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# Trypsin Activation Pathway of Rotavirus Infectivity

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**The infectivity of rotaviruses is increased by and most probably is dependent on trypsin treatment of the virus. This proteolytic treatment specifically cleaves VP4, the protein that forms the spikes on the surface of the virions, to polypeptides VP5 and VP8. This cleavage has been reported to occur in rotavirus SA114fM at two conserved, closely spaced arginine residues located at VP4 amino acids 241 and 247. In this work, we have characterized the VP4 cleavage products of rotavirus SA114S generated by in vitro treatment of the virus with increasing concentrations of trypsin and with proteases AspN and α-chymotrypsin. The VP8 and VP5 polypeptides were analyzed by gel electrophoresis and by Western blotting (immunoblotting) with antibodies raised to synthetic peptides that mimic the terminal regions of VP4 generated by the trypsin cleavage. It was shown that in addition to arginine residues 241 and 247, VP4 is cleaved at arginine residue 231. These three sites were found to have different susceptibilities to trypsin, Arg-241 > Arg-231 > Arg-247, with the enhancement of infectivity correlating with cleavage at Arg-247 rather than at Arg-231 or Arg-241. Proteases AspN and α-chymotrypsin cleaved VP4 at Asp-242 and Tyr-246, respectively, with no significant enhancement of infectivity, although this enhancement could be achieved by further treatment of the virus with trypsin. The VP4 end products of trypsin treatment were a homogeneous VP8 polypeptide comprising VP4 amino acids 1 to 231 and a heterogeneous VP5, which is formed by two polypeptide species (present at a ratio of approximately 1:5) as a result of cleavage at either Arg-241 or Arg-247. A pathway for the trypsin activation of rotavirus infectivity is proposed.**

Rotaviruses are the single most important etiologic agents of severe dehydrating infantile gastroenteritis in developed and developing countries (18). These viruses are formed by three concentric layers of proteins that enclose a genome of 11 segments of double-stranded RNA (36). The outermost layer is formed by two proteins, VP4 (776 amino acids) and VP7 (a glycoprotein of 326 amino acids) (12). VP7 forms the smooth external surface of mature virions, while dimers of VP4 form spikes that extend from this surface (2, 31). VP4 has essential functions in the viral life cycle, including receptor binding and cell penetration (12). The properties of this protein are therefore important determinants of host range, virulence, and induction of protective immunity.

It has been known for some time that the infectivity of rotaviruses is increased by, and most probably is dependent on, trypsin treatment of the virus (1, 5, 7, 37), and it has been shown that this proteolytic treatment results in the specific cleavage of VP4 to polypeptides VP8 (28 kDa) and VP5 (60 kDa) (8, 11, 13), which represent the amino- and carboxy-terminal regions of the protein, respectively (22). The cleavage of VP4 proceeds with a concomitant enhancement of viral infectivity (11, 13). However, it does not affect cell binding (8, 16, 17); rather, it has been associated with entry of the virus by direct cell membrane penetration (17, 28, 35).

We have previously shown by direct amino acid sequence analysis that during activation of the infectivity of rotavirus SA114fM by trypsin, the VP4 protein is cleaved at arginines 241 and 247, generating two VP5 polypeptide species that differ by 6 amino acids at their amino termini and are present in a 1:5 (Arg-241/Arg-247) ratio in what appear to be the end products of the digestion (22). These two arginine residues are conserved in all rotavirus VP4 sequences analyzed, although in some rotavirus strains the arginine at position 247 is either replaced by a lysine residue or located at position 246 (23, 25). In addition to these two arginines, an arginine residue at position 231 of VP4 is conserved in the great majority of the rotavirus strains studied (25). This arginine represents a potential cleavage site for trypsin that would not have been identified by the $NH_2$-terminal sequence of rotavirus SA114fM VP5, since cleavage at arginines 241 and 247 would have removed the potential $NH_2$ terminus generated by cleavage at Arg-231 (3, 24).

The mechanism of activation of rotavirus infectivity that leads to virus penetration is not known, although it is believed that the penetration of the virus may be triggered by the terminal regions newly generated by the trypsin cleavage of VP4 or by a possible conformational change in the VP4 molecule resulting from this cleavage (22). To further the understanding of this mechanism, it is important to identify all sites in VP4 that are cleaved by trypsin and to determine which of these sites is (are) directly associated to the enhancement of infectivity. In this work we have shown that in addition to arginines 241 and 247, Arg-231 is cleaved during trypsin activation of three different simian rotavirus strains, and we have found that the three cleavage sites have a different susceptibility to the protease. We have also shown that in rotavirus SA114S, the cleavage at Arg-241 is not sufficient for the enhancement of infectivity, which, instead, seems to correlate with cleavage at Arg-247.

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## MATERIALS AND METHODS

**Viruses and cells.** Rotavirus RRV was obtained from H. B. Greenberg, Stanford University, Stanford, Calif.; rotavirus SA114S (clone 3) was provided by M. K. Estes, Baylor College of Medicine, Houston, Tex.; rotavirus SA114fM was originally obtained from H. H. Malherbe, University of Texas. These three rotavirus strains are of simian origin; however, strain SA114fM has a VP4 gene that is more closely related to that of the bovine rotavirus Nebraska calf diarrhea virus (29). All rotavirus strains were propagated in MA104 cells as described previously (10).

**Virus labeling and purification.** The viruses were labeled with [$^{35}$S]methionine and purified by CsCl isopycnic centrifugation as described previously (11). To obtain unlabeled virus for Western blot (immunoblot) analysis, the virus-infected cells were harvested after the full cytopathic effect had been attained, the cell lysate was extracted with Freon, and the virus was pelleted through a 1-ml cushion of 30% sucrose in 20 mM Tris-HCl (pH 8.2)–1 mM $MgCl_2$–150 mM NaCl–10 mM $CaCl_2$ by centrifugation for 30 min at 80,000 rpm at 4°C in the TLA100.4 rotor of the tabletop Beckman Optima ultracentrifuge. The virus pellet was resuspended in the same buffer as described above and kept at 4°C until use. To obtain trypsin uncleaved labeled or unlabeled virus, the cells were washed four times with phosphate-buffered saline (pH 7.2) after adsorption of the trypsin-treated virus, and the virus preparation was kept on ice at all times during harvesting.

**Polyacrylamide gel electrophoresis.** The different species of VP5 generated by trypsin treatment of [$^{35}$S]methionine-labeled virus were resolved by electrophoresis in sodium dodecyl sulfate (SDS)–6% polyacrylamide gels with 4% cross-linkage (acrylamide-to-bisacrylamide ratio, 24:1 [wt/wt]), using the discontinuous buffer system of Laemmli as described previously (21). After electrophoresis, the gels were treated for fluorography and exposed to film at −70°C.

**Protease treatments.** Aliquots of the unlabeled virus were digested with various concentrations of diphenylcarbamyl chloride-treated trypsin (Sigma Chemical Co.) or AspN (Boehringer Mannheim; sequencing grade) for 30 min at 37°C. Each aliquot was then split into six portions. Five of these portions were used for Western blot analysis, and the sixth was used to determine the infectivity of the virus, as described previously (26). In some cases, the AspN-digested viruses were further digested with trypsin for 30 min at 37°C, as indicated. The [$^{35}$S]methionine-labeled viruses were digested with trypsin, AspN, or α-chymotrypsin (Boehringer Mannheim; sequencing grade) under the same conditions described above.

**Peptides and production of antipeptide sera.** Peptides were obtained from Research Genetics, Huntsville, Ala., and coupled to maileimide-activated keyhole limpet hemocyanin (KLH) (Pierce, Rockford, Ill.) as directed by the manufacturer. Four peptides were synthesized, representing amino acids 232 to 241 (CNVVPLSLTAR; peptide 1), 242 to 258 (CDVIHYRAQANEDIVISK; peptide 2), 248 to 265 (AQANEDIVISKTSLWKEMC; peptide 3), and 216 to 231 (CTEYINNGLPPIQNTR; peptide 4) of SA114S VP4 (see Fig. 3A). Peptides were synthesized with an extra cysteine (underlined in the peptide sequences above) at the amino (peptides 1 and 2) or carboxy (peptide 3) terminus for coupling to KLH. For peptide 4, Cys-216 originally present in the VP4 sequence was used for coupling.

For the primary immunization, rotavirus-negative BALB/c mice, 7 to 9 weeks of age, were immunized intraperitoneally with 100 μg of KLH-coupled peptide in Freund's complete adjuvant. Three booster injections were given subcutaneously, at 2-week intervals, with the same amount of KLH-coupled peptide emulsified in Freund's incomplete adjuvant. The mice were bled after the third and fourth immunizations.

**Western blot analysis.** For immunoblot analysis, the viral proteins were separated by electrophoresis in SDS–11% polyacrylamide gels (acrylamide-to-bisacrylamide ratio, 30:0.8 [wt/wt]), transferred to nitrocellulose, and incubated with the indicated dilutions of mouse antipeptide sera or monoclonal antibody (MAb) HS2 (30), kindly provided by L. Padilla-Noriega, Universidad Nacional Autónoma de México, Cuernavaca, Mexico, as previously described (4). The second antibody was peroxidase-labeled goat anti-mouse immunoglobulin G (IgG; Boehringer Mannheim) diluted 1:1,000. Antigen-antibody complexes were developed by using a chemiluminescent assay system (Amersham) and quantified by densitometry.

## RESULTS

**Analysis of the VP4 trypsin cleavage products by high-cross-linkage gels.** During the activation of infectivity of rotavirus SA114fM by trypsin, VP4, the spike protein of the virus, is cleaved at two conserved, closely spaced arginine residues located at positions 241 and 247 (22). To determine if VP4 is also cleaved at a conserved arginine residue located at position 231, [$^{35}$S]methionine-labeled rotavirus SA114fM, produced under conditions where most VP4 molecules are not cleaved, was treated in vitro with increasing concentrations of trypsin and the viral proteins were analyzed by gel electrophoresis with high-cross-linkage gels (6% polyacrylamide with 4% cross-linkage). This type of gel had been previously used by Liu et al. to resolve high-molecular-weight rotavirus proteins (21), and their experiments suggested that the VP5 species resulting from cleavage of VP4 at different arginines could also be resolved.

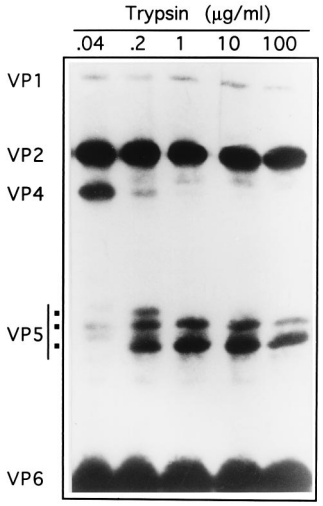


FIG. 1. Analysis of the VP5 polypeptides generated by treatment of rotavirus SA114fM with trypsin. [$^{35}$S]methionine-labeled purified virus particles were treated for 30 min at 37°C with the indicated concentrations of trypsin, and the proteins were separated by electrophoresis in 6% polyacrylamide gels with 4% cross-linkage. After electrophoresis, the gels were treated by fluorography and exposed to film at −70°C. The positions of the viral proteins are indicated on the left. The three putative VP5 species, the result of cleavage of VP4 at arginine residues 231, 241, and 247, are labeled with dots.

When the virus was treated with 0.2 μg of trypsin per ml, three bands in the region of the gel where VP5 is expected to migrate were observed (Fig. 1); upon treatment with increasing concentrations of the protease, the more slowly migrating band disappeared while the other two bands remained even at trypsin concentrations as high as 100 μg/ml. These three bands were tentatively identified as the VP5 products generated by cleavage of VP4 at arginines 231, 241, and 247. The ratio of the VP5 protein bands that putatively correspond to VP4 cleavage at arginines 241 and 247 was about 1:5 in virus treated with 1 μg of trypsin per ml or higher. This is the ratio previously found for these two VP5 products present in virus treated with 100 μg of trypsin per ml (22). These observations suggest that the two faster-migrating bands might indeed correspond to VP5-241 and VP5-247.

The putative different species of VP5 are most probably separated in the gel because of differential residual secondary structure of the proteins rather than because of differences in their molecular weight, since their migration does not correspond to that expected from their molecular weights. In this type of gel, the VP8 polypeptide runs off the gel.

It has been previously reported that treatment of rotavirus SA114fM with α-chymotrypsin generates two VP4 cleavage products that are very similar to those produced by trypsin treatment (11). Analysis of the amino acid sequence around the trypsin cleavage region (TCR) of SA114fM VP4 shows that there is only one amino acid in this region, a tyrosine residue at position 245, susceptible to cleavage by α-chymotrypsin (Fig. 2A); therefore, treatment of SA114fM with this enzyme should produce a single VP5 product, the result of cleavage at Tyr-245. As expected, when the virus was treated with α-chymotrypsin, a single band which migrated between the putative VP5-241 and VP5-247 species generated by trypsin was observed in the VP5 region of the gel (Fig. 2B).

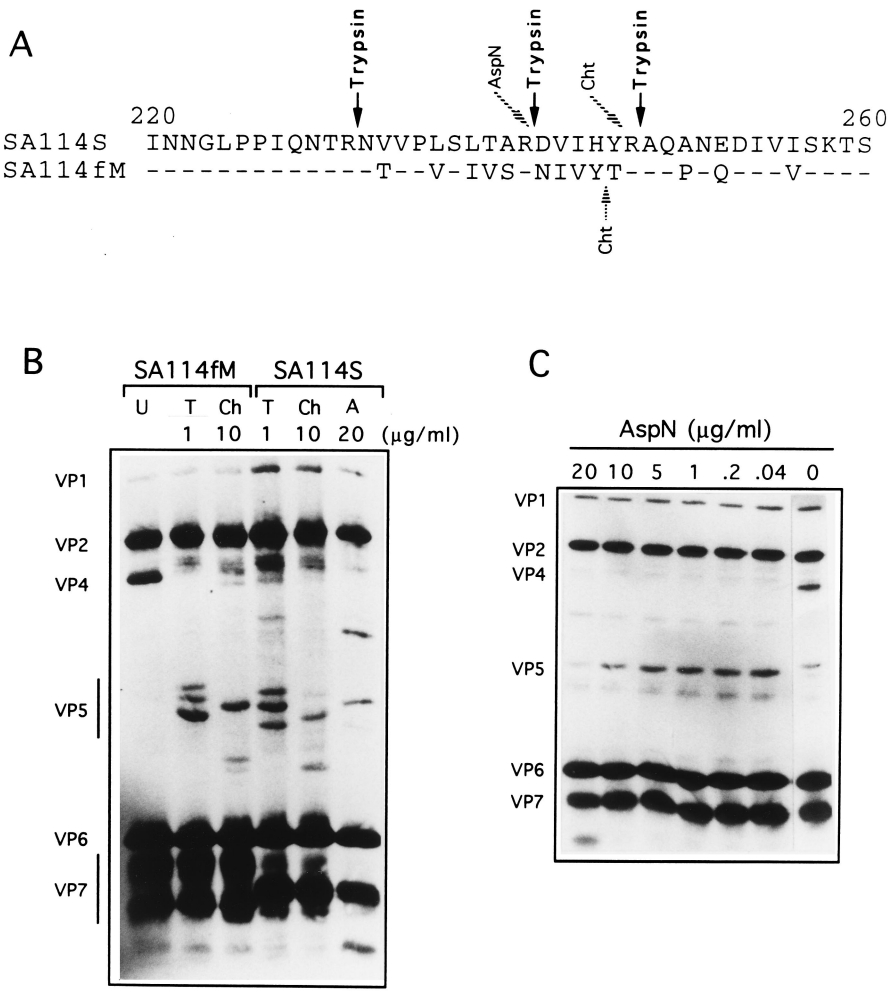


FIG. 2. Analysis of rotavirus VP4 protein with proteases having different specificities. (A) Comparison of the amino acid sequence around the VP4 trypsin cleavage region of rotavirus strains SA114S and SA114fM. Only amino acids that differ from the SA114S sequence are shown. The arrows indicate the site of cleavage of the proteases; solid arrows indicate that both VP4 proteins are cleaved, while striped arrows indicate that only one of the proteins is cleaved. The sequences of SA114S and SA114fM are from references 29 and 22, respectively. (B and C) Electrophoretic analysis of [$^{35}$S]methionine-labeled SA114S and SA114fM (B) or SA114S (C) virus particles, untreated (U) or after treatment for 30 min at 37°C with the indicated concentrations of either trypsin (T), α-chymotrypsin (Ch), or AspN (A). The concentration of trypsin needed to generate the three putative VP5 species in rotavirus SA114fM was different in this experiment from that shown in Fig. 1. This is probably because two different SA114fM virus preparations were used in these two experiments. The proteins were separated in 6% polyacrylamide gels with 4% cross-linkage. The positions of the viral proteins are indicated on the left.

The simian rotavirus SA114S VP4 protein is 83% identical to rotavirus SA114fM VP4 (27, 29). In addition to the three conserved arginines, it has in the TCR a tyrosine at position 246, and an aspartic acid at position 242 (Fig. 2A). These two amino acids should be cleaved by α-chymotrypsin and AspN, respectively, generating single VP5 species. AspN should generate a VP5 product identical to that generated by trypsin cleavage at VP4 Arg-241, since AspN specifically hydrolyzes peptide bonds located amino terminal to aspartic acid. When SA114S was treated with trypsin, three protein bands in the VP5 region were observed (Fig. 2B), as in the case of SA114fM VP4. On the other hand, treatment with α-chymotrypsin generated a single VP5 polypeptide that migrated between the putative VP5-241 and VP5-247 bands while, as expected, AspN produced a band that migrated at the same position as the putative VP5-241 trypsin product (Fig. 2B). At the AspN concentration used (20 μg/ml), the enzyme seems to cleave VP4 at sites different from Asp-242, resulting in a faint VP5 band migrating in the VP5-241 position; however, at lower concentrations (as low as 0.04 μg/ml), VP5-241 is the main VP4 product (Fig. 2C). The bands other than VP5, generated by treatment with AspN or α-chymotrypsin, probably represent cleavage products of either VP4 or the other high-molecular-weight rotavirus proteins, as has previously been described (11, 13). Taken together, all these results suggest that the gel electrophoresis under the conditions used was able to resolve the VP5 protein species generated by cleavage at different positions in the VP4 TCR and also suggest that the arginine at position 231 of VP4 is cleaved by trypsin during the activation of the infectivity of both simian rotavirus strains SA114fM and SA114S.

**Analysis of the trypsin cleavage region with antipeptide antibodies.** To confirm the findings described above and to try to elucidate the VP4 trypsin cleavage pathway that leads to the enhancement of rotavirus infectivity, we synthesized peptides that correspond to the VP4 termini newly generated by trypsin treatment of rotavirus SA114S (Fig. 3A). Antibodies to these peptides were raised in mice. The anti-peptide antibodies were used in Western blot assays (with standard 11% polyacrylamide gels) to detect the processing of SA114S VP4 when the

virus was treated with increasing amounts of trypsin. In addition, AspN was used, either alone or followed by trypsin treatment. A single preparation of SA114S was used for the experiments shown in Fig. 3B to 3F. Aliquots of the virus were digested with the different enzymes, and each aliquot was then split into six portions. Five of these portions were used for the immunoblot analysis, while the sixth portion was used to determine the infectivity of the virus (see Fig. 4). In addition to the four antipeptide sera, MAb HS2, which recognizes VP4 and its product VP5, was used in the immunoblot assays. The results obtained with the various antibodies were as follows.

**(i) MAb HS2.** MAb HS2 efficiently recognized VP4 as well as VP5 in the blot assay, and it allowed us to monitor the cleavage of VP4. As can be seen in Fig. 3B, in the range of trypsin concentrations used, the VP4 polypeptide was gradually cleaved until essentially all the protein was processed at a trypsin concentration of 25 μg/ml. On the other hand, protease AspN at 1 μg/ml cleaved most but not all of VP4, although the protein was completely cleaved upon subsequent incubation with 10 μg of trypsin per ml. Despite growth and harvesting of the virus under conditions designed to avoid the cleavage of VP4, a small amount of this protein was already processed into VP5 and VP8 in the untreated virus (Fig. 3B, C, and E, lanes 0 trypsin).

As can be seen in Fig. 3B and Figs. D to F, the VP5 polypeptide migrates as a doublet. This doublet, however, does not seem to be the result of the differential cleavage at the susceptible arginines in the TCR, since it is also observed in the virus treated with AspN, an enzyme that cleaves at a single site in the SA114S VP4 TCR (Fig. 2). In addition, the proportion of the two bands in the doublet remained constant in the virus treated with the various concentrations of trypsin or with AspN compared with untreated virus. The origin of this doublet is not clear, but the facts that VP4 is also detected as a doublet (results not shown, and barely seen in Fig. 3) and that VP8 is observed as a single band (see below) suggest that the VP4 in the virus preparation used had a heterogeneous carboxy-terminal end.

**(ii) Antibodies to peptide 4.** Antibodies to peptide 4 (VP4 amino acids 216 to 231) efficiently recognized VP4 and, as expected, VP8 but not VP5. Of interest, upon incubation of the virus with increasing concentrations of trypsin, the electrophoretic mobility of VP8 gradually shifted to a faster-migrating form compared with the migration of VP8 detected in untreated virus or virus treated with 0.04 μg of the protease per ml (Fig. 3C). This shift seemed to be essentially complete at 1 μg of trypsin per ml. Treatment with AspN produced a VP8 that migrated at a similar position to the VP8 present in untreated virus or in virus treated with low concentrations of trypsin, and this polypeptide was further cleaved by trypsin to produce the faster-migrating form of VP8. Since AspN cleaves VP4 at Asp-242, the further processing of the AspN-produced VP8 polypeptide by trypsin indicates that Arg-231 is cleaved by this enzyme; in addition, these results indicate that Arg-241 is more susceptible to trypsin cleavage than is Arg-231.

**(iii) Antibodies to peptide 3.** Antibodies to peptide 3 (VP4 amino acids 248 to 265) interacted very poorly with uncleaved VP4, although they recognized VP5 fairly well. However, the efficient interaction of these antibodies with VP5 seemed to depend on the protein having been cleaved at Arg-247, since the recognition of the VP5-241 polypeptide produced by cleavage of VP4 with AspN was rather poor (Fig. 3D) and was greatly enhanced by further incubation of the AspN-treated virus with trypsin.

At 1 μg of trypsin per ml, about 90% of VP4 is already cleaved into VP5 and VP8 (Fig. 3B), and most of VP8 seems to have already been cut at Arg-231 (Fig. 3C); however, the recognition signal of VP5 by anti-peptide 3 antibodies kept increasing with increasing concentrations of trypsin, suggesting that VP5-241 is being further cleaved at Arg-247. The cleavage at Arg-247 probably proceeds until reaching what seems to be the terminal VP5 cleavage products, consisting of about 20% of VP5-241 and 80% of VP5-247 (22) (Fig. 1).

**(iv) Antibodies to peptide 2.** A synthetic hexapeptide corresponding to amino acids 242 to 247 did not elicit a detectable antibody response; therefore, to induce antibodies directed to this region we used a longer peptide comprising amino acids 242 to 258 of VP4 (peptide 2). The anti-peptide 2 serum recognized both VP4 and VP5 reasonably well. The recognition of VP5 increased with increasing concentrations of trypsin, up to 1 μg/ml, and then decreased at higher concentrations. This decrease seems to be due to cleavage of VP5 at Arg-247, since the VP5 generated by treatment of the virus with AspN was efficiently recognized and this signal was very much diminished when the AspN-treated virus was subsequently digested with trypsin, which should further cleave VP5 at Arg-247 (Fig. 5D). Thus, the anti-peptide 2 antibodies seem to recognize VP5-241 much better than VP5-247. The preferential recognition of VP5-241 and VP5-247 by anti-peptide 2 and anti-peptide 3 antibodies, respectively, provided us with an excellent tool to analyze the kinetics of cleavage at these sites.

**(v) Antibodies to peptide 1.** Peptide 1 (VP4 amino acids 232 to 241) mimics the VP4 peptide that would be released from VP4 by trypsin cleavage at arginines 231 and 241. Since the data obtained with the other anti-peptide sera suggested that the initial cleavage of VP4 by trypsin is at Arg-241, we expected the anti-peptide 1 antibodies to detect VP8 when the virus was treated with trypsin concentrations lower that 1 μg/ml. However, VP8 was not recognized, and at trypsin concentrations ranging from 0.2 to 5 μg/ml, a weak signal with VP5 was found instead (Fig. 3F). The fact that the anti-peptide 1 serum recognized VP5 shows conclusively that trypsin can cleave VP4 at Arg-231 and also indicates that this cleavage can be the first to occur. Since the data with the anti-peptide 4 serum indicate that at low trypsin concentrations the first cleavage occurs at position 241, we interpret the signal in VP5 with the anti-peptide 1 serum as being because a proportion (probably minor) of VP4 molecules are cleaved first at 231 and are not further processed at positions 241 and 247 unless high concentrations of trypsin (25 μg/ml) are used.

The lack of reactivity of the anti-peptide 1 serum with VP8 is not because it cannot recognize the peptide when present in the carboxy terminus of VP8, since this serum reacts with VP8-241 produced by treatment of the virus with AspN (Fig. 3F, lane A). The absence of reactivity with the VP8 generated by the various concentrations of trypsin is most probably because although the first cleavage at low trypsin concentrations seems to be mainly at Arg-241, VP8 is rapidly cleaved at position 231 (as can be seen in Fig. 3C), preventing the VP8 from being recognized by the anti-peptide 1 serum. Also, the amount of 241-VP8 present in the untreated virus or in virus treated with 0.04 or 0.2 μg of trypsin per ml (Fig. 3B) is probably too little to be detected by this serum.

**Correlation between VP4 cleavage and enhancement of infectivity.** To test if the processing of VP4 with the different proteases could be correlated with the enhancement of infectivity, the infectious titer of virus samples corresponding to those analyzed by Western blotting was determined. The infectivity of the virus increased gradually when the virions were treated with increasing concentrations of trypsin, reaching a 23-fold increase when the virus was incubated with 25 μg of trypsin per ml (Fig. 4). This degree of activation indicates that

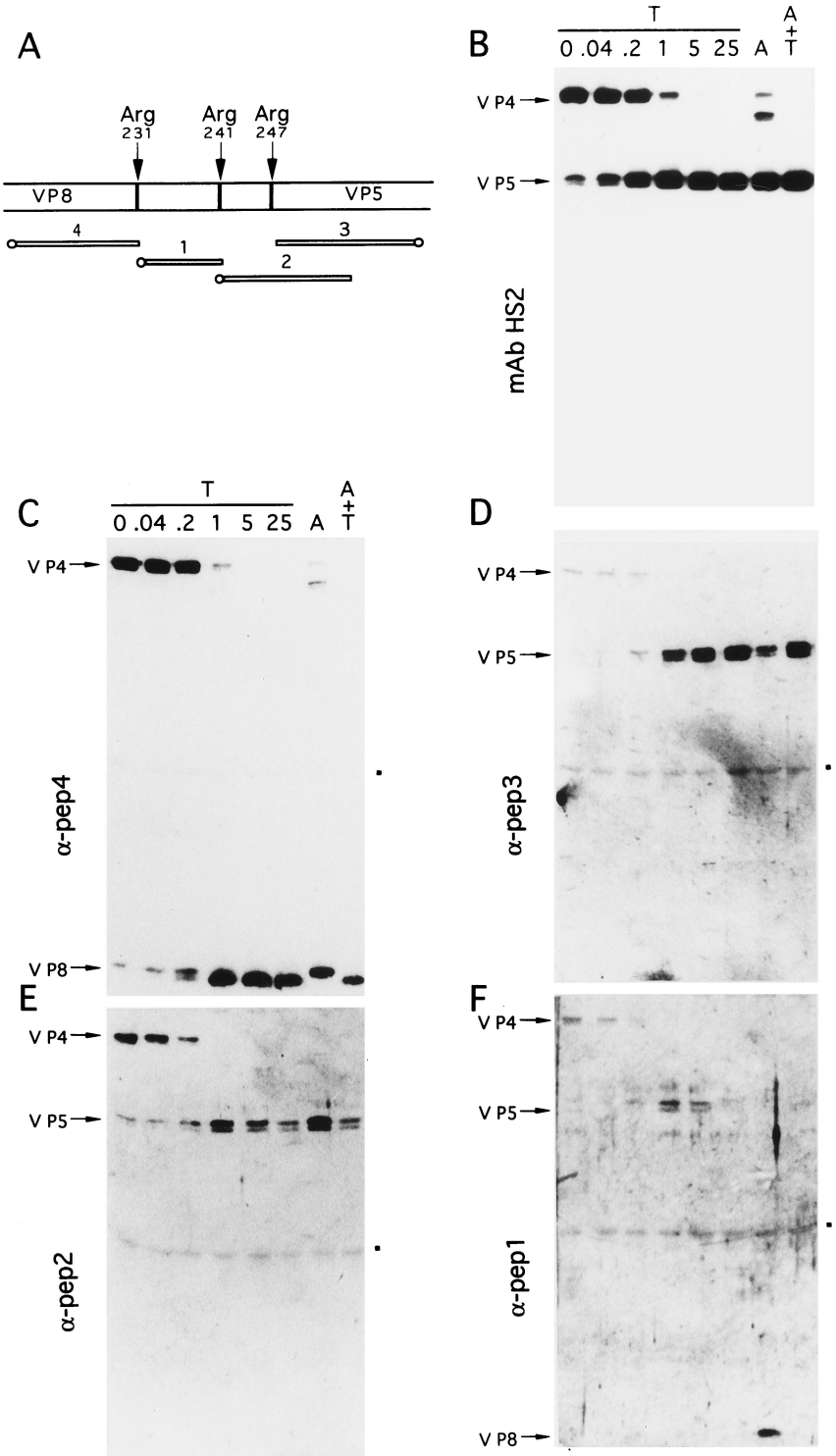


FIG. 3. Immunoblot analysis of rotavirus SA114S VP4 cleavage products. (A) Schematic representation of the trypsin cleavage region of rotavirus SA114S. The three conserved arginines are shown. The synthetic peptides that mimic the termini newly generated by trypsin treatment of the virus are indicated by open bars; the circle at one end of the bar indicates the position of the cysteine that was added for coupling the peptide to KLH. Antibodies to these peptides were produced in mice. (B to F) A single preparation of SA114S was used. Aliquots of the virus were digested for 30 min at 37°C with the indicated concentrations of trypsin (T) or 1 μg of AspN per ml (A). In addition, a portion of the virus treated with AspN was further digested with 10 μg of trypsin per ml for 30 min at 37°C. Each aliquot was then split into six equal portions; five of these portions were used for the immunoblot analysis with the antipeptide (α-pep) sera and the VP5 MAb HS2, as indicated, while the sixth portion was used to determine the infectivity of the virus (Fig. 4). Equal amounts of virus proteins were loaded in each gel; the different signals obtained for VP4, VP5, and VP8 are due to the differential recognition of these polypeptides by the various antibodies used. The virus proteins were separated by electrophoresis in standard SDS–11% polyacrylamide gels and transferred to nitrocellulose paper. The transferred proteins were incubated with serum to either peptide 1 (diluted 1:100), peptide 2 (diluted 1:500), peptide 3 (dilute 1:300), peptide 4 (diluted 1:1,000), or MAb HS2 (diluted 1:1,000), and the bound antibody was identified with a 1:1,000 dilution of a peroxidase-labeled goat anti-mouse immunoglobulin G, using a chemiluminescent substrate. The positions of the VP4 protein and its cleavage products VP5 and VP8 are indicated on the left. The weak band labeled with a dot in panels C to F corresponds to background binding to VP6, the most abundant protein of the virus.

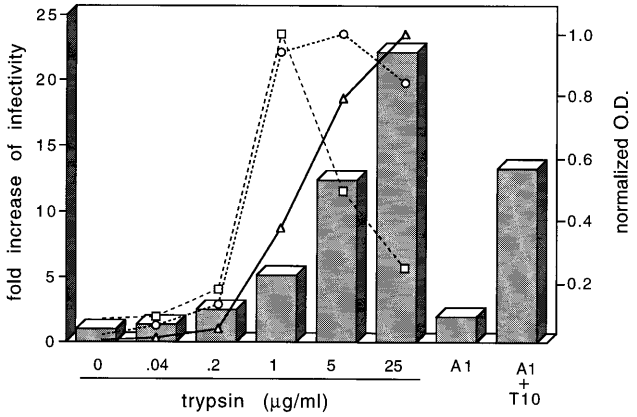


FIG. 4. Correlation between trypsin cleavage of VP4 and infectivity of rotavirus SA114S. The sixth sample of each aliquot, obtained as described in the legend to Fig. 3, was used to determine the infectivity of the virus by an immunoperoxidase focus assay (26). The vertical bars represent the fold increase in infectivity obtained with the various protease treatments. The basal infectivity (no trypsin treatment) used as the reference unit was $1.35 \times 10^7$ focus-forming units/ml. Also shown in this graph are the results of the densitometric analysis of the VP5 and VP8 bands obtained by incubation with the antipeptide 2, 3, and 4 sera in the immunoblot experiments shown in Fig. 3. The optical density (O.D.) was determined and normalized in each case to the highest signal obtained with a given antipeptide serum, and it was plotted against the trypsin concentration used to digest the virus. Symbols: ○, O.D. of antipeptide 4; △, O.D. of antipeptide 3; □, O.D. of antipeptide 2.

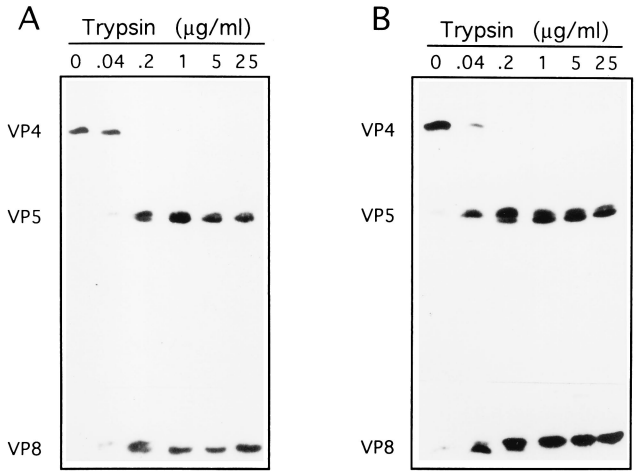


FIG. 5. Immunoblot analysis of the VP4 cleavage products of rotavirus strains SA114fM (A) and RRV (B). Virus particles were digested for 30 min at 37°C with the indicated concentrations of trypsin, and the virus proteins were separated by electrophoresis in standard SDS–11% polyacrylamide gels and transferred to nitrocellulose paper. The transferred proteins were incubated with a mixture of a 1,000-fold dilution of each antipeptide 4 serum and MAb HS2, and the bound antibody was identified as described in the legend to Fig. 3. The positions of the VP4 protein and its cleavage products VP5 and VP8 are indicated on the left.

at least 95% of the activable virions were not infectious before the cleavage of VP4. The infectivity of SA114S was not significantly enhanced by treatment with 1 μg of protease AspN per ml (twofold increase); however, cleavage with this protease did not preclude that the infectivity of the virus was augmented by subsequent treatment with trypsin (Fig. 4). In a separate experiment, the infectivity of SA114S was increased only 1.5-fold by treatment with 2 μg of α-chymotrypsin per ml; however, the infectivity of the chymotrypsin-treated virus was enhanced 12-fold by further treatment with 10 μg of trypsin per ml (data not shown). These results indicate that the additional cleavages observed in VP4, or the other viral proteins, in virus treated with either AspN or α-chymotrypsin (Fig. 2) do not inactivate the virus. The fact that AspN only marginally increased the infectivity of the virus indicates that cleavage at Arg-241 is not directly responsible for the 23-fold full enhancement of infectivity achieved with trypsin.

To determine whether cleavage of VP4 at one or more of the conserved arginines in the TCR could be correlated with the increase in infectivity, we carried out a densitometric analysis of the VP8 and VP5 products identified with the various antipeptide sera. The data obtained were normalized and plotted together with the fold increase in infectivity induced by the various trypsin concentrations (Fig. 4). For the interpretation of these data, we assumed that the antipeptide 2 and antipeptide 3 sera recognize mainly VP5-241 and VP5-247, respectively. This assumption seems to be justified, since by densitometry, each of these two VP5 polypeptides was recognized by the corresponding serum with an efficiency 7- to 10-fold higher than that achieved with the other serum. For the antipeptide 4 serum, the densitometric analysis was done on VP8-231. As can be seen, the level of VP8-231 reaches a maximum after treatment with trypsin 1 μg/ml and remains constant thereafter whereas the level of VP5-241 also reaches a maximum at this concentration but then declines steadily at higher trypsin concentrations. On the other hand, the level of VP5-247 keeps increasing with the increasing concentrations of trypsin, as the infectivity of the virus does. As shown by the reactivity with MAb HS2, it is clear that essentially all VP4 has been cleaved at 5 μg of trypsin per ml (Fig. 3B), although the infectivity still increases at 25 μg/ml (Fig. 4). These data strongly suggest that the cleavage directly responsible for the activation of infectivity is the one that occurs at Arg-247.

**VP4 cleavage of rotaviruses RRV and SA114fM.** To test if the VP4 trypsin cleavage pattern observed for SA114S was similar for other rotavirus strains, we analyzed rotaviruses SA114fM and RRV by immunoblot analysis with the antipeptide 4 serum and MAb HS2. In both strains, Arg-241 also seems to be the initial trypsin cleavage site, since the electrophoretic mobility of VP8 changed when the viruses were treated with increasing concentrations of trypsin (Fig. 5).

For rotavirus SA114fM, we could not detect any cleavage products of VP4 in the untreated virus, and the pattern of appearance of VP8 was very much like that of SA114S. A single band was observed at 0.04 μg of trypsin per ml, and then it shifted to a faster-migrating form, such that at 1 μg/ml the shift was complete. In the case of RRV, the mobility of VP8 decreased upon incubation of the virus with higher concentrations of trypsin compared with that of the VP8 band present in untreated virus or virus treated with 0.04 μg of trypsin per ml, which probably reflects a change in the secondary structure of the protein. In this virus, the sequential cleavage of VP4 at arginines 231 and 241 seems more evident than in SA114S, since at 0.04 μg of trypsin per ml, essentially all VP4 has been cleaved whereas only about 10% of VP8 seems to have been processed at Arg-231.

VP5 was observed as a doublet in both SA114fM and RRV; however, in this case, the doublet seems to be the result of trypsin cleavage, since (i) the relative abundance of the two bands changed with increasing concentrations of trypsin in both viruses; and (ii) for SA114fM, digestion with α-chymotrypsin (which cleaves at Tyr-245) of the same virus preparation shown in Fig. 5 produced a VP5 polypeptide that migrated as a single band (results not shown). A VP5 band with a slower mobility (putatively 241-VP5) appeared first and then shifted

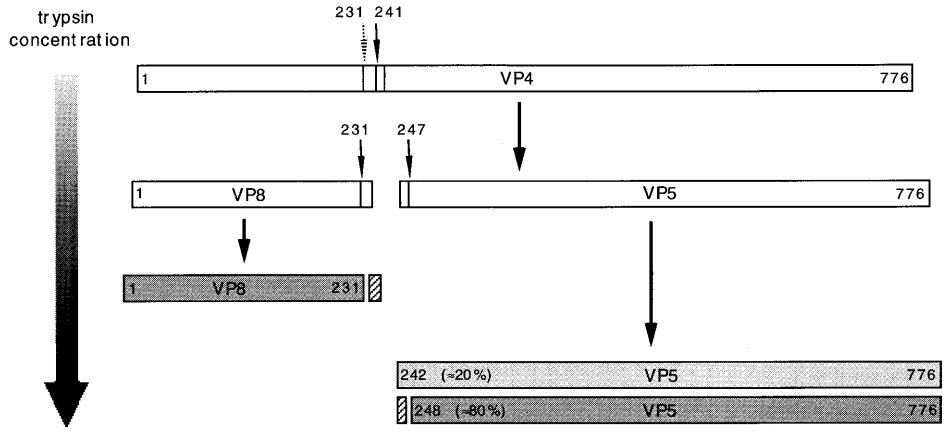


FIG. 6. Model for the major trypsin cleavage activation pathway of rotavirus SA114S infectivity. The open bars represent the uncleaved protein and its intermediate trypsin cleavage products. The arginines at positions 231, 241, and 247 are indicated by vertical lines. The first cleavage is proposed to be at Arg-241, although a small proportion of VP4 molecules could be first cleaved at Arg-231 (striped arrow). The second cleavage site is proposed to be Arg-231, while the last site to be cleaved is Arg-247. The shadowed bars are the terminal VP4 cleavage products that remain associated with the viral particles, while the hatched boxes represent the connecting peptides 232 to 241 and 242 to 247, whose fate is unknown. The numbers inside the bars represent the amino acid residue position at the amino or carboxy terminus of the corresponding polypeptide. The gradient arrow at the left indicates that increasing concentrations of trypsin are needed to cleave the least susceptible sites.

to a faster-migrating form, which presumably represents 247-VP5. As mentioned above, the sequential cleavage at arginines 241 and 247 seems more evident for RRV VP4.

## DISCUSSION

In this study, we have shown that during the enhancement of infectivity of rotavirus SA114S by trypsin, the VP4 protein is cleaved at three conserved arginines, located at positions 231, 241, and 247, extending our previous findings that SA114fM VP4 was cleaved at arginines 241 and 247 and identifying Arg-231 as a new trypsin-susceptible site. The cleavage at the three arginines appears to occur in an ordered sequence, with the most susceptible site being Arg-241. We propose that this is the preferred site for the first cleavage, followed by cleavage at Arg-231 and then by cleavage at Arg-247. Apparently, the initial cleavage can also occur at Arg-231, which seems to be the second most susceptible site. A model for the major trypsin cleavage pathway of rotavirus SA114S VP4 is shown in Fig. 6.

The terminal VP4 cleavage products generated by treatment of rotaviruses SA114S and SA114fM with trypsin concentrations higher than 1 μg/ml were a VP8 polypeptide with a homogeneous carboxy terminus (Arg-231) and a VP5 protein having a heterogeneous amino terminus, with about 20 and 80% of the molecules resulting from cleavage at VP4 arginines 241 and 247, respectively (22) (Fig. 1). Complete cleavage at Arg-247 could not be obtained even after treatment with trypsin concentrations as high as 100 μg/ml (not shown for SA114S). The structural bases that prevent all the VP4 molecules from being cleaved by trypsin at Arg-247 are not known; however, these results confirm our previous findings for SA114fM (22). The relevance of this 20% VP5-241 species for the infectivity of the virus, if any, remains to be determined.

It had been previously shown that the cleavage of VP4 by trypsin correlates with the increase in infectivity of rotavirus SA114S (11, 13). In this work, we have shown that the treatment of this virus with AspN, which cleaves VP4 at the peptide bond between Arg-241 and Asp-242 (cleavage analog to that caused by trypsin at Arg-241), does not significantly increase the infectivity of the virus, indicating that the single cleavage at this site is not sufficient for and is not directly involved in the enhancement of infectivity. This enhancement, which, rather, seems to correlate with cleavage at Arg-247, must be precise, since treatment of SA114S with α-chymotrypsin, which cleaves VP4 at Tyr-246, enhanced only marginally (this work) or did not enhance (13) the virus infectivity. The fact that the increase in infectivity correlates with cleavage at Arg-247 does not necessarily mean that activation of the virus is not dependent on cleavage at Arg-231 (see also below).

Neither of the four antipeptide sera produced had neutralization activity against rotavirus SA114S, although an enzyme-linked immunosorbent assay (ELISA) showed that only the antipeptide 2 antibodies bound to the virus (data not shown). These results are consistent with our previous findings by ELISA that antibodies to peptides comprising amino acids 220 to 233 and 258 to 271 of rotavirus SA114fM VP4 did not react with the virus and did not have neutralization activity (3).

Further experiments have to be carried out to determine to which VP5 species (VP5-241 or VP5-247) the antipeptide 2 antibodies bind in the virus particle; however, the fact that these antibodies did not neutralize the virus despite binding to it suggests that the termini newly generated by trypsin digestion are not involved in a direct interaction with the cell (although it cannot be ruled out that the carboxy terminus of VP8 might play a role in the enhancement of infectivity) and that instead, the cleavage of VP4 induces a conformational change in VP5 or in VP8 through the interaction with VP5 (since the cleavage at Arg-247 seems to be critical to activate the infectivity) that is necessary for the virus to become infectious. This proposed structural change could occur immediately after cleavage at Arg-247, or the cleavage at this position might only loosen the structure of VP4 such that the structural change occurs when the virus interacts with the cell receptor. The latter possibility seems more likely, since the difference in the structure of VP4 between trypsin-treated and untreated virus, as determined by cryoelectron microscopy, does not seem to be very striking (32). Changes in the virus surface proteins upon interaction of the virus with the cell receptor on the cell surface or after endocytosis of the receptor-virus complex have been well documented for other viruses such as poliovirus (15), influenza virus (6), human immunodeficiency virus (9), tick-borne encephalitis virus (33), reovirus (14), and paramyxovirus (20). In this context, the cleavage of VP4 by trypsin might be needed to release a lock and make the virus particle unstable,

such that upon interaction with the cell surface, the virion is able to uncoat efficiently, helping to reverse the assembly step of the surface proteins that should occur efficiently with the uncleaved VP4 during morphogenesis of the virus. In addition, the cleavage of VP4 and the proposed subsequent structural change of the protein upon contact of the virion with the cell receptor might provide the virus with a mechanism to avoid the immune selection pressure of the host against a conserved and essential domain of VP4 (needed, for example, for a secondary virus-receptor interaction [26]) that might not be exposed in the protein before the specific virus-cell interaction. This strategy to avoid the host immune system surveillance might be complementary or alternative to that proposed in the canyon hypothesis (34). The activation of virus infectivity by proteolytic cleavage of the viral spike proteins is also a strategy commonly used by enveloped viruses like paramyxoviruses, orthomyxoviruses, and retroviruses, including human immunodeficiency virus (19).

If the cleavage at Arg-247 is relevant for activation of virus infectivity, why are the three arginine residues in the TCR conserved in the great majority of the VP4 proteins sequenced so far? We propose that the cleavage at Arg-241, although not directly involved in enhancement of infectivity, is a prerequisite for the protein to be cleaved, at least efficiently, at Arg-247. In addition, cleavage at Arg-231 might be needed for an efficient cleavage at Arg-247, or the VP8-231 polypeptide could be required for the structural change, proposed to be induced by cleavage at Arg-247, to occur. This hypothesis is testable, and further experiments should be done to confirm it. The cleavage at the three VP4 conserved arginines was also found to occur in rotavirus strains SA114fM and RRV, and the most susceptible site also appeared to be Arg-241, suggesting that the proposed trypsin cleavage pathway might be the same for these viruses. It remains to be determined, however, if cleavage at Arg-247 is also directly associated with the enhancement of their infectivity and if this pathway of activation is also true for rotavirus strains isolated from different animal species, including humans.

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