The Reovirus σ1 Aspartic Acid Sandwich

A TRIMERIZATION MOTIF POISED FOR CONFORMATIONAL CHANGE*§¶

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Reovirus attachment protein σ1 mediates engagement of receptors on the surface of target cells and undergoes dramatic conformational rearrangements during viral disassembly in the endocytic pathway. The σ1 protein is a filamentous, trimeric molecule with a globular β-barrel head domain. An unusual cluster of aspartic acid residues sandwiched between hydrophobic tyrosines is located at the σ1 subunit interface. A 1.75-Å structure of the σ1 head domain now reveals two water molecules at the subunit interface that are held strictly in position and interact with neighboring residues. Structural and biochemical analyses of mutants affecting the aspartic acid sandwich indicate that these residues and the corresponding chelated water molecules act as a plug to block the free flow of solvent and stabilize the trimer. This arrangement of residues at the σ1 head trimer interface illustrates a new protein design motif that may confer conformational mobility during cell entry.

Mammalian orthoreoviruses (reoviruses)5 attach to cells by specific binding to both carbohydrate and proteinaceous receptors. For serotype 3 reoviruses, viral attachment is a multistep process initiated by low affinity binding to sialic acid followed by high affinity binding to junctional adhesion molecule-A (JAM-A) (1, 2). These steps are mediated by discrete receptor-binding domains in the attachment protein, σ1 (3), a fiber-like molecule with head-and-tail morphology (4–6). Strain type 3 Dearing (T3D) σ1 has distinct binding sites for its receptors: the head domain binds to JAM-A with high affinity (2), whereas a region in the tail has been implicated in binding to sialic acid (1, 3, 7).

Viral attachment to the cell surface by σ1 leads to internalization of the virus by receptor-mediated endocytosis that is likely clathrin-dependent (8–10). Within endosomes, virions undergo acid-dependent, proteolytic disassembly to form infectious subviral particles (8, 11). Infectious subviral particles penetrate endosomal membranes and release transcriptionally active cores into the cytoplasm (12–14). Accumulating evidence suggests that σ1 undergoes dramatic conformational changes during viral disassembly and that these changes facilitate key steps in the cell entry process (4, 15, 16).

We previously determined the structure of the C-terminal half of σ1, which comprises the head domain plus a short region of the tail (6). This structure provided clues about the interaction of the head domain with its receptor JAM-A and the nature of trimer contacts. The head domain contains a water-filled cavity formed by three eight-stranded β-barrels, one donated by each monomer. The tail region consists of three β-spiral repeats. One of the most remarkable features of the σ1 structure is a cluster of aspartic acid residues at the base of the head domain (6). Molecular dynamics studies suggest that these residues are likely to play a role in mediating conformational changes in σ1 (17). However, at 2.6 Å resolution, the structure did not allow precise placement of water molecules and visualization of contacts between amino acids with sufficient accuracy to explain how such a unique arrangement of amino acids is compatible with a higher order structure.

In this study, we determined a high resolution structure of a fragment of T3D σ1 that comprises the head domain and a single β-spiral repeat of the tail. The structure has been refined to a resolution of 1.75 Å, allowing us to discern with high clarity details of the subunit interface in the vicinity of the aspartic acid cluster. Furthermore, we have analyzed two σ1 mutants with alterations in the vicinity of the subunit interface to determine the effects on receptor binding capacity and trimer stability. Our studies suggest that the aspartic acid cluster serves as a...
molecular switch that, depending on the microenvironment, can stabilize or destabilize the formation of a trimeric structure.

**EXPERIMENTAL PROCEDURES**

**Protein Expression, Purification, and Analysis—**A cDNA encoding residues 293–455 of T3D \( \sigma 1 \) was amplified by PCR and introduced into pGEX4T-3 (GE Healthcare). Mutations were engineered using site-directed PCR with appropriate mutagenic primers. Expression was induced with 0.2 mM IPTG in *Escherichia coli* strain BL21(DE3) pLys-S cells (Novagen) at 25 °C. Bacteria were centrifuged to form a pellet, solubilized in 50 mM Tris (pH 7.8), 3 mM EDTA, 1% Triton X-100, 2 mM \( \beta \)-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride, and 100 \( \mu g/ml \) lysozyme, submitted to 50% duty-cycle sonication pulses using a Branson Sonifier 450, and centrifuged at 15,000 \( \times \) g. The soluble fraction was purified using a 5-mL GSTrapFF column (GE Healthcare) and eluted with 30 mM reduced glutathione, 2 mM \( \beta \)-mercaptoethanol, 3 mM EDTA, and 50 mM Tris (pH 8.0). Controlled tryptic protease treatment was performed overnight at 4 °C to remove the glutathione S-transferase (GST) tag. The sample was equilibrated using PD-10 desalting columns and purified further using Mono Q anion-exchange chromatography (GE Healthcare) with an increasing gradient of NaCl in 20 mM HEPES (pH 7.1). The \( \sigma 1 \)-Y313A mutant protein was cleaved with thrombin on-column and further purified by gel filtration using a Superdex 75 column (GE Healthcare). Human JAM-A (hJAM-A), expressed as a GST fusion protein, was purified as described (18). Analytical scale gel filtration was performed using a Superdex 75 column mounted on a SMART system (GE Healthcare) in 20 mM Tris (pH 7.5), 100 mM NaCl.

**Protein Crystallization and Data Collection—**Purified T3D \( \sigma 1 \) head domain was subjected to size-exclusion chromatography in 20 mM Tris (pH 7.5), 100 mM NaCl, and 0.01% sodium azide and concentrated using Millipore 5 MWCO filters to 13.6 mg/ml, as assessed by direct measurement of absorbance at 260 and 280 nm using the relationship: \( c/\text{mg/ml} = (1.55 \times A_{280 \text{ nm}}) - (0.76 \times A_{260 \text{ nm}}) \), since the T3D \( \sigma 1 \) head domain does not react linearly with either Bradford or Lowry dyes (data not shown). Crystals of native \( \sigma 1 \) protein were grown from 10–12% polyethylene glycol 8000, 0.2 M magnesium sulfate, and 0.1 M sodium cacodylate (pH 6.9) by mixing an equal amount of protein and precipitant solution. \( \sigma 1 \)-D345N was concentrated to 8 mg/ml, and crystals were grown using the same conditions used to cultivate crystals of the wild-type protein but with 20% polyethylene glycol 8000. Crystals were flash-frozen using glycerol as cryoprotectant. Data from wild-type crystals were collected at the Brookhaven National Laboratory Synchrotron (beamline X29 features high flux radiation and ADSC Quantum-315 CCD detector). Data for \( \sigma 1 \)-D345N crystals were collected at the X6S beamline of the Swiss Light Source (Villigen, Switzerland) using a MarCCD detector. Both data sets were collected from single crystals and processed with HKL2000 (19).

**Structure Determination—**Crystals of wild-type \( \sigma 1 \) belong to space group P2\(_1\) (\( a = 83.93 \text{ Å}, b = 51.38 \text{ Å}, c = 108.87 \text{ Å}, \beta = 95.66^\circ \)) and contain six molecules, forming two complete trimers, in the asymmetric unit. The structure was determined by molecular replacement using the trimeric \( \sigma 1 \) head domain (6) as a search model in AMoRe (20). Alternating rounds of model building and refinement were performed initially using the programs O (21) and CNS (22), respectively. The programs Coot (23) and Refmac5 (24) were then used to refine the model. \( \sigma 1 \)-D345N also formed crystals that belong to space group P2\(_1\), with very similar unit cell dimensions (Table 1). The structure of this protein was solved and refined to 1.85 Å using Coot and Refmac5. Structure factors and coordinates have been deposited with the Protein Data Bank with accession codes 2OJ5 and 2OJ6 for the wild-type and mutant proteins, respectively.

**Surface Plasmon Resonance—**A BIACore CM5 chip (Pharmacia Biosensor AB) was coated with mouse ascites containing monoclonal anti-GST antibody (Sigma) to 10,000 RU protein (~2000 RU antibody) by amine coupling. Purified GST or GST-hJAM-A ectodomain fusion protein at a concentration of 2 \( \mu m \) in HEPES-buffered saline (pH 7.0) were captured by injection across individual flow cells of an antibody-coated CM5 chip for 3 min at 30 \( \mu l/min \) using a BIACore 2000 (Pharmacia Biosensor AB). Purified T3D \( \sigma 1 \) head domain was injected across the conjugated chip surface at 30 \( \mu l/min \). Following \( \sigma 1 \) binding, chip surfaces were regenerated with a 20-\( \mu l \) pulse of 10 mM glycine (pH 2.5). Affinity constants for \( \sigma 1 \) binding to hJAM-A were determined using separate \( k_{on} \) and \( k_{off} \) nonlinear regression with BIAevaluation 3.0 software (Pharmacia Biosensor AB), assuming a 1:1 Langmuir binding model (25).

**RESULTS**

**Purification of the \( \sigma 1 \) Head—**The previously crystallized T3D \( \sigma 1 \) protein (6) contained a flexible linker between the C-terminal two \( \beta \)-spiral repeats of the tail. This flexibility likely contributed to the observed diffraction limit of 2.6 Å for these crystals. To obtain better diffraction crystals of T3D \( \sigma 1 \), we designed a modified construct containing the entire head domain and only the C-terminal \( \beta \)-spiral repeat of the tail (residues 293–455). We also tested an additional construct comprising only the head domain (residues 309–455), but it did not yield soluble and trimeric protein, suggesting that the C-terminal \( \beta \)-spiral contributes to trimer formation. The protein was produced with a cleavable N-terminal GST tag and purified via sequential glutathione affinity chromatography and anion exchange chromatography. Subsequent gel filtration showed that the protein elutes at an apparent molecular weight of ~45 kDa, consistent with a trimer (see Fig. 4A). N-terminal sequencing confirmed that the product released following protease treatment was the \( \sigma 1 \) head domain with two additional amino acids from the protease recognition sequence (data not shown).

**Binding of the \( \sigma 1 \) Head Domain to JAM-A—**To determine whether the purified protein folds natively, we quantitatively assessed the capacity of the \( \sigma 1 \) head domain to bind to JAM-A using surface plasmon resonance. The JAM-A ectodomain, expressed and purified as a GST fusion protein (18), was captured on a biosensor surface with a GST-specific antibody. When injected across the biosensor surface, purified \( \sigma 1 \) head bound saturaedly and reversibly to the GST-JAM-A ectodomain but not to GST alone (Fig. 1A). Kinetic
The Reovirus σ1 Aspartic Acid Sandwich

FIGURE 1. Functional and structural characterization of the σ1 head domain. A, T2D σ1 head binding to the JAM-A ectodomain. Purified σ1 head domain at 300 μM (red), 30 μM (yellow), and 3 μM (green) concentrations was injected over a biosensor surface coated with either GST or GST-JAM-A. Binding of σ1 head domain to GST was set as the baseline (gray). Binding is measured in resonance units (RU). The calculated affinity for binding to GST-JAM-A, as expressed by apparent K_{D}, is approximately 7 × 10^{-7} M. B, high resolution structure of the σ1 head. Shown is a ribbon tracing of the trimer, with the three chains drawn in red, orange, and blue. Termini are labeled in one monomer with N (N terminus) and C (C terminus). B was prepared using RIBBONS (36).

TABLE 1
Data collection and refinement statistics for the wild-type σ1 and σ1-D345N crystal structures

| Data set            | Wild-type σ1 | σ1-D345N |
|---------------------|--------------|----------|
| Diffraction data    |              |          |
| Space group         | P2_1         | P2_1     |
| Unit cell dimensions (Å) | a = 83.9 | a = 84.0 |
|                     | b = 51.4     | b = 51.6 |
|                     | c = 108.9    | c = 108.9|
| Angles (°)          | β = 95.7     | β = 95.6 |
| Resolution range (Å)| 30.1-75     | 30.1-85  |
| Completeness (%)    | 96.7 (86.7)  | 98.7 (97.9)|
| Total reflections   | 300,167      | 347,405  |
| Redundancy (%)      | 3.3 (2.6)    | 4.4 (4.0) |
| Rmerge (%)          | 27.4 (38.1)  | 22.4 (27.3)|
| Rfree (%)           | 15.7 (23.0)  | 17.5 (23.0)|
| Ramachandran plot: |              |          |
| Most favorable regions | 88.5% | 87.9% |
| Additional allowed regions | 10.0% | 10.5% |
| Generally allowed regions | 1.0% | 1.5% |
| Disallowed regions  | 0.5%         | 0.0%     |

a Data sets were collected at 100 K and a wavelength of 1.1 Å (wild-type) and 1.0 Å (D345N). Values in parentheses refer to the outermost resolution shell (1.75-1.81 Å for wild-type σ1 and 1.85–1.92 Å for D345N).

b Rmerge = \frac{\sum_h |I_h| - \langle |I_h| \rangle}{\sum_h |I_h|}, where \langle \rangle is the intensity of a reflection hkl, and \langle \rangle is the average over symmetry-related observations of hkl.

c Rmerge = \frac{\sum_h \langle F_h | \sum_h \langle F_h \rangle \rangle}{\sum_h \langle F_h \rangle}, where F_{calc} and F_{obs} are observed and calculated structure factors, respectively. Free set (38) contains 10% of the data.

d Calculated with PROCHECK (24).

analysis of the σ1-GST-JAM-A interaction using BIAevaluation 3.0 software indicated a K_{D} of ~7 × 10^{-9} M, a value that approximates the K_{D} determined for a larger fragment of σ1 (2). These results indicate that the purified σ1 head domain is functional and that the JAM-A-binding domain of σ1 lies within residues 293–455.

Overall Structure of the σ1 Head Domain at 1.75 Å Resolution—Diffraction data were collected at the National Synchrotron Light Source (Brookhaven National Laboratory) using crystals of the purified σ1 head domain. The structure was solved by molecular replacement using the previously determined lower resolution structure of a C-terminal fragment of σ1 (6) and refined to 1.75 Å (Table 1). An omit map for a portion of the refined structure calculated without model bias (supplemental Fig. 1) demonstrates that the model is accurate. Concordantly, both working and free R-factors (38) are very low (Table 1), indicating that the structure is well refined and of high quality. A total of six monomers, arranged into two almost identical trimers, are present in the asymmetric unit of the crystals. A ribbon tracing of one trimer is shown in Fig. 1B. Each σ1 monomer is composed of an eight-stranded β-barrel. The overall conformation of the monomers is very similar, with the exception of two longer and presumably flexible loops: the G-H loop at the top of each monomer and the D-E loop at its base. With the exception of His-388, all σ1 residues occupy allowed regions in the Ramachandran diagram (24). However, since the density corresponding to His-388 is unambiguous, it appears that a salt bridge to Glu-348 fixes its side chain in its observed conformation. Asp-345 is located in the generously allowed region of the Ramachandran diagram; it forms, together with Asp-346, a β-hairpin between β-strands B and C (supplemental Fig. 1). The dihedral angles classify this region as a type II′ β-hairpin (26).

Contacts in the σ1 Trimer—Trimer formation buries from solvent an area of 2292 Å² per monomer. Residues in the tail account for about 25% of this area, primarily through hydrophobic interactions between the strands of the β-spiral. The three β-barrel domains that form the head engage in a more complex pattern of interactions. Multiple contacts between the subunits at the outer edges of each monomer result in binding surfaces that extend from the base of the head to its top (Fig. 2A). However, a large cavity exists at the center of the trimer, and this cavity is surrounded by smaller regions that lack inter-subunit contacts (Fig. 2A). The central cavity measures ~15 Å in height and ~10 Å in width and contains a large number of ordered water molecules that are connected to the exterior surface through channels at the top of the trimer (Fig. 2B). The cavity probably also contains many less well ordered water molecules, which are not visible in our electron density maps. Dimensions of the channels leading toward the cavity suggest that water molecules can flow freely to the top of the trimer. In contrast, the bottom of the cavity is sealed by the three Tyr-347 side chains.

The Aspartic Acid Sandwich—An unusual cluster of aspartic acid residues lies just below the water-filled cavity. Each monomer contributes two aspartic acid residues, Asp-345 and Asp-346, to this cluster, giving rise to a total of six aspartic acid side chains that are arranged in close proximity. Asp-345 and Asp-346 are located at the very tip of a β-hairpin between β-strands B and C (Fig. 3, A and B). β-Hairpins are small structural motifs stabilized by a defined backbone hydrogen bond pattern (27).
The Reovirus σ1 Aspartic Acid Sandwich

FIGURE 2. Architecture of the σ1 head subunit interface. A. contacts between subunits in the T3D σ1 trimer. Three views, each differing by 90°, are shown. One monomer is shown as a black ribbon, and its contact area with the other two monomers is represented as a molecular surface. Residues discussed in the text are labeled. The contact area was calculated using AREAIMOL (24) as the solvent-accessible area difference between the trimeric and monomeric forms of the molecule. The difference values range from 1 Å² (dark blue) to 52 Å² (red). Regions with a high area difference have low solvent accessibility in the trimer and thus represent areas with high affinity intersubunit contacts. Surfaces with an area difference of less than 1 Å² are not shown. Calculations were performed using a point density/Å² of 1 and a solvent molecule radius of 1.4 Å. B. solvent structure at the σ1 head interface. Two monomers are shown as a surface representation; the third monomer has been removed to allow a view into the trimer interface. Ordered water molecules at the interface are represented with spheres. The waters shown in blue fill most of the central cavity between the monomers and can leave the cavity via channels leading to the top and the sides of the trimer (arrows). The two water molecules shown in red are located near the Asp-345 side chains. These waters are solvent-inaccessible. This figure was generated using PyMOL (37).

The hairpins of the three monomers face each other, with the side chains of Asp-345 making key contacts. The aspartic acids are partitioned between two layers that each contain three tyrosine residues, Tyr-347 at the top and Tyr-313 at the bottom (Fig. 3, C and D).

Each Asp-346 side chain forms a salt bridge with Arg-314 from a neighboring monomer, but the Asp-345 side chains are not engaged in any ionic interactions (with other residues or with cations) that would negate their negative charges. The accumulation of three negative charges in such a hydrophobic environment would be highly unfavorable. Although hydrogen atoms cannot be seen in our electron density maps even at the high resolution obtained (1.75 Å), the location and orientation of the carboxyl groups strongly suggests that they are protonated. A protonated state of Asp-345 is also suggested by molecular dynamics studies of σ1 (17). Each protonated carboxylate forms two hydrogen bonds. One of these involves the hydrogen atom of the Asp-345 carboxyl group and the carbonyl oxygen of a neighboring Asp-346 residue; a second is formed between the carbonyl oxygen and the backbone amide group of Asp-346 in a neighboring monomer. Since these two hydrogen bonds occur three times, and since there are few other hydrogen bonds involved in head trimerization, the aspartic acid sandwich likely makes a major contribution to trimer stability.

The cluster of aspartic acids is sandwiched between hydrophobic residues that block access of solvent molecules to the carboxylic groups. One side of this sandwich (the “top” in Fig. 3D) is formed by Val-344 and Tyr-347, the other (the “bottom” in Fig. 3D) is formed by Tyr-313 and Met-309. Two well ordered water molecules located directly on the 3-fold axis are also present in this arrangement of amino acids. These water molecules interact with Tyr-313 and Asp-345, respectively, and they are held in place by an extensive hydrogen bond network. Buried water molecules can sometimes be exchanged without major unfolding. However, residues in the immediate vicinity of the two water molecules have temperature factors that are among the lowest of the entire structure (supplemental Fig. 2), suggesting that thermal mobility is low in this area. Thus, it is unlikely that the water molecules can be exchanged.

Purification and Characterization of σ1 Y313A—To explore potential conformational changes of σ1, we generated substitutions of two key residues of the cluster, Tyr-313 (Y313A) and Asp-345 (D345N). The mutant proteins were analyzed for receptor binding properties and the capacity to form trimers. We reasoned that substitution of Tyr-313 with a smaller amino acid might allow influx of water molecules to the aspartic acid cluster, thereby causing the trimer to undergo structural changes triggered by charged Asp-345 side chains. The σ1-Y313A mutant protein was expressed as a GST fusion in bacteria, purified by glutathione affinity, proteolytically cleaved from the GST tag on-column, and further purified using gel filtration. Purified σ1-Y313A is soluble and elutes as a monomer by gel filtration chromatography (Fig. 4A). Treatment of σ1-Y313A with each of three different cross-linking reagents of various lengths (11.4 to 16.1 Å) failed to alter the chromatographic mobility of the mutant protein (data not shown). σ1-Y313A was incapable of binding to JAM-A by either gel filtration (data not shown) or surface plasmon resonance (Fig. 4B), indicating that a trimeric form of σ1 is required for JAM-A engagement. Circular dichroism spectra of σ1-Y313A show a secondary structure content similar to that of wild-type σ1, suggesting that σ1-Y313A is folded properly (data not shown). Thus, our results indicate that residue Tyr313 is required for trimerization and that the trimeric form of σ1 is essential for receptor binding.

Characterization and Structure Analysis of σ1 D345N—To determine whether structural and functional changes occur upon replacement of Asp-345 with asparagine, we engineered a D345N mutation in the wild-type T3D σ1 head construct. We anticipated that hydrogen bonds might form between the asparagine residues, stabilizing the trimer interface in much the same manner as accomplished by the protonated Asp-345 side chains. The mutant protein was puri-
The Reovirus σ1 Aspartic Acid Sandwich

fied using the strategy employed for purification of the wild-type T3D σ1 head domain. The σ1-D345N mutant forms trimers at neutral pH (Fig. 4A) and binds to JAM-A with an affinity similar to that of wild-type σ1 (Fig. 4B). Thus, σ1-D345N is biochemically and functionally indistinguishable from the wild-type protein.

To test whether the D345N mutation in the T3D σ1 head domain alters the conformation of the aspartic acid sandwich, we determined the structure of σ1-D345N. The mutant protein was crystallized using conditions similar to those employed to crystallize the wild-type protein. We collected a complete data set from the σ1-D345N crystals to 1.85 Å and solved the structure by molecular replacement using the wild-type protein structure as a model (Table 1). The σ1-D345N mutant crystallized as a trimer with a structure that is nearly identical to that of the wild-type T3D σ1 head domain (Fig. 1B). At the subunit interface, the two water molecules observed just above and below residues 345 are at almost exactly the same position in σ1-D345N (Fig. 5). The amino groups of Asn-345 form the same hydrogen bond pattern as the hydroxyl group of protonated Asp-345. Thus, the mutant structure provides evidence that in trimeric, wild-type σ1 all Asp-345 residues must be protonated.

DISCUSSION

Formation of σ1 Trimmers—Although a C-terminal fragment of σ1 has been crystallized (6), further characterization at an atomic level of resolution has enhanced an understanding of its trimeric nature. Interactions that form the trimer are highly complex. The base of the trimer is held firmly together by hydrophobic interactions, a standard means of inducing the formation of oligomeric structures. In contrast, contacts at the center and top of the trimer involve interrupted surfaces, cavities filled with water molecules, trapped individual water molecules, very few hydrophobic contacts, and protonated side chains that form hydrogen bonds. These types of interactions are unusual for protein–protein contacts. We think that the σ1 head is designed to exist as both monomeric and trimeric species. In fact, the mutation of only one residue, Y313A, results in soluble, folded protein that is entirely monomeric. A single substitution at the base of the trimer therefore suffices to completely abolish trimer formation, indicating that the lower affinity contacts at the center and top of the σ1 head are not sufficient for stabilization of the trimer.

Comparison of deduced amino acid sequences of σ1 proteins from prototype and field-isolate reovirus strains reveals that Asp-345 and Asp-346 are highly conserved (6, 28). Additionally, hydrophobic residues that form the top and bottom of the aspartic acid sandwich show a high degree of conservation. Phenyl ring-containing side chains are found at positions 313 and 347 in a σ1 sequence alignment. For example, strain type 1 Lang contains phenylalanine and tryptophan residues, and strain type 2 Jones contains tyrosine and tryptophan residues at positions corresponding to Tyr-313 and Tyr-347 in T3D σ1. Furthermore, a hydrophobic residue (isoleucine, leucine, methionine, or valine) is found at position 309, and a valine is absolutely conserved at position 344. The striking level of sequence conservation at these positions suggests that the aspartic acid sandwich serves an essential function in reovirus replication.

Engagement of JAM-A—Our analysis shows that the σ1-Y313A mutant is soluble and monomeric. Furthermore, since its CD spectrum is similar to that of wild-type σ1, σ1-Y313A appears to be properly folded. However, the mutant
protein does not bind to JAM-A. The most likely interpretation of these findings is that a trimeric form of \( \sigma1 \) is required for engagement of JAM-A. We envision two possible explanations for these results. First, the JAM-A binding site may extend across more than one monomer. Second, the surface structure of the JAM-A-binding region may be stable only in the context of a trimer. We think it unlikely that JAM-A makes a direct contact with Tyr-313 or with residues in close proximity. Tyr-313 is not exposed to solvent in the trimeric wild-type protein and would be unlikely to encounter JAM-A at its position in the head trimer interior.

The structure of \( \sigma1 \) is closely related to that of the adenovirus attachment protein, fiber (6). Moreover, the receptors for reovirus and adenovirus, JAM-A and coxsackievirus and adenovirus receptor, respectively, also share significant structural and functional homology and may engage their viral ligands in a similar manner (29). We note that the adenovirus fiber knob binds to its receptor coxsackievirus and adenovirus receptor via contacts between two knob subunits and a single receptor molecule. If this mode of binding is conserved in \( \sigma1 \), it would be affected by alterations at the trimer interface.

We observed a single magnesium ion adjacent to residues Asp-365 and Glu-419 at the center of the concave surface of \( \sigma1 \) (data not shown). This surface of \( \sigma1 \) mimics the JAM-A dimer interface and has been proposed to participate in interactions with JAM-A (29, 30). The side chains of Asp-365 and Glu-419 are exposed to solvent and exist in close spatial proximity to side chains of Arg-427 and Arg-429. These residues would be positioned to engage in salt-bridge interactions with charged residues in the receptor. In support of this idea, our preliminary analysis of point mutants of JAM-A suggests that acidic and basic residues in the JAM-A dimer interface are required for high-affinity interactions with \( \sigma1 \).

A Unique Cluster of Aspartic Acid Residues at the Head Tri-mer Interface—The structure of \( \sigma1 \) reveals a unique cluster of solvent-inaccessible, conserved aspartic acid residues at the head trimer interface. A key aspect of these residues is that they are located at the very tip of a \( \beta \)-hairpin. As judged by its temperature factors (supplemental Fig. 2), the \( \beta \)-hairpin is rigid and possesses limited mobility. Three lines of evidence support the conclusion that the side chains of Asp-345 must be protonated and would be unlikely to encounter JAM-A at its position in the head trimer interior.

The Reovirus \( \sigma1 \) Aspartic Acid Sandwich

FIGURE 4. Functional analysis of \( \sigma1 \) mutants. A, gel filtration of T3D \( \sigma1 \) head mutants. Purified wild-type T3D \( \sigma1 \) head (blue), \( \sigma1 \)-D345N (red), and \( \sigma1 \)-Y313A (yellow) constructs were applied to a Superdex 75 gel filtration column (GE Healthcare) in 20 mM Tris (pH 7.5), 100 mM NaCl. The D345N mutant elutes at the same time as the wild-type protein, indicating that it forms a trimer, whereas the Y313A mutant shows a significantly smaller molecular weight that corresponds to monomeric protein. B, binding of T3D \( \sigma1 \) head mutants to the JAM-A ectodomain. Purified point mutants of the \( \sigma1 \) head domain at 300 nM (red), 30 nM (yellow), and 3 nM (green) concentrations were injected across a biosensor surface coated with either GST or GST-JAM-A. Binding of \( \sigma1 \) head point mutants to GST was set as the base line (gray line). Binding is measured in resonance units (RU). The identity of the mutants is indicated. The calculated affinity for binding of \( \sigma1 \)-D345N to GST-JAM-A, expressed as apparent \( K_D \), is approximately \( 1 \times 10^{-8} \) M.

FIGURE 5. Structure of \( \sigma1 \)-D345N. Superposition of wild-type T3D \( \sigma1 \) and \( \sigma1 \)-D345N at the aspartic acid cluster. Ribbon drawings of the wild-type and \( \sigma1 \)-D345N backbones are shown in gray, oxygen atoms are shown in red, and nitrogen atoms are shown in blue. The Asn-345 side chains of \( \sigma1 \)-D345N (green) show the same conformation as those of wild-type \( \sigma1 \) (yellow). The water molecules above and below the cluster also occupy virtually identical positions in both molecules. This figure was generated using PyMOL (37).

\( ^6 \) K. M. Guglielmi and T. S. Dermody, unpublished observations.
to allow formation of the trimer. First, molecular dynamics studies show that the introduction of negative charges at the Asp-345 side chains destabilizes the trimer, causing partial separation of the three chains at the base of the \( \sigma_1 \) head (17). Second, the mutant \( \sigma_1 \)-D345N protein assembles into a trimeric structure that is indistinguishable from that of the wild-type protein. Remarkably, the arrangement of water molecules in the vicinity of residue 345 in both the wild-type and mutant structures is identical. Since the asparagine side chain is an excellent mimic for a protonated aspartic acid but not for a charged aspartate, the structural similarities between the two proteins argue strongly that the wild-type protein contains protonated Asp-345 residues. Third, substitution of a residue that shields Asp-345 from solvent, Y313A, results in monomeric protein. It is likely that mutation of Tyr-313 renders Asp-345 solvent-accessible, causing it to lose its proton at neutral pH.

We performed gel-filtration experiments using purified \( \sigma_1 \) head domain under conditions of low, neutral, and high pH (data not shown). The \( \sigma_1 \) head eluted as a trimer under all conditions tested. These results provide additional evidence that the aspartic acids are protonated and inaccessible to solvent in the assembled trimer.

The hypothesis that Asp-345 in \( \sigma_1 \) is protonated is also supported by the finding of a similar cluster of aspartic acids in the G protein of vesicular stomatitis virus (VSV) (31). Although the global architecture of VSV G differs substantially from reovirus \( \sigma_1 \), VSV G also forms a trimer that features three aspartic acids that face each other at the trimer interface. The location and orientation of the aspartic acid clusters in VSV G and \( \sigma_1 \) are in fact surprisingly similar (Fig. 6). In both cases, hydrogen bonds are thought to mediate interactions between protonated aspartic acid side chains. These hydrogen bonds lie in a plane that is perpendicular to the trimer axes, and deprotonation would lead to destabilization of the trimer. In both molecules, the aspartic acids emanate from well ordered backbone structures with low mobility (an \( \alpha \)-helix in VSV G and a \( \beta \)-hairpin in \( \sigma_1 \)). Most interestingly, both clusters contain trapped water molecules that form hydrogen bonds with the protonated carboxylate groups.

What are the implications of the aspartic acid cluster for \( \sigma_1 \) function in viral attachment and cell entry? Both VSV and reovirus enter cells via the endosomal pathway and thus encounter a low pH environment during the entry process. The VSV G structure has been interpreted as a low-pH conformer of the molecule (31). In keeping with this conclusion, it is possible that the \( \sigma_1 \) structure reported here also represents a form of the protein found at low pH. Both VSV G and reovirus \( \sigma_1 \) were crystallized at close-to-neutral pH, but the conditions used for crystallization are far from physiologic in both cases and may easily create an environment that favors protonated aspartic acids. Thus, the aspartic acid cluster may act as a molecular switch that disfavors trimerization when charged but favors trimerization when the protein encounters a low pH environment that allows it to be protonated. Because the aspartic acids project from rigid structural motifs, they may prevent trimerization in environments that favor deprotonation.

Conformational changes of viral proteins in response to ligand binding or exposure to acidic pH are well documented.

**FIGURE 6. Comparison of aspartic acid clusters in reovirus \( \sigma_1 \) and VSV G.** Views into the aspartic acid clusters of \( \sigma_1 \) (A) and VSV G (B). In both cases, the view is along the 3-fold axis. Ribbon drawings of the complete trimers are shown on the left in each case to depict the location of the clusters (red circles). Ribbon tracings of the backbones are shown in gray, carbon atoms of aspartic acid residues Asp-268 (VSV G) and Asp-345 and Asp-346 (\( \sigma_1 \)) are shown in orange, and oxygen atoms are shown in red. Hydrogen bonds are represented with dotted lines. This figure was generated using PyMOL (37).
These changes allow viruses to expose previously hidden epitopes for ligand binding and membrane penetration. For example, the influenza virus hemagglutinin undergoes a massive rearrangement upon exposure to acidic pH. This conformational change is enabled by prior proteolytic cleavage and leads to the formation of $\alpha$-helical coiled-coil structures that expose a hydrophobic fusion peptide. Importantly, the hemagglutinin structure at neutral pH represents a metastable form of the protein, as the low pH conformer, once formed, is stable even at neutral pH (35). The conspicuous location of Asp-345 at the protein, as the low pH conformer, once formed, is stable glutinin structure at neutral pH represents a metastable form of the protein. Placed in the context of the entire virion, such a conformational change might facilitate events during reovirus entry subsequent to viral attachment, such as internalization into the endocytic pathway and proteolytic disassembly to form infectious subviral particles.

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REFERENCES

1. Barton, E. S., Connolly, J. L., Forrest, J. C., Chappell, J. D., and Dermody, T. S. (2001) J. Biol. Chem. 276, 2200–2211
2. Barton, E. S., Forrest, J. C., Connolly, J. L., Chappell, J. D., Liu, Y., Schnell, F., Nusrat, A., Parkos, C. A., and Dermody, T. S. (2001) Cell 104, 441–451
3. Chappell, J. D., Duong, J. L., Wright, B. W., and Dermody, T. S. (2000) J. Virol. 74, 8472–8479
4. Furlong, D. B., Nibert, M. L., and Fields, B. N. (1988) J. Virol. 62, 246–256
5. Fraser, R. D. B., Furlong, D. B., Trus, B. L., Nibert, M. L., Fields, B. N., and Steven, A. C. (1990) J. Virol. 64, 2990–3000
6. Chappell, J. D., Prota, A., Dermody, T. S., and Stehle, T. (2002) EMBO J. 21, 1–11
7. Chappell, J. D., Gunn, V. L., Wetzel, J. D., Baer, G. S., and Dermody, T. S. (1997) J. Virol. 71, 8334–8341
8. Sturzenbecker, L. J., Nibert, M. L., Furlong, D. B., and Fields, B. N. (1987) J. Virol. 61, 2351–2361
9. Baer, G. S., Ebert, D. H., Chung, C. I., Erickson, A. H., and Dermody, T. S. (1999) J. Virol. 73, 9532–9543
10. Ehrlich, M., Boll, W., Van Oijen, A., Hariharan, R., Chandran, K., Nibert, M. L., and Kirchhausen, T. (2004) Cell 118, 591–605
11. Baer, G. S., and Dermody, T. S. (1997) J. Virol. 71, 4921–4928
12. Chandran, K., Farsetta, D. L., and Nibert, M. L. (2002) J. Virol. 76, 9920–9933
13. Chandran, K., Parker, J. S., Ehrlich, M., Kirchhausen, T., and Nibert, M. L. (2003) J. Virol. 77, 13361–13375
14. Odegard, A. L., Chandran, K., Zhang, X., Parker, J. S., Baker, T. S., and Nibert, M. L. (2004) J. Virol. 78, 8732–8745
15. Dryden, K. A., Wang, G., Yeager, M., Nibert, M. L., Coombs, K. M., Furlong, D. B., Fields, B. N., and Baker, T. S. (1993) J. Cell Biol. 122, 1023–1041
16. Nibert, M. L., Chappell, J. D., and Dermody, T. S. (1995) J. Virol. 69, 5057–5067
17. Cavalli, A., Prota, A. E., Stehle, T., Dermody, T. S., Recanatini, M., Folkers, G., and Scapozza, L. (2004) Biophys. J. 86, 3423–3431
18. Prota, A. E., Campbell, J. A., Schelling, P., Forrest, J. C., Peters, T. R., Watson, M. J., Aurrand-Lions, M., Imhof, B., Dermody, T. S., and Stehle, T. (2003) Proc. Natl. Acad. Sci. U. S. A. 100, 5366–5371
19. Otwinowski, Z., and Minor, W. (1997) Methods Enzymol. 276, 307–326
20. Navaza, J. (1994) Acta Crystallogr. Sect. A 50, 157–163
21. Jones, T. A., Zhou, J. Y., Cowan, S. W., and Kjeldgaard, M. (1991) Acta Crystallogr. Sect. A 47, 110–119
22. Brünger, A. T., Adams, P. D., Clore, G. M., Delano, W. L., Gros, P., Grosse-Kunstleve, R. W., Jiang, J. S., Kuszewski, J., Nilges, M., Pannu, N. S., Read, R. J., Rice, L. M., Simonson, T., and Warren, G. L. (1998) Acta Crystallogr. Sect. D Biol. Crystallogr. 54, 905–921
23. Emsley, P., and Cowtan, K. (2004) Acta Crystallogr. Sect. D Biol. Crystallogr. 60, 2126–2132
24. Collaborative Computational Project, Number 4 (1994) Acta Crystallogr. Sect. D Biol. Crystallogr. 50, 760–763
25. Karlsson, R., and Falt, A. (1997) J. Immunol. Methods 200, 121–133
26. Sibanda, B. L., and Thornton, J. M. (1991) Methods Enzymol. 202, 59–82
27. Blanco, F., Ramírez-Alvarado, M., and Serrano, L. (1998) Curr. Opin. Struct. Biol. 8, 107–111
28. Campbell, J. A., Shelling, P., Wetzel, J. D., Johnson, E. M., Wilson, G. L., Forrest, J. C., Aurrand-Lions, M., Imhof, B., Stehle, T., and Dermody, T. S. (2005) J. Virol. 79, 7967–7978
29. Stehle, T., and Dermody, T. S. (2004) Viral Immunol. 17, 129–143
30. Forrest, J. C., Campbell, J. A., Schelling, P., Stehle, T., and Dermody, T. S. (2003) J. Biol. Chem. 278, 48434–48444
31. Roche, S., Bressanelli, S., Rey, F. A., and Gaudin, Y. (2006) Science 313, 187–191
32. Bullough, P. A., Hugheson, F. M., Skehel, J. J., and Wiley, D. C. (1994) Nature 371, 37–43
33. Kwong, P. D., Wyatt, R. Robinson, J., Sweet, R. W., Sodroski, J., and Hendrickson, W. A. (1998) Nature 393, 648–659
34. Chen, B., Yogan, E. M., Gong, H., Skehel, J. J., Wiley, D. C., and Harrison, S. C. (2005) Nature 433, 834–841
35. Chen, J., Wharton, S. A., Weissenhorn, W., Calder, L. J., Hugheson, F. M., Skehel, J. J., and Wiley, D. C. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 12205–12209
36. Carson, M. (1987) J. Mol. Biol. 5, 103–106
37. DeLano, W. L. (2002) The PyMOL Molecular Graphics System, DeLano Scientific, San Carlos, CA
38. Brünger, A. T. (1992) Nature 355, 472–475