A and B) and double-shelled (lanes C and D) simian rotavirus SA11 particles purified from MA104 cells infected and cultivated in the absence (lanes A and C) and presence (lanes B and D) of 10 μg (4 U) of trypsin per ml. The polypeptides in double-shelled particles were labeled with [^{3}H]glucosamine (10 μCi/ml) (lane E). Asterisks indicate cleavage products. MW, Molecular weight.*

polypeptide pattern which recently has also been described by Espejo et al. (9). These particles contained seven polypeptides (Fig. 1, lane C, and Fig. 2, lane A), namely, VP1 (125,000 daltons), VP2 (94,000 daltons), VP3 (88,000 daltons), VP6 (41,000 daltons), VP7 (38,000 daltons), VP7a (36,000 daltons), and VP9 (27,000 daltons), which was present in minor amounts. VP9 was not detected in our previous study (15), and it is often difficult to detect in virus preparations. However, as Fig. 1 and 2 show, VP9 can be found consistently after long exposures of fluorograms of radiolabeled virus polypeptides. Other polypeptides observed after long exposures of fluorograms were not found consistently enough to label them as viral polypeptides, but they could have represented additional cleavage products.

An analysis of the double-shelled particles obtained from virus grown in the presence of trypsin yielded a polypeptide pattern similar to the pattern which we have described previously (15). Double-shelled particles grown in the presence of trypsin contained the following 10 polypeptides (Fig. 1, lane D): VP1 (125,000 daltons), VP2 (94,000 daltons), minor amounts of VP3* (88,000 daltons) and VP4* (84,000 daltons), VP5* (60,000 daltons), VP6 (41,000 daltons), VP7 and 7a (both glycoproteins; 38,000 and 36,000 daltons, respectively [Fig. 1, lane E]), and two small polypeptides, VP8* (28,000 daltons) and VP9 (27,000 daltons). Analyses of independently prepared radiolabeled preparations and preparations stained with Coomassie brilliant blue confirmed these differences in the rotavirus polypeptide patterns produced under different culture conditions. The most obvious differences in the pattern of the double-shelled particles cultivated without trypsin were the appearance of the major outer capsid polypeptide VP3 and the lack of VP5* and VP8*. However, it should be noted that a small amount of VP5* was detected in all of the purified virus preparations examined.

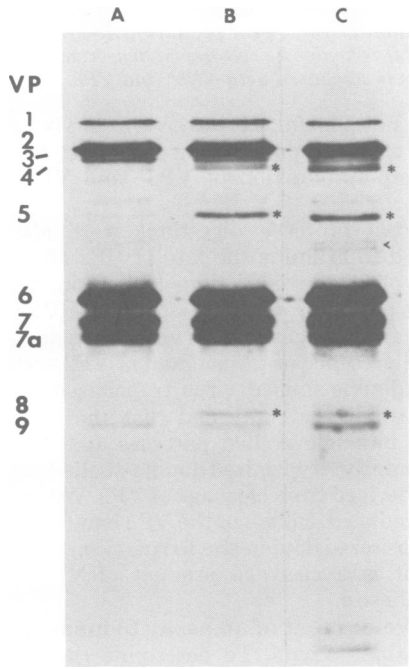

FIG. 2. *Specific cleavage of SA11 virion polypeptides after in vitro trypsin treatment. [^{35}S]methionine-labeled double-shelled particles were electrophoresed in 12% polyacrylamide gels after treatment with buffer (lane A), 0.10 μg (0.04 U) of trypsin per ml (lane B), or 2.5 μg (1.0 U) of trypsin per ml (lane C). Asterisks indicate cleavage products. Arrowhead indicates the 53,000-dalton cleavage products.*

These results showed that specific modification of rotavirus polypeptides occurred with trypsin treatment. The observed differences in the polypeptide patterns were confirmed by in vitro trypsin treatment of purified double-shelled virus particles from virus grown in the absence of trypsin. Trypsin treatment resulted in the disappearance of VP3 and the appearance of VP5* and VP8* (see below) (Fig. 2). Depending on the conditions of the in vitro treatment (duration and amount of enzyme added), minor amounts of an 84,000-dalton polypeptide (VP4*) could also appear, and minor amounts of an 88,000-dalton polypeptide (VP3 or VP3*) and an 84,000-dalton polypeptide (VP4*) remained. In vitro treatments could result in the appearance of polypeptides of 53,000 daltons and several small polypeptides having molecular weights less than 30,000. The occurrence of minor amounts of these polypeptides (88,000, 84,000, 53,000, and <30,000 daltons), which had the same molecular weights as components in the single-shelled trypsinized particles, suggested that they arose from secondary proteolytic alterations.

Production of double-shelled particles containing uncleaved polypeptides. Typical viral yields were quite low when the virus was grown in the absence of trypsin. For example, yields of double-shelled virus grown in the absence and presence of trypsin were approximately 25 and 100 μg per 850-cm^2 roller bottle, respectively. Previous experiments suggested that trypsin acts upon assembled viruses in the extracellular environment to activate particles that can then successfully reinitiate infection (10). Therefore, we reasoned that virus particles without cleaved proteins should be produced in

single-cycle conditions by using high multiplicities of infection. To obtain high multiplicities of infection, virus inocula were preactivated with trypsin. After adsorption, the trypsin was removed with extensive washing. These conditions resulted in the production of double-shelled particles containing uncleaved proteins in greater quantities (50 to 100 μg/roller bottle). The proteins remained uncleaved provided that proteolysis was prevented by adding the protease inhibitor Trasylol to the medium before the cells were lysed and by keeping the extracts cold (4°C) during all steps of the purification.

Precursor-product relationships of the rotavirus polypeptides. Radiolabeled rotavirus preparations containing uncleaved polypeptides were treated in vitro with buffer or with tolylsulfonyl phenylalanyl chloromethyl ketone-treated trypsin (10 μg/ml for 30 min) and then analyzed on cylindrical sodium dodecyl sulfate-polyacrylamide gels to quantitate the percentage of each polypeptide present before and after trypsin treatment. These analyses confirmed the disappearance of the VP3, and the radioactivity from VP3 was recovered in VP5* and VP8* (data not shown). These analyses also revealed a 40% decrease in the amount of the VP2, but the fate of the radioactivity lost from VP2 was not determined.

Interestingly, the other major viral polypeptides (VP6 [41,000 daltons] and the glycoproteins VP7 and VP7a [38,000 and 36,000 daltons, respectively]) were not altered significantly by trypsin treatment of intact particles, confirming the trypsin resistance of the other rotavirus polypeptides in assembled particles. In contrast, trypsin treatment of unassembled polypeptides (obtained from cell lysates or in vitro translation products) resulted in the nonspecific cleavage of both the structural and the nonstructural virus-specific proteins (data not shown).

Partial protease mapping of the postulated precursor and product polypeptides (VP3 and VP5*) was performed to confirm the relationship between these proteins. VP3 and VP5* yielded similar patterns (Fig. 3) when they were digested with alpha-chymotrypsin. Figure 3 also shows the partial protease pattern of the 53,000-dalton rotavirus polypeptide synthesized in vitro from the transcript of RNA segment 5 (25; manuscript in preparation). These data showed the 53,000-dalton polypeptide was a distinct polypeptide and not, as suggested by others (25, 27), a precursor of the viral structural polypeptide (VP5*). The partial protease maps for VP8* resembled the VP3 maps but were not sufficiently complex to identify this polypeptide conclusively as a cleavage product by this method

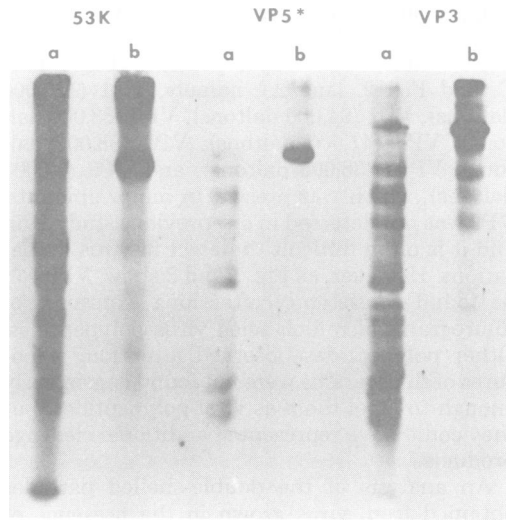

FIG. 3. *Comparison of partial peptide patterns of SA11 precursor and trypsin cleavage polypeptides. Partial peptide patterns were resolved in 15% polyacrylamide gels after digestion of polypeptides with 1.0 mg of alpha-chymotrypsin per ml (lanes a) and 0.1 mg of alpha-chymotrypsin per ml (lanes b). The 53,000-dalton SA11 gene product (53K) synthesized in a rabbit reticulocyte cell-free system from viral transcripts is compared with VP5* and VP3.*

(data not shown). However, the in vivo and in vitro cleavage data strongly support this relationship (production of VP5* and VP8* from VP3).

Partial protease digestions were also performed to examine the fate of VP2 after exposures of virus preparations to trypsin. Figure 4 shows partial protease digests of VP2, VP3*, and VP4* from single-shelled virus particles grown in the presence of trypsin and of VP2 and VP4* from double-shelled virus trypsinized in vitro. These patterns confirmed that the VP3* and VP4* in single-shelled particles and the VP4* remaining in trypsinized double-shelled particles were derived from cleavage of VP2. We have not yet produced enough of the VP3 band remaining in trypsinized double-shelled particles to confirm that it is a cleavage product of VP2, as we hypothesize.

Development of an assay to measure "activatable" virus. To determine the effect of the protease on virus infectivity, we optimized conditions to measure the amounts of infectious and activatable viruses present in virus preparations. Initial experiments revealed a 2- to 10-fold enhancement of infectivity after trypsin treatment of particles documented to contain uncleaved polypeptides. We postulated that the levels of enhancement measured were underes-

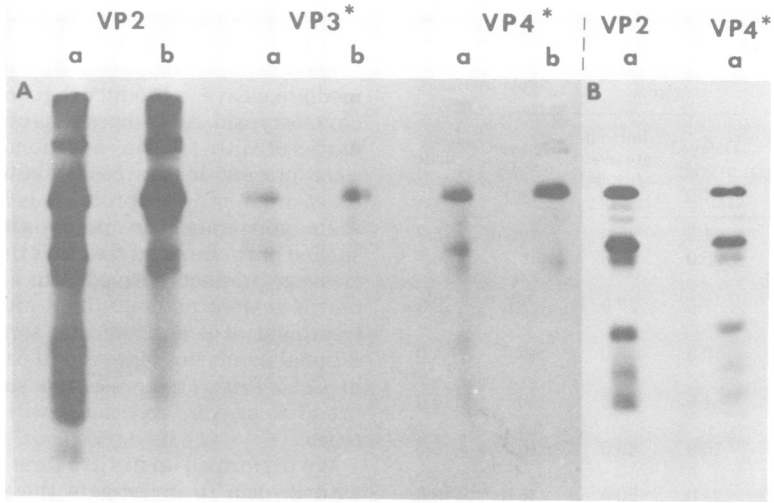

FIG. 4. *Partial peptide patterns of SA11 polypeptides VP2, VP3*, and VP4* from single-shelled virus grown in the presence of trypsin (A) and of VP2 and VP4* generated after in vitro treatment of double-shelled virus with 10 μg of tolylsulfonyl phenylalanyl chloromethyl ketone-treated trypsin per ml (B). The peptide patterns shown were from polypeptides digested with 20 μg of V8 protease per ml (lanes a) and 2 μg of V8 protease per ml (lanes b).*

timated since untrypsinized virus was activated by pancreatin in the overlay of the standard rotavirus plaque assay.

Since trypsin was effective in activating virus early in the infectious cycle, we performed assays in which the inoculum was carefully removed after adsorption and the agar overlay was added immediately (standard immediate overlay) or after a 4-h postadsorption period (delayed overlay) when the infected cells were maintained under a liquid medium. These experiments showed a significant difference in the viral titers obtained when either crude stocks (data not shown) or purified double-shelled virus particles were assayed (Table 1). Lower titers were obtained in the delayed overlay assay, which presumably represented the true titers of the infectious virus present in the virus preparations. The higher titers found in the immediate overlay assay presumably represented both the infectivity of the original infectious virus in the preparation and the infectivity of the virus activated by pancreatin in the overlay. This interpretation was consistent with the observation that trypsin treatment of virus before the plaque assay resulted in an enhancement of viral infectivity in the delayed assay system that approached or equaled the titer observed under the immediate assay conditions.

As Table 1 shows, the infectivity of the virus preparation (1.5×10^8 PFU/ml) was enhanced maximally (55×10^8 PFU/ml) after in vitro activation and assay by the immediate overlay method. The fact that the titer obtained by the delayed overlay method (25×10^8 PFU/ml) approached but did not equal this titer suggested that the in vitro activation by trypsin was not complete in this experiment. Complete activation was achieved in most experiments (Table 2).

Reproducible results were obtained readily in these two assay systems, and the final titers of activated virus were equal with both assays, providing the inocula on the duplicate plates for the immediate and delayed overlays were removed with washing at the same time. Good quantitation of the amount of activatable virus in a preparation could be obtained if a buffer-treated virus sample and a high-concentration trypsin-activated sample were assayed by the delayed overlay method. Using this assay, we showed that the majority of the rotavirus particles produced in the absence of exogenously added enzymes were not infectious. For example, in preparations which contained uncleaved VP3, ratios of physical particles to infectious particles ranged from 500 to 700, whereas these ratios ranged from 15 to 100 in trypsin-activated preparations.

Correlation of cleavage of VP3 with enhancement of infectivity. To test whether the observed polypeptide cleavage could be correlated with the enhancement of viral infectivity, we compared the titers and polypeptide patterns of untreated and protease-treated virus preparations. The effects of proteases which are

TABLE 1. *Influence of protease activation and assay conditions on rotavirus titers*

Treatment[a]	Titer (PFU/ml, $\times 10^{-8}$)		Fold increase[b]	
	Delayed overlay assay	Immediate overlay assay	Delayed overlay assay	Immediate overlay assay
Buffer	1.5	8.6	Control	5.7
Trypsin (10.0 μg/ml)	25.0	55.0[c]	16.7	36.7
Trypsin (1.0 μg/ml)	7.5	27.0	5.0	19.3
Chymotrypsin (5.0 μg/ml)	0.5	6.0	−2.7	4.0
Chymotrypsin (0.5 μg/ml)	1.6	7.5	0	5.0
Pancreatin (1:60)	16.0	40.0	10.7	26.7
Elastase (10.0 μg/ml)	24.0	20.0	16.0	13.0
Elastase (5.0 μg/ml)	3.3	9.6	2.2	6.4

[a] [^{35}S]methionine-labeled double-shelled virus was treated with enzyme or buffer and incubated at 37°C for 30 min. A sample was removed for dilution for the immediate infectivity assay, and another sample was put into electrophoresis buffer for analysis in 12% sodium dodecyl sulfate–polyacrylamide gels. All infectivity samples were assayed in triplicate.

[b] Increase above the titer obtained when the virus was incubated in buffer and assayed by the delayed overlay method.

[c] Maximum titer in this assay.

known to enhance infectivity (trypsin, elastase, and pancreatin) and of a protease which does not enhance inectivity (chymotrypsin) were quantitated by plaque assays and sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

Table 1 shows representative results from one experiment in which we examined enzyme treatments of purified double-shelled virus, and Fig. 5 shows the corresponding changes in polypeptide patterns. Trypsin, pancreatin, or elastase treatment resulted in a concentration-dependent enhancement of infectivity, cleavage of VP3, and appearance of VP5*. The smaller VP8* cleavage product (molecular weight, 28,000) generated by trypsin treatment was not present after elastase or pancreatin treatment. In contrast, other low-molecular-weight components were observed with these enzymes, suggesting that the 60,000-molecular-weight polypeptide (VP5*) was the cleavage product of biological importance.

Treatment of particles with chymotrypsin resulted in cleavage of VP3, but there was no concomitant enhancement of infectivity. At low enzyme concentrations, the chymotrypsin cleavage products included a polypeptide having a molecular weight similar to that of VP5*, but this polypeptide was absent at higher enzyme concentrations, suggesting that it was an intermediate cleavage product that was degraded by chymotrypsin. After digestions of double-shelled particles with trypsin, additional polypeptides were present in the 53,000-dalton region; the exact origin of these products is not known, but their appearance in preparations of single-shelled particles suggested that they represented cleavage products derived from VP2. Since these particles were not repurified after the in vitro treatment, it is possible that some of these additional bands not observed in particles purified in CsCl (Fig. 1) represented soluble cleavage products or were associated with unstable particles.

We performed an in vitro time course analysis with trypsin to investigate the relative importance of the observed polypeptides in the enhancement of infectivity. Treatment with 10 μg

TABLE 2. *Kinetics of rotavirus infectivity increases after treatment with trypsin in vitro*

Treatment[a]	Length of incubation at 37°C (min)	Gel profile shown in:		Titer (PFU/ml, $\times 10^{-8}$)	
		Fig.	Lane	Delayed overlay assay	Immediate overlay assay
Buffer	0	6	A	4.6	19
TPCK-trypsin (10 μg/ml)	<1	6	B	20.0	38
	5	6	C	40.0	42
	15	6	D	39.0	42
	30	6	E	42.0	36
	60	6	F	37.0	44
	180	6	G	ND[b]	ND
Buffer	30	7	A	2.0	5
TPCK-trypsin (2.5 μg/ml)	<1	7	B	4.2	ND
	5	7	C	7.5	ND
	15	7	D	7.5	ND
	120	7	E	16.0	ND
TPCK-trypsin (10 μg/ml)	30	7	F	27.0	24

[a] Samples (2 μg) of purified double-shelled SA11 were treated with the buffer or with tolylsulfonyl phenylalanyl chloromethyl ketone-treated trypsin (TPCK-trypsin) and incubated at 37°C. After the indicated times, one portion was put into electrophoresis sample buffer for analyses on gels (see Fig. 6 and 7) and another portion was diluted in 0.05 M Tris-hydrochloride–0.15 M NaCl (pH 7.4) for plaque assays.

[b] ND, Not done.

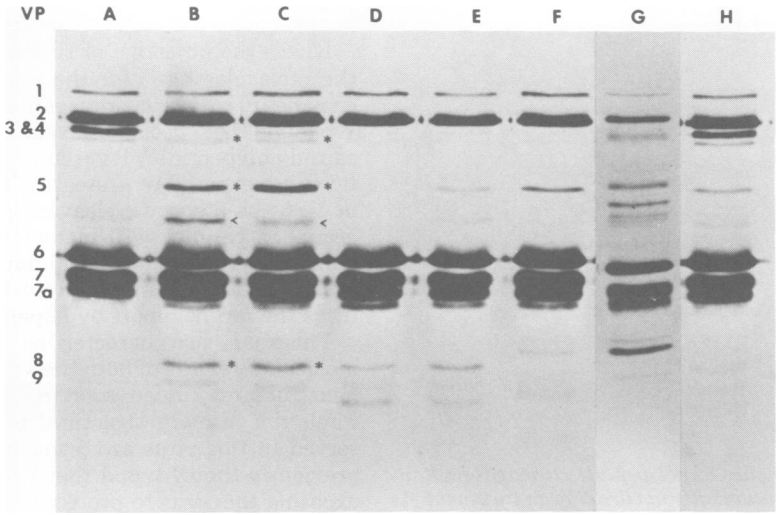

FIG. 5. *Correlation of polypeptide cleavage with infectivity activation after in vitro enzymatic treatment of [^{35}S]methionine-labeled SA11 particles containing uncleaved VP3. Samples (2 μg) of purified double-shelled particles (30,000 cpm) were electrophoresed in 12% polyacrylamide gels after treatment with buffer (lane A), 10 μg of tolylsulfonyl phenylalanyl chloromethyl ketone-treated trypsin per ml (lane B), 1 μg of tolylsulfonyl phenylalanyl chloromethyl ketone-treated trypsin per ml (lane C), 5 μg of tolylsulfonyl lysyl chloromethyl ketone-treated chymotrypsin per ml (lane D), 0.5 μg of tolylsulfonyl lysyl chloromethyl ketone-treated chymotrypsin per ml (lane E), a 1:60 dilution of oxoid pancreatin (lane F), 10 μg of elastase per ml (lane G), and 5 μg of elastase per ml (lane H) for 30 min at 37°C. The infectivity titers obtained with duplicates of these samples are shown in Table 1. Asterisks indicate characterized cleavage products. Arrowheads indicate the 53,000-dalton cleavage product.*

of trypsin per ml (Fig. 6 and Table 2) showed that infectivity activation was achieved within 5 min and was associated with the disappearance of VP3. When the original VP3 was no longer detectable (180 min), VP3* and VP4* appeared, again suggesting that these polypeptides were secondary cleavage products of VP2.

The addition of 2.5 μg of trypsin per ml (Fig. 7 and Table 2) resulted in the commencement (<1 min) of cleavage of VP3 and the concomitant appearance of VP5*, VP8*, and VP4*, and, over time, the amount of the 88,000-dalton protein (VP3 or perhaps VP3*) decreased. Enhancement of infectivity was observed at <1 and 5 min, and after 120 min, and the level of activation was correlated with the appearance of VP5* and the complete disappearance of VP3. These data were consistent with the hypothesis that cleavage of VP3 was important for infectivity. Whether cleavage of VP2 to VP4* contributes to rotavirus infectivity remains to be determined.

Site and origin of the proteases required for cleavage activation. Our previous experiments (10) predicted that no cleavage products (VP5* or VP8*) would be found in rotavirus-infected cells. The polypeptide patterns in infected cells were examined after 1-h pulses with [^{35}S]methionine performed throughout the virus

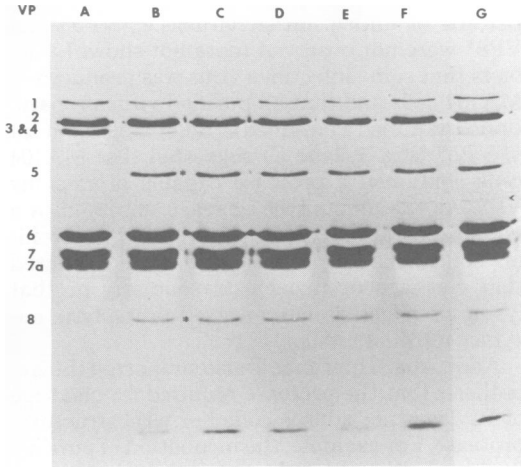

FIG. 6. *Kinetics of polypeptide cleavages with infectivity activation after in vitro trypsin treatment. As described in the legend to Fig. 5, SA11 particles were treated with buffer (lane A) or with 10.0 μg (2.8 U) of tolylsulfonyl phenylalanyl chloromethyl ketone-treated trypsin per ml (lanes B through G) for different lengths of time at 37°C. The treatments, durations of treatment, and titers as determined by the delayed overlay and immediate overlay assays are shown in Table 2. The polypeptide profiles resolved in 12% polyacrylamide gels are shown for each time point.*

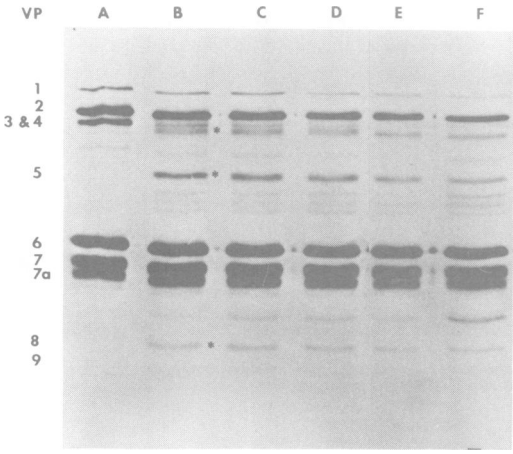

FIG. 7. *Kinetics of polypeptide cleavages with infectivity activation after in vitro trypsin treatment. As described in the legend to Fig. 5, SA11 particles were treated with buffer (lane A) or with 2.5 μg (0.7 U) (lanes B through E) or with 10 μg (2.8 U) (lane F) of tolylsulfonyl phenylalanyl chloromethyl ketone-treated trypsin per ml for different lengths of time at 37°C. The treatments, durations of treatment, and titers are shown in Table 2. The polypeptide profiles resolved in 12% polyacrylamide gels are shown for each time point.*

replication cycle (1 to 12 h postinfection). At each time point, the polypeptide pattern was the pattern of untrypsinized virus; i.e., VP5* and VP8* were not observed (data not shown). The facts that some infectious virus was produced in MA104 cells and that all purified virus preparations examined contained a detectable amount of VP5* (Fig. 1, lane C) suggested that MA104 cells contained a protease capable of cleaving VP3. As the amount of cleavage observed in a virus preparation could be minimized by avoiding conditions for protease activity, it appeared that cleavage occurred extracellularly or that lysing of infected cells released proteolytic enzymes into the media.

Additional experiments also supported the hypothesis that the protease required for cleavage of VP3 was not a virus-coded or viral structural protease. For example, the incubation of purified double-shelled particles containing the uncleaved polypeptide at 37°C for 30 min either (i) alone, (ii) after heat treatment (45 or 60°C for 1 min), or (iii) after treatment with agents known to disrupt virus particles [1% sodium dodecyl sulfate or 0.2 mM ethylene glycol-bis(β-aminoethyl ether)-*N,N*-tetraacetic acid] did not result in any observable polypeptide cleavages. Finally, cleavage of VP3 was not observed in in vitro translation products made either in wheat germ lysates or in rabbit reticulocyte translation systems (data not shown).

DISCUSSION

It was the objective of this study to identify the molecular basis for the role of proteolytic enzymes in rotavirus propagation and detection. We found that specific cleavage of a major outer capsid polypeptide VP3 occurs when intact particles are exposed to proteases. The appearance of VP5* as a specific cleavage product of VP3 occurs in parallel with increases in infectivity, demonstrating the biological importance of this event. Therefore, this study extends and confirms the recent report by Espejo et al. (9).

This study also characterized the number and molecular weights of both the structural and the nonstructural simian rotavirus gene products. First, the primary structural polypeptides observed in this virus are fewer in number than previously thought, and they can be altered by exposing the virus to proteolytic enzymes. Second, this study confirms the report by Espejo et al. (9) of the existence of the major outer capsid polypeptide VP3. This polypeptide is a primary gene product, as shown by protease map identity with the 88,000-dalton polypeptide synthesized in vitro from genome segment 4 (manuscript in preparation). Our findings also clarify the origin of the high-molecular-weight viral structural polypeptides. Confusion regarding these polypeptides has centered around the fact that the primary 88,000-dalton gene product (the major outer capsid polypeptide VP3) has a molecular weight identical to the VP3 previously identified as a minor polypeptide in both single-shelled and double-shelled trypsinized viruses (15). We now recognize that the previously described VP3, which is found as a minor band in single-shelled and double-shelled particles exposed to trypsin, is a secondary cleavage product of VP2. The minor structural polypeptide previously designated VP4 similarly represents a cleavage product of VP2. Therefore, we agree with Espejo et al. (9) that VP4 is not a primary structural component of the virion, nor is it a primary gene product. A new nomenclature to distinguish these proteins of similar molecular weights is needed to identify the primary gene products, the precursors, and the cleavage products clearly. We suggest an asterisk to identify the cleavage products.

This study also shows that minor polypeptide bands having molecular weights of approximately 53,000 and smaller products having molecular weights of less than 30,000 can be found after in vitro treatments of both double-shelled and single-shelled particles, suggesting that a series of cleavages may occur during prolonged protease exposure. The exact origin and significance of these minor bands cannot yet be established since the low level of radioactivity in these

bands has precluded identification by peptide mapping. However, the identities of these bands can be studied in the future by using monospecific antisera to the individual structural polypeptides.

All of these results emphasize the importance of a cautious interpretation (based on molecular weight determinations alone) of the origins and relatedness of the polypeptide bands found in virus preparations or in infected cells. For example, the 53,000-dalton polypeptide synthesized in cell-free protein synthesis systems and found in infected cells is not related to either VP5* or to the 53,000-dalton secondary structural cleavage product characterized in this study. It has been hypothesized (25, 27) that a polypeptide designated O_{1A} and observed in infected cells is a precursor of O_1 or VP5*, but the protease mapping patterns of the polypeptides in this study confirm the speculation of Espejo et al. (9) that these proteins are distinct. Biochemical mapping data (25; manuscript in preparation) showed that SA11 RNA segment 5 codes for the 53,000-dalton protein and that this is a nonstructural polypeptide found in infected cells (15). The recognition that VP4*, VP5*, and VP8* are cleavage products also explains the fact that they were not translated in vitro by SA11 mRNA (15) or SA11 denatured ds RNA (25).

The additional cleavage of the 94,000-dalton protein is of interest in that it suggests that either this inner capsid polypeptide is exposed after the removal of VP3 or it may extend into the outer layer of the virus capsid. Although the biological activity, if any, of this polypeptide remains unknown, the extension of an inner capsid protein through the outer capsid could explain the extensive cross-reactivities observed in serological assays (including neutralization and immunoelectron microscopic tests) between heterologous rotavirus types. A precedent for this possibility has been reported for the related reoviruses, in which the core λ2 protein is exposed on the reovirus particle surface (12).

The similarities between the biological significance of the cleavage of VP3 for rotavirus infectivity and the documented cleavage activation of the F and HA proteins of paramyxoviruses and myxoviruses, respectively, are striking (5). Besides the fact that the rotaviruses are not enveloped, the major distinction between the rotavirus system and these model systems is that rotavirus VP3 is not a glycoprotein and rotavirus outer capsid glycoproteins VP7 and VP7a show no alterations after protease treatment (based upon quantitative recovery and migration in sodium dodecyl sulfate-polyacrylamide gels). The significance of this cleavage for other rotavirus-specific biological functions (e.g., hemagglutination or virulence) remains to be determined. However, a postulated correlation between cleavage activation of infectivity and virulence may be relevant to prevention or therapy and may aid in attempts to cultivate the more fastidious rotavirus strains (including the human strains). The importance of cleavage activation for rotavirus growth will be confirmed if the reassortants able to rescue noncultivable human rotavirus (11) contain the bovine genome segment encoding VP3, which should also be cleaved to enhance infectivity.

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LITERATURE CITED

1. **Almeida, J. D., T. Hall, J. E. Banatvala, B. M. Totterdell, and I. L. Chrystie.** 1978. The effect of trypsin on the growth of rotavirus. J. Gen. Virol. **40**:213–218.
2. **Babiuk, L. A., K. Mohammed, L. Spence, M. Fauvel, and R. Petro.** 1977. Rotavirus isolation and cultivation in the presence of trypsin. J. Clin. Microbiol. **6**:610–617.
3. **Barnett, B. B., R. S. Spendlove, and M. K. Clark.** 1979. Effect of enzymes on rotavirus infectivity. J. Clin. Microbiol. **10**:111–113.
4. **Bridger, J. C., and G. N. Woode.** 1976. Characterization of two particle types of calf rotavirus. J. Gen. Virol. **31**: 245–250.
5. **Choppin, P. W., and A. Schied.** 1980. The role of viral glycoproteins in adsorption, penetration and pathogenicity of viruses. Rev. Infect. Dis. **2**:40–61.
6. **Clark, S. C., B. B. Barnett, and R. S. Spendlove.** 1979. Production of high-titer bovine rotavirus with trypsin. J. Clin. Microbiol. **9**:413–417.
7. **Cleveland, D. W., S. G. Fischer, M. W. Kirschner, and U. K. Laemmli.** 1977. Peptide mapping by limited proteolysis in sodium dodecyl sulfate and analysis by gel electrophoresis. J. Biol. Chem. **252**:1102–1106.
8. **Cohen, J., J. LaPorte, A. Charpilienne, and R. Scherrer.** 1979. Activation of rotavirus RNA polymerase by calcium chelation. Arch. Virol. **60**:177–186.
9. **Espejo, R. T., S. Lopez, and C. Arias.** 1981. Structural polypeptides of simian rotavirus SA11 and the effect of trypsin. J. Virol. **37**:156–160.
10. **Graham, D., and M. K. Estes.** 1980. Proteolytic enhancement of rotavirus infectivity: biologic mechanisms. Virology **101**:432–439.
11. **Greenberg, H. B., A. R. Kalica, R. G. Wyatt, R. W. Jones, A. Z. Kapikian, and R. M. Chanock.** 1981. Rescue of noncultivatable human rotavirus by gene reassortment during mixed infection with ts mutants of a cultivatable bovine rotavirus. Proc. Natl. Acad. Sci. U.S.A. **78**:420–424.
12. **Hayes, E. C., P. W. K. Lee, S. E. Miller, and W. K. Joklik.** 1981. The interaction of a series of hybridoma IgGs with reovirus particles. Virology **108**:147–155.
13. **Kalica, A. R., and T. S. Theodore.** 1979. Polypeptides of simian rotavirus (SA-11) determined by a continuous polyacrylamide gel electrophoresis method. J. Gen. Virol. **43**:463–466.
14. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4.

Nature (London) **227**:680–685.

15. **Mason, B. B., D. Y. Graham, and M. K. Estes.** 1980. In vitro transcription and translation of simian rotavirus SA11 gene products. J. Virol. **33**:1111–1121.
16. **Matsuno, S., and A. Mukoyama.** 1979. Polypeptides of bovine rotavirus. J. Gen. Virol. **43**:309–316.
17. **McNulty, M. S.** 1979. Morphology and chemical composition of rotaviruses, p. 111–139. *In* F. Bricoat and R. Scherrer (ed.), Viral enteritis in humans and animals. Colloque INSERM, vol. 90. Institut National de la Santé et de la Recherche Médicale, Paris.
18. **Monette, J. P. L., M. M. Carpio, R. A. Kapitany, and L. A. Babiuk.** 1979. Polypeptide analysis of bovine rotavirus, p. 249–262. *In* S. D. Acres (ed.), Veterinary Infectious Disease Organization Proceedings of the Second International Symposium on Neonatal Diarrhea. Saskatoon, Canada.
19. **Newman, J. F. E., F. Brown, J. C. Bridger, and G. N. Woode.** 1975. Characterisation of a rotavirus. Nature (London) **258**:631–633.
20. **Obijeski, J. F., E. L. Palmer, and M. L. Martin.** 1977. Biochemical characterization of infantile gastroenteritis (IGV). J. Gen. Virol. **34**:485–497.
21. **Rodger, S. M., R. D. Schnagl, and I. H. Holmes.** 1975. Biochemical and biophysical characteristics of diarrhea viruses of human and calf origin. J. Virol. **16**:1229–1235.
22. **Rodger, S. M., R. D. Schnagl, and I. H. Holmes.** 1977. Further biochemical characterization, including the detection of surface glycoproteins, of human, calf, and simian rotaviruses. J. Virol. **24**:91–98.
23. **Sharp, D. G.** 1974. Physical assay of purified viruses, particularly the small ones, p. 264–265. *In* C. J. Arceneaux (ed.), Proceedings of the 32nd Annual Meeting of the Electron Microscopy Society of America. Claitor's Publishing Div., Baton Rouge, La.
24. **Smith, E. M., M. K. Estes, D. Y. Graham, and C. P. Gerba.** 1979. A plaque assay for the simian rotavirus SA11. J. Gen. Virol. **43**:515–521.
25. **Smith, N. L., I. Lazdins, and I. H. Holmes.** 1980. Coding assignments of double-stranded RNA segments of SA11 rotavirus established by in vitro translation. J. Virol. **33**:976–982.
26. **Theil, K. W., E. H. Bohl, and A. G. Agnes.** 1977. Cell culture propagation of porcine rotavirus (reovirus-like agent). Am. J. Vet. Res. **38**:1765–1768.
27. **Thouless, M. E.** 1979. Rotavirus polypeptides. J. Gen. Virol. **44**:187–197.
28. **Todd, D., and M. S. McNulty.** 1977. Biochemical studies on a reovirus-like agent (rotavirus) from lambs. J. Virol. **21**:1215–1218.