New insights into rotavirus entry machinery: stabilization of rotavirus spike conformation is independent of trypsin cleavage

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Running head: Three-dimensional structure of rotavirus uncleaved spike

Abstract

The infectivity of rotavirus, the main causative agent of childhood diarrhea, is dependent on activation of the extracellular viral particles by trypsin-like proteases in the host intestinal lumen. This step entails proteolytic cleavage of the VP4 spike protein into its mature products, VP8* and VP5*. Previous cryo-electron microscopy (cryo-EM) analysis of trypsin-activated particles showed well-resolved spikes, although no density was identified for the spikes in uncleaved particles; these data suggested that trypsin activation triggers important conformational changes that give rise to the rigid, entry-competent spike. The nature of these structural changes is not well understood, due to lack of data relative to the uncleaved spike structure. Here we used cryo-EM and cryo-electron tomography (cryo-ET) to characterize the structure of the uncleaved virion in two model rotavirus strains. Cryo-EM three-dimensional reconstruction of uncleaved virions showed spikes with a structure compatible with the atomic model of the cleaved spike, and indistinguishable from that of digested particles. Cryo-ET and subvolume average, combined with classification methods, resolved the presence of non-icosahedral structures, providing a model for the complete structure of the uncleaved spike. Despite the similar rigid structure observed for uncleaved and cleaved particles, trypsin activation is necessary for successful infection. These observations suggest that the spike precursor protein must be proteolytically processed, not to achieve a rigid conformation, but to allow the conformational changes that drive virus entry.

Author summary

Rotavirus is responsible for more than 400,000 annual infant deaths worldwide. Its viral particle bears 60 protuberant spikes that constitute the machinery responsible for virus binding to and entry into the host cell. For efficient infection, the protein molecules that build the spike must be cleaved. Despite the importance of this activation step, the nature of the changes induced in the spike structure is unknown. According to the current hypothesis, the uncleaved spike is very flexible, and activation stabilizes the spike in an entry-competent conformation. Here we used distinct electron microscopy techniques to determine the structure of the uncleaved particle in two model rotavirus strains. Our results provide a complete structure of the uncleaved spike and demonstrate that cleaved and uncleaved spikes have similar conformations, indicating that proteolytic processing is not involved in stabilization of the spike. We suggest that spike processing is important for infection since it is necessary to allow the spike domain movements involved in rotavirus entry.

Introduction

To initiate infection, viruses must overcome the complex membranous system that surrounds and resides within the cell. The ability of the virus to penetrate this barrier is one of the elements that define virulence and host range. Entry into the host cell is thus a key factor in viral infectivity, and a natural target for the design of efficient strategies against virus infections [1].

Rotaviruses are non-enveloped, double-stranded (ds)RNA viruses of the Reoviridae family; they infect only vertebrates, via the oral-fecal route. Their replication is generally limited to terminally differentiated enterocytes of the intestinal tract, with severe gastroenteritis restricted in the great majority of cases to the young [2]. In humans, rotavirus infection is the leading cause of medical gastroenteritis in children under five years of age [3,4].

The rotavirus mature virion is a complex triple-layered particle (TLP) built around its inner capsid, a T=1 icosahedral shell made of 60 asymmetric dimers of the VP2 protein [5,6]. Inside this core, each of the eleven dsRNA segments of the viral genome is associated, below the five-fold symmetry axes, with one copy of the RNA-dependent RNA polymerase VP1, and the RNA capping enzyme VP3 [7,8]. The inner core is surrounded by a thick shell formed by 260 trimers of the VP6 protein ordered in an icosahedral T=13 symmetry [5,6,9]. This double-layered particle (DLP) constitutes the rotavirus transcriptional machinery and, characteristically of Reoviridae family members, it does not disassemble during viral infection. Rotavirus infection is effectively initiated when the DLP is released into the cytoplasm and begins synthesis of the viral transcripts.

The DLP are not infective, however, as they lack the ability to identify, bind and penetrate target cells; those functions reside in the external layer of the mature TLP [10,11]. This external shell is formed by 260 trimers of the VP7 glycoprotein, ordered in a T=13

icosahedral lattice. Each VP7 trimer rests on one of the VP6 trimers of the underlying DLP, anchored to small protrusions of the VP6 layer by its flexible N-terminal arm [9,12]. Sixty spikes, formed by trimers of the VP4 protein, project from the VP7 shell. They are anchored in depressions in the VP6 layer that surround the five-fold axes, clamped by the VP7 shell that partially covers their base [6].

To become fully infectious, cell-released TLP must be digested by trypsin-like proteases from the intestinal lumen [13,14]. This activation step cleaves the VP4 protein after three defined sites (Arg^{231}, Arg^{241} and Arg^{247}) to produce two main fragments, the N-terminal VP8* and the larger C-terminal VP5*. Proteolytic processing of the spikes is thought to occur through an ordered cleavage cascade that culminates in scission at Arg^{247}. Cleavage after this residue is essential for membrane interactions and infectivity [15,16].

Analysis of the near-atomic structure of the cleaved TLP shows that the three VP4 molecules that form the spikes, termed VP4A-C [6], are organized into a complex structure that is held in place by non-covalent interactions among its components (Figure 1). Structural and biochemical data have allowed formulation of a model for rotavirus entry in which VP4 plays a role similar to that of fusion proteins during enveloped virus entry [11,17-20]. Receptor binding and attachment take place through the distal lectin domains of the two VP8* molecules of chains A and B. Probably triggered by this binding, the spike components are reorganized into an extended intermediate in which hydrophobic loops of the three VP5* β-barrels, previously covered by the lectin domains (chains A and B) or by the spike body (chain C), are inserted into the target cell membrane. An additional unknown triggering event would provoke the transition from this extended intermediate to a folded-back structure, in which the hydrophobic loops now point toward the virus particle. The remarkable similarity of the initial form, the extended intermediate, and the folded-back conformation to analogous structures involved in membrane fusion of enveloped viruses suggests that the energy

106 released by these vast conformational changes is used by rotaviruses to disrupt the cell
107 membrane.

108 In this model, as with the fusion proteins of some enveloped viruses, proteolytic cleavage
109 of the rotavirus spike protein VP4 into VP8* and VP5* primes rotavirus TLP for efficient
110 infectivity [11,14]. Despite the availability of the atomic structure of the trypsinized rotavirus
111 TLP, understanding of the structural changes involved in this process has been hampered by
112 the lack of information regarding the structure of the undigested spike. Available single
113 particle cryo-electron microscopy (cryo-EM) reconstructions of undigested TLP show no
114 density for VP4 projecting from the VP7 shell [21]. This suggests that proteolytic processing
115 of VP4 triggers undetermined structural changes in the spike that result in a more stable, rigid
116 spike structure, as described by the atomic model, that mediates rotavirus entry.

117 Here we used cryo-EM and cryo-ET to study the structure of the undigested rotavirus
118 spike. Cryo-EM results showed that the structure of the uncleaved TLP of rotavirus strains
119 SA11 and OSU is indistinguishable from that of the trypsin-digested particle, and concurs
120 with the previously resolved near-atomic structure of the mature TLP. Cryo-ET analyses
121 provided new insight into the organization of the uncleaved spike, and a model for its
122 complete structure.

123

Results

Purification and characterization of TLP

Rotavirus TLP were purified from cells infected with rotavirus strain SA11, in the presence of trypsin (TR-TLP), or in its absence in medium supplemented with the protease inhibitor leupeptin (NTR-TLP). Purified particles were characterized by Coomassie blue staining of SDS-PAGE gels and by western blot analysis using an antibody specific for protein VP4/VP5*. In NTR-TLP samples, the VP4 spike protein was detected mainly as the 98 kDa unprocessed precursor form (Figure 2A, S1), whereas in TR-TLP, the trypsin proteolytic products VP8* (28 kDa) and VP5* (55 kDa) account for most of the VP4 protein mass (Figure 2B, S1). During virus purification, TLP spike stability depends both on viral strain and the proteolytic state of its VP4 components [22]. To estimate the amount of spike protein preserved in our preparations, we quantified VP4 (in NTR-TLP) or its product VP5* (in TR-TLP) relative to protein VP6. Densitometric analysis of the Coomassie-stained gels produced values of 70 ± 3% and 85 ± 6% of the stoichiometric amount for NTR- and TR-TLP, respectively, which indicates that most spike protein was maintained to a similar extent in both samples.

Cryo-EM analysis of NTR- and TR-TLP (Figure 2C, D) showed a homogeneous population of well-preserved rotavirus particles; in some, VP4 (or VP8*/VP5*) spikes can be visualized projecting from the virion surface (Figure 2C, D, arrowheads).

Single particle analysis of NTR- and TR-TLP

Three-dimensional reconstructions (3DR) were calculated for NTR- and TR-TLP with a resolution of 12.8 Å and 11.9 Å, respectively (Figure 3), obtained at a Fourier shell correlation (FSC) threshold of 0.3 (Figure S2). At the resolution achieved, the 3DR for NTR-TLP (Figure 3A-C) and TR-TLP (Figure 3D-F) were virtually indistinguishable; a difference map calculated between them showed no significant differences. The molecular architecture

149 of the spike in both particles (Figure 3C, F) is consistent with previous structural studies for
150 TR-TLP [6,23], in which the cleaved spike adopts a distinctive, rigid bilobulate shape divided
151 into a head, body, stalk and foot domains [2] (Figure 1, S3). Comparison of the relative
152 density of spikes in both density maps using VP2-VP6-VP7 shell density as a reference
153 (Figure 3B, E; arrowheads) showed an equivalent occupancy level (58 and 50% for NTR- and
154 TR-TLP, respectively).

155 The almost identical structure observed for NTR and TR spikes (Figure 3C, F) contrasts
156 with a previous report for the SA11-4F rotavirus strain, in which the spikes were resolved in
157 the cryo-EM 3DR of the TR-TLP, but were undetectable for undigested TLP [21]. This
158 difference prompted us to verify whether the presence of the protease inhibitor leupeptin or
159 the purification method affected our results. To exclude these possibilities, NTR-TLP were
160 produced in the absence of leupeptin, following the purification method described previously
161 [21]. Analysis of these capsids produced a virion map equivalent to previous NTR- and TR-
162 TLP (Figure S2 and S4). To ascertain whether these differences are strain-specific, NTR- and
163 TR-TLP were produced from the porcine strain OSU [24] and analyzed (Figure S5, S6, S7).
164 Cryo-EM 3DR of OSU NTR- and TR-TLP are essentially indistinguishable from each other.
165 There were no significant differences between the OSU strain density maps or when they
166 were compared with the 3DR of the SA11 particles.

167 These results show that spikes formed by trimers of undigested VP4 have a conformation
168 similar to that of the trypsin-digested entry-primed rotavirus particles. For the simian and
169 porcine strains analyzed in this study, ordering of the VP4 spike in a rigid bilobulate
170 conformation is independent of trypsin digestion.

171 **Infectivity assays of NTR- and TR-TLP**

172 To study the effect of trypsin cleavage on infectivity, we determined the specific
173 infectivity of both TLP types, before and after trypsin treatment (Figure 4). *In vitro* trypsin

174 treatment of NTR-TLP resulted in disappearance of the VP4 band and appearance of
175 proteolytic products VP8* and VP5*; there were no detectable changes in the protein profile
176 following similar treatment of TR-TLP (Figure 4A, B, grey arrows). Due to the trypsin
177 dependence for rotavirus plaque formation, the specific infectivity of mock-treated TLP was
178 evaluated in a focus-forming assay in the absence of trypsin, in which expression of the viral
179 protein NSP4 was used to detect infected cells (Figure 4C, D). The background infectivity in
180 NTR-TLP preparations is attributed mainly to the activity of proteases released during cell
181 lysis [14]. Undigested NTR-TLP had ~1.5 logarithmic units lower specific infectivity than
182 undigested TR-TLP (1.89 for SA11, $p < 0.02$; 1.56 for OSU, $p < 0.005$). The specific
183 infectivity of trypsin-treated TLP was determined in a plaque-forming assay that directly tests
184 for infective particles able produce infective progeny. In this assay, trypsin-activated NTR-
185 and TR-TLP showed specific infectivity levels with no significant differences (Figure 4E, F).
186 The results demonstrate that trypsin treatment enhances NTR-TLP specific infectivity to a
187 level similar to that of trypsin-activated TR-TLP, as shown for several rotavirus strains
188 [14,22]. Although we detected no structural differences between NTR- and TR-TLP by
189 single particle cryo-EM 3DR, proteolytic processing of VP4 is necessary for efficient viral
190 infectivity.

191 **Cryo-electron tomography of NTR- and TR-TLP**

192 The densities for the spikes in single particle cryo-EM analysis of NTR- and TR-TLP can
193 be assigned to most of the three VP4 polypeptide chains (A, B and C) that compose the
194 spikes, with the exception of the lectin domain of the VP4-C molecule (Figure 1). It is
195 suggested that this lectin domain is lost during TR-TLP preparation [6]. In NTR spikes, it is
196 covalently bound to the rest of the VP4 molecule, which indicates that the density for this
197 domain is smeared due to the icosahedral averaging imposed during 3DR. We used cryo-ET
198 to overcome this limitation and study the structure of SA11 strain NTR-TLP, a strategy used

199 successfully to analyze surface proteins of enveloped viruses and non-symmetric elements in
200 icosahedral viruses [25,26]. We collected several cryo-tomographic series of projection
201 images of SA11 NTR- and TR-TLP covering the angular range from -66º to +66º.
202 Tomograms from these tilt-series were reconstructed to obtain the averaged map of 607 and
203 242 single NTR and TR particles, respectively (Figure 5A-D).

204 Virtual sections from individual particles showed an electron-dense region near the
205 geometric center of some particles (~30% and ~15% of NTR and TR, respectively, Figure 5A,
206 C, white arrowheads), which can only be associated with the genomic dsRNA. This structure
207 is reminiscent of the dsRNA condensation detected by cryo-EM analysis of TR-TLP in the
208 presence of ammonium ions at high pH [27], although the biological importance of this
209 feature is not clear. Projecting densities that correspond to the virion spikes were clearly
210 visible on the outer particle surface of both samples (Figure 5A, C, black arrowheads).

211 **Subtomogram averaging**

212 Subtomograms of individual virions were extracted (Figure 5B, D), aligned and averaged,
213 considering icosahedral symmetry to optimize determination of the origin and orientation of
214 each virion subvolume. In accordance with our results in single particle cryo-EM 3DR
215 (Figure 3), the spikes in the final averaged density maps of the NTR- and TR-TLP showed no
216 significant differences (Figure 5).

217 This alignment process not only generated an average structure for the virions, but also
218 allowed determination of the origin and orientation of each particle subvolume relative to the
219 average density. We used this information, combined with knowledge of each spike position
220 in the average structure and of their icosahedral symmetry relationships, to computationally
221 extract and orient 36,420 and 14,580 spike subtomograms from the original non-symmetrized
222 densities of the NTR- and TR-TLP, respectively. The extracted spike subtomograms were
223 then reference-free classified using a maximum-likelihood algorithm that takes into account

224 the missing wedge information [28]. In this process, no spike density was detected projecting
225 from the VP7 shell for 28% of the NTR and 40% of the TR spike subtomograms, which could
226 correspond to positions where spikes have been lost and those in which they are flexible or
227 disordered. For the remaining subtomograms, the classification process converged to two
228 classes for the subtomograms of the NTR spikes, whereas there was only one class in the TR
229 spike subtomograms (Figure 6A, B). Positions in which no density was detected and those
230 assigned to a class were distributed randomly at the virus particle surface.

231 Class 1 contains the majority of the NTR and TR spike subtomograms (47% for NTR,
232 60% for TR) and yielded averages that greatly resemble the structure of the NTR- and TR-
233 TLP spikes obtained when icosahedral symmetry was imposed on the 3DR from cryo-EM
234 (Figure 3) or cryo-ET data (Figure 5). Fitting of the atomic coordinates of trypsinized VP4
235 [6] into class 1 averages showed good agreement for the spike stalk, body and head (Figure
236 6C). The lectin domain of the VP4-C molecule is the only VP4 region not accounted for by
237 either the atomic model or the density maps. Although for the TR spike it could be argued
238 that this lectin domain is lost after proteolytic cleavage, the existence of this class for the NTR
239 subtomograms, in which the lectin domain is covalently bound to the remainder of the VP4-C
240 molecule, indicates that this domain is more flexible in this molecule than the equivalent
241 domain of the other VP4 molecules. Class 2 was detected only for NTR spikes, and contained
242 25% of the subtomograms. The averaged spike density is similar to that of class 1, with an
243 additional bulge at the base of the spike stalk (Figure 6A, arrowheads). When the atomic
244 coordinates of the TR VP4 (Figure 6D) were fitted to this averaged volume, the unassigned
245 bulge density was compatible in size and shape with a single lectin domain (Figure 6E). The
246 terminal residues of this domain (Leu^{65} and Leu^{224}, cyan and yellow spheres, respectively, in
247 Figure 6E) were located just above the last defined residues in the atomic model for the VP4-
248 C flanking regions (Lys^{29} and Glu^{264}, purple and orange spheres, respectively, in Figure 6C-

E) [6]. The evidence suggests that this density corresponds to the lectin domain of the VP4-C molecule that contributes to the spike stalk, and that the average density for class 2 subtomograms reflects the complete structure of the undigested rotavirus spike.

Discussion

Recent reports suggest that the conformational changes undergone by the rotavirus spike protein VP4 are the main driving force behind membrane disruption and virus entry into the host cell [11,17,20]. For this process to be efficient, the rotavirus particle must be primed by proteolytic cleavage of VP4 into its mature products, VP8* and VP5*. Despite the availability of an atomic structure for the trypsinized rotavirus particle, our understanding of the molecular mechanisms that underlie the proteolytic enhancement of rotavirus infectivity has been hindered by the lack of a structure of the undigested spike. The aim of this study was to determine, using cryo-EM and cryo-ET, the structure of the uncleaved rotavirus to improve comprehension of the structural mechanisms underlying the proteolytic enhancement of rotavirus infectivity.

Single particle cryo-EM reconstructions of the NTR- and TR-TLP of the simian SA11 and porcine OSU rotavirus strains yielded structures that showed no differences among them and were consistent with the near-atomic structure of the rotavirus RRV strain TR-TLP [6]. This indicates that the overall spike conformation achieved by the three complete undigested VP4 molecules is essentially maintained after trypsin activation. The interactions of the unprocessed VP4 molecules with the VP6 and VP7 trimers during assembly in the six-coordinated cavity are thus sufficient to configure these molecules in the A, B or C conformations, according to their relative position. This structure for the undigested spike is consistent with the available structural data for the TR-TLP particle, since the length of the trypsin-released segment of the loops is sufficient to bridge the observed C and N termini of VP8* and VP5*, respectively [6]. This architecture would also explain the differential trypsin sensitivity of unassembled and spike-assembled VP4 [29], as most putative trypsin-sensitive sites are protected in the latter, with the exception of the known cleavage sites Arg^{230}, Arg^{241}, Arg^{247} and the proposed cleavage site at Lys^{29} in VP8* of VP4-C [6]. The observation of an

equivalent structure at the cell interaction domain of cleaved and uncleaved spikes is also consistent with the finding that both particle types bind similarly to cell membranes [30].

Although trypsin cleavage has only a limited effect on spike structure, infectivity experiments showed that protease cleavage of the spike protein is essential for high levels of specific viral infectivity. These data suggest that whereas the fold shared by NTR and TR particles is attachment-competent, the spike must be primed by cleavage for further conformational changes. In this dependence on proteolytic activation, as well as in the nature of the conformational changes during viral entry, VP4 has a striking similarity to some membrane fusion proteins of enveloped viruses [11,17]. The class 1 viral fusion glycoproteins are synthesized as precursors that require protease activation for virus infectivity [31,32]. This necessary activation step is associated with relatively little structural rearrangement of the fusion protein, similar to our observations for rotavirus. For example, the atomic structures of the uncleaved and cleaved forms of the influenza prefusion hemagglutinin protein are largely superimposable, except for the 19-residue segment that includes the protease cleavage site [33,34]. In the uncleaved form, these residues are in an exposed loop; after cleavage, the fusion peptide in the newly-formed N terminus is buried in a nearby hydrophobic cavity. In the paramyxovirus parainfluenza virus 5, structural movements between the cleaved and uncleaved forms of the fusion protein are also limited to the residues that compose and surround the protease recognition site; since the residues that compose the hydrophobic fusion peptide are already packed in the uncleaved structure, the conformational changes are even more subtle [35].

The cryo-EM-derived atomic structure of the rotavirus TR-TLP does not account for the lectin domain of VP4-C [6]. Results were similar for the NTR- and TR-TLP 3DR of both strains used in this study. The absence of density for this domain, which in NTR-TLP is covalently bound to the rest of the VP4-C subunit, indicates greater flexibility of this part of

303 the molecule, resulting in loss of the density due to the icosahedral averaging imposed during
304 3DR. The classification of cryo-ET subvolumes allowed us to overcome this limitation, and
305 the averages for the distinct classes provides a deeper understanding of the spike structure.
306 The atomic model of the TR-TLP spike was fitted with good agreement to the averaged
307 density of class 1, the most abundant class for NTR-TLP spikes and the only class detected in
308 TR-TLP spikes. Neither the averaged maps for class 1 nor the atomic coordinates detect the
309 VP4-C lectin domain. This domain is also absent in all 3DR derived from cryo-EM single
310 particle analysis, as well as the in the icosahedrally averaged maps of virion volumes obtained
311 by cryo-ET. In the case of NTR-TLP, this domain is covalently linked to the remainder of the
312 VP4-C molecule; classes 1 and 2 thus contain three complete VP4 molecules in NTR
313 samples. Nonetheless, we only identified a density compatible with this domain in the class 2
314 averaged maps, observed only in NTR-TLP subtomograms. This apparent contradiction
315 could be a result of VP4-C lectin domain flexibility. As illustrated in Figure 7, spike
316 subtomograms in which this domain is located near a central position (25%, white asterisk in
317 Figure 7, top) generate the class 2 averaged density. Class 1 subvolumes (47%) are composed
318 of densities in which the lectin domain swings away from this central position (black asterisks
319 in Figure 7, top) and whose averaging results in disappearance of the density. In the case of
320 the spike subtomograms from TR-TLP, the lack of a class equivalent to class 2 might indicate
321 that trypsin digestion further increases lectin domain flexibility or, as suggested by analysis of
322 the TR-TLP atomic structure, that a second cleavage in Lys^{29} releases the domain from the
323 spike (Figure 7, bottom) [6].
324 In both samples, a fraction of the subtomograms (28% of NTR and 40% of TR) rendered
325 averaged maps that show no density in the regions in which the spikes are located (Figure 7,
326 No Spike density). These subtomograms would include positions in which the spike is very
327 flexible, has been lost, or its structure has been damaged due to the purification conditions

328 (particularly the organic extraction procedure [22]).. Biochemical analysis of the TLP
329 preparations (Figure 2) shows that a percentage of the predicted VP4 molecules is not present
330 in the purified NTR (30%) or TR (15%) particles. Although we cannot rule out the possibility
331 that all volumes in these fractions correspond to positions at which the spike has been lost, the
332 large percentage of subtomograms included in this group, especially in the case of TR-TLP
333 positions, suggests that they also include positions in which the spike is present but is highly
334 flexible and whose density is lost after averaging (Figure 7). It can also be argued that the
335 greater percentage of tomograms in this fraction in TR-TLP reflects a greater degree of
336 freedom induced by trypsin cleavage of VP4 into its products, VP8* and VP5*. The
337 redundancy derived from the virion icosahedral symmetry could explain its tolerance for the
338 presence of defective spikes in the particle. Indeed, the relatively low occupancy observed in
339 *in vitro* recoated particles is sufficient for a very efficient infectivity [36].

340 Previous cryo-EM single particle analysis of the NTR-TLP structure did not detect a
341 density corresponding to the spike structure [21]. These different results could be attributed
342 to the nature of the SA11-4F strain used in the previous 3DR, which is unique in its ability to
343 produce small clear plaques in the absence of trypsin, and for its extreme sensitivity to
344 protease digestion [37]. These characteristics arose from the reassortment of a segment 4 that
345 encodes a VP4 gene from a bovine rotavirus on a SA11 genetic background [38,39]. The
346 molecular basis of the distinct behavior of SA11 and SA11-4F strains is thus probably due to
347 changes in the sequence of the reassorted VP4 molecule (129 changes in a total of 776
348 residues). The large number of variations makes it impossible to determine the amino acids
349 involved. Nonetheless, the VP4 region that interacts with VP6, which mediates spike foot
350 recruitment and trimerization, is conserved. There are several changes in the VP7-contacting
351 region of VP4, which dictates how the spike projects, as well as in the preceding linker, which
352 is not resolved in the atomic structure [6]. The SA11 and OSU strains used in this study show

more standard behavior, with no evidence of interspecies reassortment. Based on our results, we hypothesize that whereas most NTR-TLP spikes in SA11 and OSU strains are stabilized, the majority of VP4 molecules in the SA11-4F strain are not stabilized, and the spike density is lost when it is averaged and icosahedral symmetry is imposed. We suggest that the structures we observed for SA11 and OSU uncleaved spikes represent a more general architecture, and that SA11-4F is an extreme example of uncleaved spike flexibility.

In summary, our cryo-EM studies of two unrelated model rotavirus strains show that in the absence of trypsin cleavage, most of the three VP4 molecules that compose the spike adopt a stable conformation, similar to that of the mature cleaved TLP. Cryo-ET results reinforce this conclusion and, additionally, evidence the great flexibility of the lectin domain of the VP4-C chain, providing us with a model for the complete structure of the uncleaved spike. Our findings indicate that cleavage of the spike proteins is important for infectivity because it influences later events, probably those conformational changes proposed to mediate membrane disruption.

Materials and Methods

Virus production, purification and titration. The monkey epithelial cell line MA104 (ECACC 85102918) was cultured in MEM with 10% fetal calf serum and used between passages 7 and 24. The simian agent 11 rotavirus strain (SA11, [39,40]) was obtained from Dr. J. Buesa, (University of Valencia, Valencia, Spain), and the Ohio State University porcine strain (OSU; [24]) from Dr. O. Burrone (ICGEB, Trieste, Italy). Both strains were cloned by four successive plaque isolation steps in MA104 cells. The clones SA11-C4111 and OSU-C5111 were selected and amplified. cDNAs of the complete genome segments of the two viral clones were obtained following procedures described by Potgieter et al. [41], cloned in the plasmid pJET1.2 (Fermentas) and sequenced. Given the complex history of the SA11 family of viruses [39,42], we analyzed the genome sequence of the SA11-C4111 strain. The results showed a SA11-like genome very similar to that of the N5 strain [43], with no evidence of genomic reassortment. The amplified viruses were used within three passages of the last plaque isolation step. For simplicity, here we refer to these clones as SA11 and OSU.

TLP were produced and purified by ultracentrifugation in CsCl gradients as described by Patton et al. [53], with minor modifications (see supporting information). In parallel experiments, aliquots of the TLP samples were vitrified (below), treated with SDS-PAGE sample buffer for biochemical characterization or analyzed for infectivity. Viral infectivity was determined by plaque assays and fluorescent focus assays, essentially as described by Arnold et al. [44] with minor modifications (see supporting information).

Cryo-electron microscopy. NTR and TR-TLP samples (5 μl) were applied to one side of acetone-washed Quantifoil R 2/2 holey grids, blotted, and plunged into liquid ethane using a Leica EM CPC cryo-fixation unit. Cryo-EM images were recorded in low-dose conditions (~10 $e^-/Å^2$), in a Tecnai G2 electron microscope operating at 200 kV. For SA11 NTR and TR samples, micrographs were recorded at a nominal magnification of 50,000X. SA11 NTR-

393 TLP produced in the absence of leupeptin and OSU samples were imaged on a FEI Eagle 4k
394 CCD at a detector magnification of 67,873X (2.21 Å/pixel sampling rate).
395 **Image processing.** General image processing operations were performed using Bsoft [45]
396 and Xmipp [46] software packages. Graphic representations were produced by UCSF
397 Chimera [47]. Micrographs were digitized using a Nikon Super CoolScan 9000 ED at a
398 6.35 μm step size, or a Zeiss TD scanner at a 7 μm step size, to yield 1.27 Å or 1.4 Å pixel
399 size in the specimen, respectively. X3d [48] was used to manually select 3,802, 2,980, 2,150,
400 4,100 and 2,100 individual images for SA11 NTR-, SA11 TR-, SA11 NTR - leupeptin, OSU
401 NTR- and OSU TR-TLP, respectively. Defocus was double determined with bshow [45] and
402 CTFfind [49], and the CTF were corrected in the images by flipping phases in the required
403 lobes. The published structure of the rotavirus VP7-recoated particle [12], low-pass filtered
404 to 30 Å, was used for the initial determination of the origin and orientation of each particle for
405 all samples. As these recoated particles lack VP4, any model bias at the spike density is
406 avoided. Xmipp iterative projection matching was carried out to determine and refine the
407 origin and orientation of each particle. Reconstructions were computed using interpolation in
408 Fourier space. After each iteration, resolution was assessed by FSC, applying a correlation
409 limit of 0.5 between two independent reconstructions. The final reconstructions combined
410 3,421 images for SA11 NTR, 2,682 for SA11 TR, 1,935 for SA11 NTR - leupeptin, 3,690 for
411 OSU NTR and 1,890 for OSU TR. Amplitude decay was corrected by adjusting the spatial
412 frequency components of the cryo-EM maps to the decay profile of the atomic map of
413 rotavirus TLP (PDBs 3N09 and 2GH8). This adjustment was applied in the frequency range
414 from 340 Å to the maximum resolution achieved, and a soft low-pass filter was applied.
415 Amplitude decay was also calculated and corrected with Embfactor [50], with similar results.
416 **Cryo-electron tomography.** Samples were mixed with 10 nm gold particles and vitrified as
417 described above. Tomographic tilt-series were recorded in a Tecnai G2 electron microscope

operating at 200 kV on a FEI Eagle 4k CCD using the FEI Xplore3D software at a detector magnification of 32,609X (4.6 Å/pixel sampling rate) every 1.5 degrees. Images were acquired with a defocus ranging from 5 to 8 μm, and an accumulated total dose from 90 to 120 e/Å. Tilted series were processed using IMOD [51] and CTF corrected using TOMOCTF [52]. A final number of 4 and 3 tomograms were reconstructed for SA11 NTR and TR samples, respectively, using unfiltered weighted back projection algorithms implemented in the TOMO3D package [53] to recover all the high frequencies for subvolume averaging process.

Subtomogram averaging and classification. IMOD software was used to manually select, and extract 607 and 243 virions from SA11 NTR and TR samples, respectively. Subvolumes were aligned and averaged using the MLTomo routine [28] from Xmipp, considering icosahedral symmetry to optimize the origin and orientation determination at this step. This process was performed using the previously obtained cryo-EM 3DR as initial template, as well as in a reference-free manner. Both approaches yielded equivalent results. The final averaged volumes were used to determine the 3D position of the spikes. This information, together with the origin and orientation assigned to each particle, were used to automatically extract 36,420 and 14,520 subvolumes for each spike in the original asymmetric tomograms of SA11 NTR and TR, respectively. The spike subtomograms were classified and averaged with MLTomo. Independently of the initial number of classes used, the classification of the NTR spikes converged to 3 groups containing 10,084 (28%, no spike density), 17,281 (47%, class 1) and 9,055 (25%, class 2) volumes, whereas classification of the SA11 TR spikes converged to two groups with 5,810 (40%, no spike density) and 8,710 (60%, class 1) volumes.

Accession codes. Sequences corresponding to the different SA-C4111 and OSU-C5111 genomic segments are deposited in GenBank (accession numbers in Supplemental Table 1).

443 The 3DR and averaged subtomograms are deposited in the Electron Microscopy Data Bank
444 (EMDB; accession codes in Supplemental Table 2).
445

446 **Acknowledgements**

447 We thank J.A. Melero for critical reading of the manuscript, M.C. Terrón, R. Marabini and

448 J.J. Fernández for technical assistance and C. Mark for editorial assistance.

449

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576

577 **Figure legends**

578 **Figure 1. Atomic model of the VP4 spike.** Ribbon representation (top) of the atomic
579 structure of the VP4 spike (PDB entry 3IYU) color-coded to represent the spike foot (green),
580 stalk (red), body (red) and head (purple). As indicated (VP4-A, -B and -C), each of the
581 subunits is highlighted in color, while the remaining molecules are shown in grey. The
582 amino- and carboxy-terminal regions of the three molecules, located contacting one another
583 beneath the VP7 layer [6], contribute equally to the trimeric foot of the structure. The
584 asymmetric central stalk is formed mainly by the VP5* β-barrel domain of VP4-C, which lies
585 almost parallel to the particle surface. The distal part of the spike, constructed by equal
586 contributions of the A and B molecules, contains the body (built by the VP5* β-barrel
587 domains) and the globular heads (formed by VP8* lectin domains). The unusual structure of
588 VP4-C appears to arise from asymmetries on the VP7 trimers that surround the base of the
589 spike, which allows for distinct interactions with the base of the VP5*-C β-barrel. The
590 globular VP8* lectin domain of the VP4-C molecule is not accounted for in the resolved
591 structure, and is presumed to be released during proteolysis. Diagram of the organization of
592 the VP4 chain (bottom), color-coded as in top. The VP4 proteolytic products (VP5* and
593 VP8*) and domains are labeled. Residues delimiting domains and trypsin cleavage sites are
594 indicated.

595 **Figure 2. Analysis of SA11 NTR- and TR-TLP by SDS-PAGE and cryo-electron**
596 **microscopy.** (A, B) Coomassie blue-stained SDS-PAGE gels of purified SA11 TLP grown in
597 the absence (A) or presence (B) of trypsin. Positions of rotavirus structural proteins (VP) are
598 indicated. Unprocessed spike protein VP4 and its proteolytic products VP8* and VP5* are
599 highlighted in grey. (C, D) Cryo-electron micrographs of NTR (C) and TR (D) particles.
600 Arrowheads indicate examples of clearly defined spikes projecting from the virion surface.
601 The bar represents 50 nm.

602 **Figure 3. Single particle three-dimensional structures of SA11 NTR- and TR-TLP.** (A,
603 D) Surface-shaded representation of the outer surfaces of NTR (A) and TR (D) particles,
604 viewed along an icosahedral 2-fold axis. The surfaces are radially color-coded to represent
605 VP4 or VP8*/VP5* spikes (red), VP7 (yellow), and VP6 (blue). The density is contoured at
606 1 σ above the mean. The bar represents 100 Å. (B, E) Transverse sections, 2.8 Å thick,
607 taken from the maps of NTR- (B) and TR-TLP (E), parallel but displaced 34 Å from the
608 central section, viewed along a 2-fold axis (darker, denser). Arrows indicate spikes in the
609 surface-shaded representations in A and D and their corresponding densities in B and E. For
610 the NTR map, the relative density of the spike contained ~50% of the shell density; for the TR
611 map, relative density was ~55%. (C, F) Close up view of the NTR (C) and TR (F) spike
612 represented as in A and D.
613 **Figure 4. Infectivity assay of *in vitro* trypsin-treated TLP.** (A, B) Coomassie blue-stained
614 SDS-PAGE gels of purified SA11 (A) and OSU (B) TLP grown in the absence (NTR) or
615 presence (TR) of trypsin. Samples were mock-incubated (-Trp) or incubated *in vitro* with 100
616 BAEE units/ml of trypsin (+Trp) (30 min, 37ºC). The positions of the structural proteins
617 (VP) are indicated. The unprocessed spike protein VP4 and its proteolytic products VP8* and
618 VP5* are highlighted in grey. (C, D) Determination of specific infectivity of SA11 (C) and
619 OSU (D) TLP by fluorescent focus assay in the absence of trypsin. (E, F) Determination of
620 infectivity of SA11 (E) and OSU (F) TLP by plaque-forming assay in the presence of trypsin.
621 Data are shown as mean ± SD. FFU, focus-forming units. PFU, plaque-forming units.
622 * $p < 0.02$, ** $p < 0.005$.
623 **Figure 5. 3DR of SA11 TLP from cryo-electron tomography.** (A, C) Slice through the xy
624 plane of the reconstructed cryo-electron tomograms of NTR- (A) and TR-TLP (C). The bar
625 represents 100 nm. White arrowheads indicate examples of electrodense structures inside
626 particles. Black arrowheads indicate examples of spikes on the outer particle surface of the

virions. (B, D) Gallery of central slices through extracted NTR- (B) and TR-TLP (D). (E, G) Surface-rendered model of the averaged NTR- (E) and TR-TLP (G) calculated from the extracted subtomograms and viewed along an icosahedral 2-fold axis. The bar represents 100 Å. (F, H) Close up view of the NTR (C) and TR (F) spike represented as in E and G.

Figure 6. Tomogram averaging and classification of NTR and TR spikes. (A, B) Surface-rendered model of averaged tomograms, reference-free classified, for NTR (A) and TR (B) spikes. Top and middle rows show two side views of the averages related by a 90 degree rotation. The bottom row shows the top view of the averages. Arrowheads indicate an extra density at the base of the spike stalk in the class 2 NTR average, which is absent in NTR and TR class 1 averages. (C) Surface-rendered model of the class 1 TR spike fitted with the VP4 atomic model (PDB entry 3IYU). VP8* molecules A and B are in purple, VP5* molecules A and B are in red, and VP5* C is in orange. The last resolved residue of VP8* (Lys^{29}, purple) and the first resolved residue of VP5* (Glu^{264}, orange) are indicated by spheres for the VP4-C molecule. (D) Surface-rendered model of the class 2 NTR spike fitted with the VP4 atomic model and represented as in C. (E) Close up view of the NTR spike fitted with a single VP8* lectin domain superimposed on the extra density detected at the base of the stalk. First (Leu^{65}, cyan) and last (Leu^{224}, yellow) residues for the fitted domain are represented as spheres (arrowheads).

Figure 7. Model of the conformational states of the rotavirus spike in NTR- and TR-TLP. NTR spikes have a flexible VP4-C lectin domain at the base of the stalk (top, left). NTR Class 1 represents the average of more distal positions (black asterisks) and class 2 is generated by the average of more central positions (white asterisk) of the flexible lectin domain. The proteolytic processing of spike components releases the VP4-C lectin domain (if an additional cleavage at Lys^{29} is produced) or increases its flexibility in TR class 1 spikes (bottom, left). The fraction of NTR (top, right) and TR (bottom, left) subvolumes in which no

652 spike density is detected could correspond to a mixture of positions without spikes (or with
653 damaged spikes) and with highly flexible spikes.
654

655 **SUPPORTING INFORMATION**

656 **Materials and Methods**

657 **Production and purification of rotavirus TLP.** TLP were prepared by infecting 3-day post-
658 confluent monolayers of MA104 cells with a multiplicity of infection of 25 PFU/cell. Viruses
659 were activated by digestion with 100 BAEE U/ml of TPCK-treated trypsin (17,768 BAEE
660 U/mg of protein; TPCK Trypsin, Thermo Scientific Pierce; 30 min, 37ºC). Prior to
661 adsorption, MA104 monolayers were washed twice with MEM medium and the virus
662 inoculum was added and allowed to adsorb (90 min, 37ºC). Cell monolayers were then
663 washed twice with MEM. For the preparation of TLP in the presence of trypsin (trypsinized
664 rotavirus; TR), MEM containing 10 BAEE U/ml TPCK-trypsin was added. For the
665 preparation of rotavirus TLP with intact VP4 (non-trypsinized rotavirus, NTR), cell
666 monolayers were washed three times with MEM after virus adsorption and MEM containing
667 0.5 μg of the protease inhibitor leupeptin was added. Cells and extracellular media were
668 harvested 24 h post-infection (hpi) and separated by centrifugation (12,000xg, 30 min, 4ºC).
669 The supernatant was adjusted to 10% PEG-8000 and 2.3% NaCl, incubated (overnight, 4ºC)
670 with gentle agitation, centrifuged (10000xg, 30 min, 4ºC), and the pelleted virus was
671 resuspended in a small volume of TBS (25 mM Tris base, 0.7 mM Na_2PO_4, 5.6 mM glucose,
672 136.9 mM NaCl, 5.1 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$) [54]. The pellet of infected cells
673 was resuspended in a small volume of medium, frozen and thawed three times to release cell-
674 associated virus, and clarified by centrifugation (12,000xg, 30 min, 4ºC). The virus-
675 containing supernatant was pooled with the PEG-8000-concentrated virus and semi-purified
676 through a cushion of 40% sucrose in TBS by centrifugation (70,000xg, 2.5 h, 4ºC). The pellet
677 was resuspended in TBS and extracted twice with 1,1,1,2,3,4,4,5,5,5-decafluoropentane
678 (Sigma-Aldrich). Highly purified TLP were isolated from this extract by double band
679 isolation in CsCl gradients as described by Patton et al. [54]. Purified TLP were dialyzed

680 extensively against TBS and stored at 4ºC. TR- and NTR-TLP were also purified following
681 the procedure described by Crawford et al. [21], without the use of protein inhibitors for
682 purification of NTR-TLP. Samples were resolved by SDS-PAGE and stained with
683 Coomassie blue or analyzed by western blot with an anti-VP4 antibody which recognizes
684 VP5* and the precursor VP4, (a kind gift from Dr. C. Eichwald, Virologisches Institut,
685 Universität Zürich, Switzerland). The amount of TLP in the preparations was determined by
686 densitometric analysis of Coomassie stained SDS-PAGE gels, assuming that VP6 correspond
687 to 37% of total viral protein mass.
688 **Determination of viral infectivity.** Viral infectivity was determined by plaque assays and
689 fluorescent focus assays essentially as described by Arnold et al. [44]. NTR and TR-TLP
690 were treated (30 min, 37ºC) with 0 or 100 U/ml TPCK-trypsin. A sample of activated and
691 mock-activated virus was treated immediately with Laemmli sample buffer (5 min, 100ºC),
692 resolved in a 12% SDS-PAGE gel and Coomassie-stained.
693 For plaque assays, triplicate samples of viruses activated with 100 U/ml TPCK-trypsin
694 were serially diluted and adsorbed to confluent MA104 monolayers (90 min, 37ºC);
695 monolayers were washed and overlaid with 0.6% noble agar in MEM medium with 10 U/ml
696 TPCK-trypsin. Plaques were fixed at 4-5 days after infection with 4% formaldehyde and
697 stained with crystal violet.
698 For fluorescent focus assays, triplicate samples of mock-activated viruses were serially
699 diluted and adsorbed to confluent MA104 monolayers (90 min, 37ºC). Following virus
700 adsorption, the inoculum was removed and monolayers washed three times with MEM before
701 addition of MEM with 2% fetal calf serum. Infection was allowed to continue for 16 h, after
702 which infected cells were washed with cold PBS and methanol-fixed. Infected cells were
703 detected by incubation with a 1:500 dilution of rabbit antiserum to a purified protein
704 containing the 140 C-terminal amino acids of the SA11-C4111 NSP4 protein fused to a 6xHis

705 tag (Davids Biotechnology, Regensburg, Germany). Bound antibodies were detected by
706 incubation with Alexa568-conjugated goat anti-rabbit secondary antibody (1:500; Life
707 Technologies). Cells were imagined with a Leica SP5 confocal microscope. Images were
708 analyzed using Fiji software [55]. Statistical analysis was performed using Student's t-test for
709 unpaired data. Data are expressed as mean ± SD.
710

Figure S1. Western Blot analysis of SA11 and OSU NTR- and TR-TLP. (A) Coomassie blue-stained SDS-PAGE gel of purified SA11 and OSU NTR- and TR-TLP. Positions of structural viral proteins (VP) are indicated. Position of unprocessed spike protein VP4, and its products VP5* and VP8* are highlighted (grey). (B) Western blot analysis. A gel similar to that in A was immunoblotted with an anti-VP4 antibody that recognizes the precursor VP4 and its product VP5*.

Figure S2. Assessment of the resolution cryo-EM 3DR of SA11 NTR and TR TLP. (A) FSC resolution curves were calculated for SA11 NTR-TLP (blue, continuous line), NTR-TLP grown in absence of leupeptin (purple, dashed line) and TR-TLP (blue, dashed line). For the 0.5 threshold the values for SA11 NTR-, TR- and NTR-TLP without leupeptin were 15.4, 14.5 and 15.9 Å, respectively; values for the 0.3 threshold were 12.8, 11.9 and 13.3 Å, respectively.

Figure S3. Fit of the atomic coordinates into the cryo-EM map. (A) Scheme of the NTR spike and its interaction with VP6 and VP7 shells. Proteins and color coding are indicated. (B) Superposition of the atomic coordinates of RRV TR-TLP (PDBs 3N09 and 2GH8) [6] and the SA11 NTR-TLP cryo-EM map. Coordinates and densities are color-coded as in A.

Figure S4. Biochemical and structural analysis of SA11 NTR-TLP grown in the absence of leupeptin. (A) Coomassie blue-stained SDS-PAGE gels of purified SA11 TLP grown in the absence of trypsin and leupeptin. Positions of structural viral proteins (VP) are indicated. Position of unprocessed spike protein VP4 is highlighted in grey. (B) Cryo-electron micrograph of vitrified particles. The bar represents 50 nm. (C) Surface-shaded representation of the outer surface of the 3DR, viewed along an icosahedral 2-fold axis. The surface is radially color-coded to represent VP4 (red), VP7 (yellow) and VP6 (blue). The density is contoured at 1 σ above the mean. (D) Transverse sections, 2.8 Å thick, taken from the maps parallel but displaced 34 Å from the central section, viewed along a 2-fold axis (darker, denser). Arrows indicate spikes in the surface-shaded representations in C and their corresponding densities in D. (E) Close-up view of the spike represented as in C.

Figure S5. Analysis of OSU NTR- and TR-TLP by SDS-PAGE and cryo-EM. (A, C) Coomassie blue-stained SDS-PAGE gels of purified OSU TLP grown in the absence (A) or presence (C) of trypsin. Positions of structural viral proteins (VP) are indicated. Unprocessed spike protein VP4 and its proteolytic products VP8* and VP5* are highlighted in grey. (B, D) Cryo-electron micrographs of NTR (B) and TR (D) particles. The bar represents 50 nm.

Figure S6. Assessment of the resolution cryo-EM 3DR of OSU NTR and TR TLP. FSC resolution curves were calculated for OSU NTR-TLP (red, continuous line) and TR-TLP (red, dashed line). For the 0.5 threshold, values for OSU NTR- and TR-TLP were 16.2 and 17.4 Å, respectively; values for the 0.3 threshold were 14.3 and 15.4 Å, respectively.

Figure S7. Single-particle three-dimensional structures of OSU NTR- and TR-TLP. (A, D) Surface-shaded representation of the outer surfaces of NTR (A) and TR (D) particles, viewed along an icosahedral 2-fold axis. The surfaces are radially color-coded to represent VP4 or VP8*/VP5* spikes (red), VP7 (yellow) and VP6 (blue). The density is contoured at 1σ above the mean. The bar represents 100 Å. (B, E) Transverse sections, 2.8 Å thick, taken from the maps of NTR (B) and TR (E) TLP, parallel but displaced 34 Å from the central section, viewed along a 2-fold axis (darker, denser). Arrows indicate spikes in the surface-shaded representations in C and D and their corresponding densities in B and E. (C, F) Close up view of the NTR (C) and TR (F) spike represented as in A and D.

Supplemental Table 1. GenBank accession numbers for SA-C4111 and OSU-C5111 genomic segments

Strain	Segment	Accession number
SA-C4111	1	KJ450831
	2	KJ450832
	3	KJ450833
	4	KJ450834
	5	KJ450835
	6	KJ450836
	7	KJ450837
	8	KJ450838
	9	KJ450839
	10	KJ450840
	11	KJ450841
OSU-C5111	1	KJ450842
	2	KJ450843
	3	KJ450844
	4	KJ450845
	5	KJ450846
	6	KJ450847
	7	KJ450848
	8	KJ450849
	9	KJ450850
	10	KJ450851
	11	KJ450852

762 **Supplemental Table 2. Accession codes for density maps**

Strain	Accession code
SA11 NTR-TLP	EMD-2573
SA11 TR-TLP	EMD-2574
SA NTR-TLP -leupeptin	EMD-2575
OSU NTR-TLP	EMD-2576
OSU TR-TLP	EMD-2577
Class 1 NTR spike	EMD-2578
Class 2 NTR spike	EMD-2579
Class 1 TR spike	EMD-2580

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