A Zinc Ion Controls Assembly and Stability of the Major Capsid Protein of Rotavirus

Inge Erk, Jean-Claude Huet, Mariela Duarte, Stéphane Duquerroy, Felix Rey, Jean Cohen, and Jean Lepault

Virologie Moléculaire et Structurale, UMR CNRS-INRA 2472, F-91198 Gif-sur-Yvette, and Unité de Biochimie et Structure des Protéines, INRA, Domaine de Vilvert, F-78352 Jouy-en-Josas, France

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The recent determination of the crystal structure of VP6, the major capsid protein of rotavirus, revealed a trimer containing a central zinc ion coordinated by histidine 153 from each of the three subunits. The role of the zinc ion in the functions of VP6 was investigated by site-directed mutagenesis. The mutation of histidine 153 into a serine (H153S and H153S/S339H) did not prevent the formation of VP6 trimers. At pH < 7.0, about the pK of histidine, wild-type and mutated VP6 proteins display similar properties, giving rise to identical tubular and spherical assemblies. However, at pH > 7.0, histidine 153 mutant proteins did not assemble into the characteristic 45-nm-diameter tubes, in contrast to wild-type VP6. These observations showed that under conditions in which histidine residues are not charged, the properties of VP6 depended on the presence of the centrally coordinated zinc atom in the trimer. Indeed, wild-type VP6 depleted of the zinc ion by a high concentration (100 mM) of a metal-chelating agent behaved like the H153S mutant proteins. The susceptibility of wild-type VP6 to proteases is greatly increased in the absence of zinc. NH2-terminal sequencing of the proteolytic fragments showed that they all contained the β-sheet-rich VP6 head domain, which appeared to be less sensitive to protease activity than the α-helical basal domain. Finally, the mutant proteins assembled well on cores, as demonstrated by both electron microscopy and rescue of transcriptase activity. Zinc is thus not necessary for the transcription activity. All of these observations suggest that, in solution, VP6 trimers present a structural flexibility that is controlled by the presence of a zinc ion.

Rotaviruses, pathogens of both animals and humans, are members of the Reoviridae family. They have an icosahedral capsid composed of three layers of proteins and are thus generally referred to as triple-layer particles. The genome of rotaviruses is composed of 11 segments of double-stranded RNA. All but segment 11 code for a single protein. Six structural proteins participate in the architecture of the virus (VP1, VP2, VP3, VP4, VP6, and VP7). VP2 forms the internal layer of the virion, VP6 forms the middle one, and VP7 and VP4 form the external one. VP4, the spike, participates in the attachment of the virion to the target cell (6). The middle and external layers display quasi-equivalence symmetry (3) and belong to the T=13 icosahedral class. The internal layer belongs to the T=1 class and presents two independent monomers in the asymmetric unit. The geometry of rotavirus VP2 is similar to that of the corresponding protein in orbiviruses (10) and reovirus (22). The three-dimensional structures of rotavirus particles have been determined by electron cryomicroscopy and image reconstruction to increasing resolutions (20, 24, 25, 21, 14). All of the major capsid proteins have been localized in the electron microscopic reconstruction. VP1 and VP3 are present in small molar ratios within the viral particle and possess RNA polymerase and guanylyltransferase and methyltransferase activities, respectively (19). Triple-layer particles do not present any transcription activity. However, when the external layer is removed and the particle is transformed into a double-layer particle (DLP), transcription is activated. In vivo, the activation takes place during cell entry when the virion loses its external layer. VP6, the major protein of the rotavirus capsid, is required for the transcription activity. Solubilization of VP6 from the DLP produces cores that are transcription incompetent (2). VP6 thus plays a crucial role in the virus cycle and may have more than one function: capsid formation and activation of the transcription activity of the viral particles.

Recently, the atomic structure of VP6 has been determined (17). Two domains, termed the base and the head, form VP6. While the base is rich in α-helices, the head domain mainly contains β-sheets. A zinc ion is located on the threefold axis of the trimer and is coordinated to histidine 153 of each of the VP6 monomers. It has also been shown that, depending on pH and ionic strength, VP6 self-assembles into helical or spherical particles having diameters varying from 45 to 100 nm (18, 15). To investigate the role of the zinc ion in the assembly and stability of VP6, we engineered site-directed mutations at position 153, replacing the histidine with serine. We found that the zinc ion is not essential for either trimerization of VP6 or transcription activity. However, the sensitivity of mutant VP6 proteins to proteases is strongly increased. In addition, although they self-assemble into helical and spherical particles, the small helical assemblies having a diameter of 45 nm are not formed. Similarly, wild-type VP6 depleted of zinc with a metal-chelating agent is more sensitive to protease activity and does not form small helical particles. These findings suggest that the...
basal domain of VP6 presents a structural flexibility that depends on both pH and the presence of zinc ions.

**MATERIALS AND METHODS**

**Viruses and cells.** A derivative of *Autographa californica* nuclear polyhedrosis virus containing the wild-type rotavirus VP6-encoding gene (bovine strain RF) has been previously described (12). Mutations in the VP6-encoding gene were constructed by using the QuikChange kit from Stratagene (Amsterdam, The Netherlands). Donor plasmids were transposed into DH10BAC cells, and the recombinant bacmid DNA was used for lipofection of Sf9 cells. Mutated nucleotides in recombinant baculoviruses were verified by sequencing the corresponding donor plasmid.

Sf9 cells cultured as monolayers at 28°C in Hink’s medium supplemented with 10% fetal calf serum were infected with the recombinant baculovirus and harvested 3 days postinfection. *Spodoptera frugiperda* caterpillars infected with the recombinant baculovirus were also used. At 3 days postinfection, the caterpillars were sacrificed (6).

DLPs, cores, and virus-like particles made of VP2 and VP6 (VLP2/6) were prepared as previously described (5, 12) and purified by centrifugation through CsCl gradients.

**VP6 purification.** Purification of VP6 from caterpillars was done as previously described (4). Infected Sf9 cells were harvested, and the clarified supernatant was centrifuged at 4°C for 35 min at 35,000 rpm in a Beckman 45Ti rotor. The pellet was resuspended in 50 mM N-acetylglucosamine (NAG) buffer (pH 5.6) and then adjusted to 0.3 M NaCl and centrifuged for 10 min at 14,000 × g in an Eppendorf tube. The supernatant contained semipurified VP6 and was dialyzed overnight against distilled water at room temperature. Under these conditions, VP6 formed large assemblies that were recovered by centrifugation and used further.

**Electrophoresis and zymographs.** Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) was performed essentially as previously described (13). In general, 12% polyacrylamide gels were used. Low- and high-molecular-mass standards were purchased from Bio-Rad.

SDS-PAGE zymography was performed by using a modification of the method previously described (11). Ten percent polyacrylamide gels containing 1% gelatin were used. After electrophoresis, gels were rinsed overnight in a buffer containing 20 mM HEPES, pH 7.2, and 1% Tween 20 (Sigma) and then stained with Coomassie blue.

**Partial proteolysis.** Partial digestions of VP6 were performed with trypsin from either Roche (Meylan, France) or Promega (Charbonnières, France). The latter was chymotrypsin free. VP6 in 50 mM Tris-HCl (pH 8.0)–50 mM NaCl was incubated with trypsin at 37°C for 1 h. The VP6/trypsin weight ratio was varied from 5 × 10−3 to 5 × 10−4 in 10-fold dilution steps. The reactions were stopped by addition of Complete EDTA-free tablets (Roche) or by boiling, and the mixtures were analyzed by SDS-PAGE. Contaminant proteases in partially purified VP6 preparations were inhibited with Complete EDTA-free tablets.

**NH2-terminal sequencing.** Samples separated by electrophoresis were transferred onto polyvinylidene difluoride membrane (ProBlott; Applied Biosystems) by passive absorption as previously described (7). Briefly, after staining and destaining of the gel, the bands of interest were excised and dried in a Speedvac. The gel pieces were then reswollen in 50 μL of a 2% SDS solution in 0.2 M Tris-HCl, pH 8.5, for 30 min. After swelling, 250 μL of high-performance liquid chromatography-grade water was added and a piece of polyvinylidene difluoride membrane (4 by 4 mm) soaked in methanol was immersed in the solution. After 2 days at room temperature (23°C) with gentle vortexing, the gel piece and the solution were clear and the membrane was blue. The membrane was washed five times with 1 mL of 10% methanol with vortexing.

**Transcription activity.** The transcription assay was performed as previously described (4). Reconstituted DLPs were prepared as previously described (2) and purified again by CsCl gradient centrifugation.

**Electron microscopy.** Electron microscopy was performed on an Applied Biosystems Precise 494HT instrument with the reagents and methods recommended by the manufacturer.

**RESULTS**

**Assembly properties of mutant VP6 proteins.** VP6 mutated at position 153 by replacing the histidine residue with a serine (H153S) displayed assembly properties similar to those of wild-type VP6 as long as the pH was kept below about 7. In the presence of calcium (≥150 mM), free trimers were observed (Fig. 1a). In the absence of calcium, at pHs 3 to 5, spheres or parts thereof were observed (Fig. 1b), and at pHs 6 to 7, large tubes 75 nm in diameter were visible (Fig. 1c). At pHs of >7, in contrast to wild-type VP6, large assemblies were not observed with the mutant VP6 proteins. In particular, small tubes having a diameter of 45 nm were never detected with the mutant proteins. Instead, small isometric particles with dimensions smaller than those of the wild-type trimer were observed (Fig. 1d). When analyzed by SDS-PAGE, the samples maintained at pHs of >7.0 displayed a band corresponding to a molecular mass of 23 kDa (insert in Fig. 1d) and not to a molecular mass of 45 kDa, as found when the samples were maintained at an acidic pH (insert in Fig. 1b). VP6 samples are thus cleaved at an alkaline pH and did not self-assemble to reform 75-nm-diameter tubes or spheres when the pH was lowered. This stands in contrast to the pH-dependent reversibility of wild-type VP6 assembly (15). From these observations, two conclusions can be drawn. First, at pHs of >7.0, the mutant adopts a conformation different from that of wild-type VP6. Second and consequently, the mutant proteins display an increased sensitivity to proteases. Under these conditions, proteinolysis prevents self-assembly of VP6 fragments on a return to an acidic pH. When commercially available protease inhibitor cocktails were included to block the degradation of H153 mutant proteins at pHs of >7 (SDS-PAGE insert in Fig. 1e), no formation of 45-nm-diameter tubes, only disordered aggregates, occurred (Fig. 1e). The absence of tubular assembly at pHs of >7 is therefore not due to VP6 degradation.

**Protease activity associated with VP6 samples.** Our results suggested that VP6 samples contained protease activity. To verify this hypothesis, we analyzed the samples on zymographs (11). Strong protease activity was indeed detected in VP6 samples purified from the baculovirus-insect cell expression system (Fig. 2, lane 2). The protease activity was fully inactivated by boiling (Fig. 2, lane 3) and never detected in DLP and VLP2/6 samples (Fig. 2, lanes 5 and 6, respectively). To purify VP6, we performed size exclusion chromatography and analyzed the main peaks by zymography. Three peaks did digest the gelatin; however, the VP6 peak did not contain any protease activity (data not shown). These results clearly show that the protease activity could be attributed not to VP6 itself but to some contaminant.

**Anomalous migration of unboiled VP6 on SDS-PAGE.** SDS-PAGE of boiled wild-type VP6 is characterized by the presence of a band located at a position corresponding to a protein with an apparent molecular mass of 45 kDa (Fig. 3a, lane 2). When samples were not boiled, VP6 was found at positions corresponding to higher molecular weights. The positions depend on the experimental conditions of the loading buffer.
containing VP6 (ionic strength and temperature [Fig. 3a, lanes 3 to 5]). Apparent molecular masses typically varied from 60 to 180 kDa. When the ionic strength was low, and as shown earlier (8), unboiled VP6 gave a major band located at a position corresponding to 150 kDa, about three times the molecular mass of the VP6 monomer (Fig. 3a, lane 5). A very faint band at about 35 kDa identified as VP6 by terminal sequencing (see below) was detectable in boiled samples (Fig. 3a, band in lane 2 identified by an asterisk). The time for which the sample was incubated at 37°C determined the intensity of the 35-kDa band (compare lanes 2 and 3 in Fig. 3b). In addition, the longer VP6 solutions were kept at room temperature, the slower the
FIG. 2. Zymograph of VP6 samples. Lanes: 1, molecular mass markers (St); 2 and 3, VP6 samples (VP6) not boiled (nb) and boiled (b), respectively; 4, Staphylococcus aureus V8 protease control (SAV8); 5 and 6, purified DLP and VLP2/6, respectively. The molecular masses (kilodaltons) of some markers are indicated on the left.

FIG. 3. SDS-PAGE of VP6 samples. (a) Effects of buffer conditions. Lanes: 1, molecular mass markers; 2, boiled VP6; 3, 4, and 5, unboiled samples; 3, VP6 in 50 mM MOPS, pH 6.0; 4, VP6 in 50 mM NaCl–10 mM Tris-HCl, pH 7.5, incubated for 30 min at 37°C; 5, VP6 in water incubated for 30 min at 37°C. (b) Cleavage of VP6 incubated at 37°C in the presence of SDS. Lanes: 1, molecular mass markers; 2, boiled VP6 sample in 50 mM MOPS [pH 6.0]–150 mM NaCl; 3, same VP6 sample incubated for 1 h at 37°C in the presence of 2% SDS and then boiled; 4 and 5, unboiled forms of the samples displayed in lanes 2 and 3, respectively. The molecular masses of some markers are indicated. The asterisks show the positions of a 35-kDa VP6 fragment resulting from protease activity.

unboiled samples migrated (Fig. 3b, compare lanes 4 and 5). Together, these results suggest that denaturation of VP6 trimers by SDS is a slow process and demonstrate that VP6 samples were contaminated by proteases that are active in SDS solutions. To better analyze the process of VP6 digestion, we studied the effect of the addition of a purified protease (trypsin) to the samples.

Partial proteolysis of VP6 by trypsin. In Fig. 4a, the wild-type VP6/trypsin weight ratio was varied from 5 × 10^1 to 5 × 10^6 with a 10-fold increase at each step. In all cases, a major band was present at a position corresponding to 45 kDa (Fig. 4a). A minor band migrating at about 23 kDa was visible (Fig. 4a). The intensity of this band was proportional to the amount of trypsin added (indicated by dots). Weak bands at 35 kDa (Fig. 4a) and at 14 kDa (triple asterisk in Fig. 4a) were visible in some experiments. In control experiments, VP6 present in DLPs was not digested, even at the lowest VP6/trypsin ratio (Fig. 4d). SDS-PAGE of the H153S mutant VP6 protein incubated with different amounts of trypsin (Fig. 4b) showed that the sensitivity of the mutant protein to proteases is much higher than that of the wild-type protein. When the VP6/trypsin weight ratio was less than 5,000, the 45-kDa VP6 band disappeared and a band close to 23 kDa was observed. At lower concentrations of the enzyme, as for wild-type VP6, bands at 35 and 14 kDa were observed. Comparison of SDS-PAGE analyses of the wild-type (Fig. 4a) and H153S mutant (Fig. 4b) VP6 proteins in the presence of variable concentrations of trypsin showed that the H153S mutation increased the sensitivity of VP6 to proteases by at least 3 orders of magnitude.

In VP6 from group B and C rotaviruses, position 153 is a serine and residue 339 is a histidine. Because residues 153 and 339 are close together, it has been proposed (17) that histidine 339 could coordinate a zinc ion. To study this hypothesis, we constructed the H153S/S339H double-mutant protein. Results of SDS-PAGE analysis of this mutant in the presence of various amounts of trypsin (data not shown) were similar to those obtained with the H153S mutant protein (Fig. 4b).

Single and double-mutant VP6 proteins, which are unable to coordinate a zinc atom, have similar sensitivities to proteolysis. In agreement with this conclusion, preliminary results of atomic absorption spectroscopy suggest that the zinc concentration was lower for both mutant VP6 solutions than for wild-type VP6 and that there is no significant difference between the two mutant proteins.

Zinc-depleted wild-type and H153S mutant VP6 proteins display similar properties. Our data suggested that the different properties of VP6, resistance to protease and tube assembly, depended on the coordination of a zinc ion to histidine 153. To confirm our observations, we tried to chelate zinc from purified VP6. Our success was monitored by both sensitivity to trypsin and the absence of 45-nm tubes as determined by electron microscopic observations. At pH 7.0, zinc could not be removed from VP6 at a concentration of EDTA as high as 100 mM. After such treatment, VP6 was still assembled into 45-nm tubes. When the EDTA treatment was carried out at pH 4.6, VP6 assembled into spherical particles (Fig. 5a). When the EDTA was dialyzed out and the pH was adjusted to 6.0, large tubes were formed (Fig. 5b). However, when the pH was returned to 7.5, no 45-nm tubular assemblies, only VP6 trimers, were observed (Fig. 5c). In addition, the sample displayed a sensitivity to trypsin similar to that of the H153S mutant protein (Fig. 4c). Finally, addition of 6 mM zinc restored the ability of VP6 trimers to form 45-nm tubes (Fig. 5d).

Identification of proteolysis products. The N-terminal end of VP6 and of three proteolytic fragments migrating in SDS-PAGE as proteins with 35-, 23-, and 14-kDa molecular masses (Fig. 4) were analyzed. While the N terminus of VP6 was blocked, all three fragments were not and thus have been sequenced. The 35-kDa fragment started at glutamic acid residue 103. A minor cleavage site was also detected at asparagine 107. The 23-kDa band started at threonine 151 and serine 153 for the wild-type and mutant (H153S and H153S/S339H) pro-
FIG. 4. Partial trypsin digestion of wild-type and mutant VP6 proteins. (a) Wild-type VP6. Lanes: 1, molecular mass markers; 2 to 7, purified VP6 treated with decreasing amounts of trypsin for 1 h at 37°C. In lane 2, the VP6/trypsin mass ratio is 50; it was multiplied by a factor of 10 in each of the successive lanes. The concentration of VP6 was about 3 mg/ml in all cases. (b) Partial digestion of H153S VP6 mutant by trypsin. Lanes: 1, molecular mass markers; 2 to 7, samples treated with decreasing amounts of trypsin as described above. (c) Wild-type VP6 depleted of divalent ions by treatment with 100 mM EDTA (pH 4.6). Lanes: 1 molecular mass markers; 2 to 7, samples treated with decreasing amounts of trypsin at pH 8.0 as described above. The concentration of VP6 was about 1 mg/ml. (d) DLP treated with trypsin under conditions similar to those of lane 2. The faint band located at about 14 kDa resulted from a very mild degradation of VP6. The main cleavage products are indicated by asterisks (*, 35 kDa; **, 23 kDa; ***, 14 kDa). The band corresponding to trypsin is indicated by a dot.

DISCUSSION

VP6 migrates at different positions on SDS-PAGE, depending on the ionic strength, pH, and temperature of the loading buffer. Indeed, unboiled VP6 is found in bands corresponding to proteins having apparent molecular masses varying from 60 to 180 kDa. In the presence of salt, a short incubation of VP6 in SDS-containing loading buffer resulted in a low apparent molecular mass (60 kDa). The longer VP6 is maintained at 37°C, the more VP6 migrates as a protein with an apparent molecular mass of 150 kDa. This observation shows that denaturation of VP6 in a 2% SDS solution at 37°C is a slow process that may require hours. Results of electrophoresis of unboiled samples thus need to be carefully interpreted. However, the fact that unboiled VP6 displays a band at a position corresponding to about 150 kDa strongly suggests that VP6 forms trimers even in 2% SDS solutions (23). Indeed, a trimer appears as the thermodynamically most stable form of VP6 under all of the conditions so far studied: the viral particle (20, 24), helical tubes (15), and three-dimensional crystals (17).

Our analysis of mutant VP6 proteins shows that histidine 153 is not crucial for the formation of VP6 trimers. Partially purified VP6 may undergo proteolytic cleavage when kept in SDS-containing solutions at 37°C. Under these conditions, SDS-PAGE of VP6 shows a band at 35 kDa, unlike the 45 kDa of the VP6 molecules that have been rapidly boiled after SDS addition. This observation suggests that VP6 samples were contaminated by proteases that are active in SDS solutions and that wild-type VP6 needs to be denatured by SDS for proteolysis. By using zymographs and size exclusion chromatography, we showed that the proteolysis arises from protease contaminants that are copurified with VP6. The use of commercially available protease inhibitor cocktails is thus essential during VP6 preparation.

Formation of DLPs by assembly of VP6 to VP2 on purified cores renders VP6 less sensitive, if not insensitive, to trypsin, at least under the harshest condition tested (i.e., a VP6/trypsin weight ratio of 50 for 2 h at 37°C). Under these conditions, purified VP6 is cleaved by trypsin. These observations indicate that interaction between VP2 and VP6 in the DLP prevents proteolysis by hindering access to the cleavage site and/or by stabilizing the VP6 trimer. The atomic structure of VP6 has revealed that histidine 153 of each of the monomers forming the VP6 trimer coordinates a zinc ion that appears to considerably stabilize the VP6 trimer. Mutation of histidine 153 into serine prevents this zinc coordination and could be thought of as a factor that destabilizes the VP6 trimer. Mutation of histidine 153 into serine prevents this zinc coordination and could be thought of as a factor that destabilizes the VP6 trimer. Indeed, we observed that the sensitivity of H153S mutant VP6 to proteolysis by contaminating proteases or trypsin was increased, suggesting that structural destabilization of and associated conformational changes in VP6 are the major causes of its degradation by proteases. The absence of small-tube assembly at pHs of >7.0 confirms that the wild-type and H153S mutant forms...
have different conformations in solution. The different conformations of VP6 are a consequence of the presence or absence of a zinc ion coordinated to histidine 153. This conclusion is confirmed by the finding that the histidine 153 mutant and wild-type VP6 depleted of the zinc ion have similar properties.

There are two major cleavage site areas located at positions 103 to 107 and 148 to 153. Sites 103, 107, and 148 are typical for trypsin: an arginine is present at residues 102, 106, and 147. The cleavage sites at positions 151 and 153 appear to be characteristic of chymotrypsin: a phenylalanine residue occupies both positions 150 and 152. This atypical trypsin digestion site likely arises from chymotrypsin-like contaminants in some of the trypsin batches used, as well as in the VP6 preparations. While wild-type VP6 is cleaved at position 151, the H153S mutant form is mainly cleaved at position 153. We attribute this difference to an increase in the accessibility of residue 153 in the absence of zinc. Taking into account the fact that the 23- and 14-kDa fragments start at residues 151 and 153, it can be concluded that the head domain of VP6 (residues 145 to 334 [17]) mainly constitutes the trypsin-resistant fragments. The α-helix-rich base domain of VP6 is sensitive to proteases, in contrast to the β-strand-rich head domain.

The H153S/S339H double-mutant form and the H153S single-mutant form have similar protease susceptibilities and assembly characteristics. This observation may suggest that histidine 339 of VP6 of group B and C rotaviruses is not coordinated to a zinc atom. However, because of other significant sequence differences, this conclusion has yet to be demonstrated. The zinc coordination may indeed require a high concentration of zinc because of the low affinity of mutant VP6 or greater homology than that introduced by a simple double mutation.

Wild-type VP6 and the H153S mutant form rescue transcription activity of rotavirus cores. This observation indicates that the histidine 153 mutation does not alter the interactions between VP6 and VP2 and that zinc is not essential for transcription.

VP7 of orbiviruses (9, 10) and μ1 of reovirus (16) are structurally related to rotavirus VP6 (17). Two domains, one rich in α-helices (base) and the other rich in β-strands (head), form these proteins. All of these proteins present a right-handed twist around a threefold axis. The head domain appears as an insertion in the basal domain. VP7 of orbivirus has a sensitivity to proteases similar to that of VP6 of rotavirus, resulting in digestion of the basal domain (7). In the case of μ1 of reovirus, the cleavage of the head and basal domains is associated with
virus penetration into the host cell (16). It can thus be postu-
lated that cleavage between the head and basal domains is a
property of a common ancestor of these three genera.

In conclusion, we have shown that VP6 in solution is cleaved
by proteases and that the proteolytic sensitivity is increased
when a zinc ion does not coordinate the subunits forming a
VP6 trimer. These results suggest that the basal domains of
VP6 display a structural flexibility that may play a role during
rotavirus morphogenesis.

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