Sigma 1 Protein of Mammalian Reoviruses Extends from the Surfaces of Viral Particles

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Electron microscopy revealed structures consisting of long fibers topped with knobs extending from the surfaces of virions of mammalian reoviruses. The morphology of these structures was reminiscent of the fiber protein of adenovirus. Fibers were also seen extending from the reovirus top component and intermediate subviral particles but not from cores, suggesting that the fibers consist of either the μ 1C or σ 1 outer capsid protein. Amino acid sequence analysis predicts that the reovirus cell attachment protein σ 1 contains an extended fiber domain (R. Bassel-Duby, A. Jayasuriya, D. Chatterjee, N. Sonenberg, J. V. Maizell, Jr., and B. N. Fields, *Nature [London]* 315:421-423, 1985). When σ 1 protein was released from viral particles with mild heat and subsequently obtained in isolation, it was found to have a morphology identical to that of the fiber structures seen extending from the viral particles. The identification of an extended form of σ 1 has important implications for its function in cell attachment. Other evidence suggests that σ 1 protein may occur in virions in both an extended and an unextended state.

The S1 gene of mammalian reoviruses determines the pattern of reovirus spread (13, 33) and its tropism for particular target cells (22, 30, 38) in the host. The structural product of the S1 gene, the σ 1 protein, is the viral cell attachment protein responsible for binding reovirus to specific receptors on the surfaces of cells (7, 14, 21, 37). Many of the biologic properties associated with the S1 gene are thought to be determined by the interaction of the σ 1 protein with receptors on target cells in the brain and other organs of the host, as well as with receptors on host immune cells (for a review, see reference 27).

To gain insight into how the σ 1 protein mediates the binding of virus to receptors and determines cell tropism, our laboratory and others have recently sequenced the S1 gene of the type 3 (T3) Dearing strain of reovirus (2, 6, 20). The nucleotide sequence predicts a σ 1 protein that contains at least two distinct structural regions (2). Heptad repeats of hydrophobic residues in an amino-terminal portion of the protein are consistent with this region occurring in the form of an extended coiled-coil α helix (24). The carboxy-terminal portion of σ 1, in which amino acid sequence changes are found in neutralization-resistant mutants and which is therefore thought to contain the cell-receptor-binding domain (4), does not possess any such apparent feature as the regular heptad repeat and is expected to have a complex globular structure. On the basis of this analysis, the σ 1 protein may be predicted to have an identifiable morphology, that of a fiber topped with a knob.

In this study, we used the electron microscope to identify long fiber structures extending from the surfaces of virions of reovirus strains T3 Dearing, type 2 (T2) Jones, and type 1 (T1) Lang. We concentrated on characterizing the T2 Jones fiber because it is frequently observed. We observed that these fibers are present, extending from top component particles and intermediate subviral particles (ISVPs) but not from cores of strain T2 Jones. The fibers, as well as σ 1 protein, are lost from viral particles on mild heating. We isolated the σ 1 protein released by heat from particles of T2

Jones and found it to appear as a long fiber with a knob at one end. Lastly, we made observations that suggest that the σ 1 protein can occur in virions in either an extended or an unextended state. These findings confirm predictions concerning the structure of the σ 1 protein and have important implications for the function of σ 1 as the reovirus cell attachment protein.

MATERIALS AND METHODS

Cells and viruses. Stock cultures of mouse L cells were grown in suspension in Joklik modified minimal essential medium (Irvine Scientific, Santa Ana, Calif.) supplemented with 5% fetal bovine serum (HyClone Laboratories, Logan, Utah) and 2 mM glutamine (Irvine Scientific). Second- and third-passage L-cell lysate stocks of plaque-purified reovirus strains T1 Lang, T2 Jones, and T3 Dearing were used in these experiments.

Virus purification. L cells at a concentration of 1×10^7 to 2×10^7 cells per ml in Joklik modified minimal essential medium supplemented with 4% fetal bovine serum, 2 mM glutamine, and 1% penicillin-streptomycin solution (Irvine Scientific) were inoculated with viral cell lysate stocks to give a multiplicity of infection of 5 to 20 PFU per cell. Viral adsorption was continued for 2 h at 35°C with occasional agitation. Cells were then diluted to a concentration of 5×10^5 cells per ml with supplemented Joklik medium and maintained in Spinner cultures at 35°C. After 2.5 to 4 days, cells were harvested by centrifugation and suspended in homogenization buffer (10 mM Tris (pH 7.5), 250 mM NaCl). Routinely, a culture that had contained 2×10^8 cells before incubation was suspended in 10 ml of homogenization buffer at this step. Harvested cells were often stored at -70°C before continuing. To obtain virus labeled with ³⁵S, Tran³⁵S label (ICN Radiochemicals, Irvine, Calif.) was added to the culture at 12 to 20 h postinfection at an activity of 5 to 12.5 μ Ci/ml and the culture was otherwise treated as described.

Viral particles were extracted from the infected cells by sonication and Freon extraction. All sonications lasted 1 to 2 min and were done with a sonicator (model W-225R; Heat Systems-Ultrasonics, Plainview, N.Y.) fitted with a standard

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tapered microtip and used at low energy levels. Cells were kept cooled on ice between all steps in the procedure. Cells in homogenization buffer were first disrupted by sonication. Sodium desoxycholate (0.1%; Difco Laboratories, Detroit, Mich.) was added, and the mixture was incubated for 30 min on ice. An equal volume of Freon (1,1,2-trichloro-1,2,2-trifluoroethane; Fisher Scientific Co., Fair Lawn, N.J.) was then added, and the mixture was again sonicated to form a complete emulsion. The aqueous and organic phases were separated by centrifugation, and the aqueous phase was decanted into a new tube. An equal volume of Freon was again added to the aqueous phase, and the mixture was sonicated to form an emulsion and subjected to centrifugation to separate the two phases. The aqueous phase was collected with a Pasteur pipette and layered on top of a 7-ml preformed CsCl gradient (ρ , 1.25 to 1.45 g/cm³). Centrifugation was carried out at 5°C in a 28.1 rotor (Beckman Instruments, Inc., Palo Alto, Calif.) spun at 26,000 rpm for 2 to 4 h. Virion and top component bands were harvested separately by dripping from the tube bottom. Harvested bands were extensively dialyzed against virion storage buffer (10 mM Tris [pH 7.5], 150 mM NaCl, 10 mM MgCl₂) and stored at 4°C until use. Virion particle concentrations were determined from optical density measurements at 260 nm by using the conversion factor of Smith et al. (29).

ISVPs and cores. ISVPs and cores of reovirus T2 Jones were obtained by treating purified virions in vitro with 200 μ g of α -chymotrypsin per ml (Sigma Chemical Co., St. Louis, Mo.). All treatments occurred in virion storage buffer at 37°C. T2 Jones virions at a concentration of 5×10^{12} particles per ml were digested for 15 min to obtain ISVPs; T2 Jones virions at a concentration of 2×10^{13} particles per ml were digested for 60 min to obtain cores. Digestions were ended by the addition of 1 to 2 mM phenylmethylsulfonyl fluoride (Boehringer GmbH, Mannheim, Federal Republic of Germany) and removal of the treatment mixture to ice. ISVPs and cores were isolated from the treatment mixtures by centrifugation in preformed CsCl gradients (ρ , 1.25 to 1.45 g/cm³ and 1.25 to 1.50 g/cm³, respectively). Centrifugation was carried out at 5°C in a 50.1 rotor (Beckman) spun at 35,000 rpm for 60 to 90 min. The isolated subviral particles were dialyzed against virion storage buffer and stored until use at 4°C.

Plaque assays. Viral samples were subjected to serial 1:10 dilutions in phosphate-buffered saline (pH 7.5; 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na₂HPO₄, 1.5 mM KH₂PO₄) containing 1 mM MgCl₂. Samples (100 μ l) of dilutions were inoculated in duplicate onto semiconfluent monolayers of L cells in six-well cluster plates, and virus adsorption was allowed to proceed for 1.5 to 2 h at 37°C in a 5% CO₂ incubator. Monolayers were then overlaid with 2 ml of 199 medium containing Hanks salts (Irvine Scientific) supplemented with 17 mM NaHCO₃, 4% fetal bovine serum, and 2 mM glutamine and including 1% penicillin-streptomycin solution, 50 U of nystatin per ml (E. R. Squibb & Sons, Princeton, N.J.), and 1% Bacto-Agar (Difco Laboratories, Detroit, Mich.). Incubation was continued at 37°C and 5% CO₂. Monolayers were fed with an identical overlay on day 3 postinfection and, in the case of assays with reovirus T2 Jones (which forms plaques slowly), also on day 6. Monolayers were given a final overlay that included 0.04% neutral red (Fisher Scientific) on either day 6 (for T1 Lang and T3 Dearing) or day 9 (for T2 Jones), and plaques were counted the next day. Plaque titers used for determining particle-to-PFU ratios in this study were the averages obtained from two or more determinations for each sample.

Particle densities. ³⁵S-labeled viral particles in small volumes of virion storage buffer (≤ 100 μ l) were layered on the tops of 4.5-ml preformed CsCl gradients and subjected to centrifugation at 5°C in a 50.1 rotor spun at 35,000 rpm for 90 min. CsCl gradients (ρ , 1.20 to 1.45 g/cm³) were used for density measurements of virions, top component, and ISVPs; CsCl gradients (ρ , 1.25 to 1.50 g/cm³) were used for cores. Spun gradients were harvested by dripping into fractions of approximately 200 μ l. Fractions containing viral particles were identified by analysis for radioactivity in an LS-233 liquid scintillation counter (Beckman). Densities of the fractions were determined by measuring their refractive indices with an Abbe-3L refractometer (Bausch and Lomb, Inc., Rochester, N.Y.).

SDS-polyacrylamide gel electrophoresis. Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis, Coomassie staining, and fluorography were done as described elsewhere (A. K. Jayasuriya, M. L. Nibert, and B. N. Fields, submitted for publication). In this study, 5 to 10% gradient gels were found to resolve the reovirus proteins and double-stranded (ds) RNA segments in a nonoverlapping fashion and were therefore sometimes used in place of the 5 to 20% gels. Silver staining was done by using a modification of the procedure of Merrill et al. (18). Each illustrated gel has been cropped at the top of the resolving gel and just below the level of the dye front.

CTEM. Negative staining was done by a miniaturization of the method of Valentine and Green (34) by using 40- to 100- μ l samples containing purified virions at a concentration of 5×10^{11} to 2×10^{12} particles per ml in virion storage buffer. Pictured micrographs were obtained by using 0.5% uranyl formate for staining. Conventional transmission electron microscopy (CTEM) micrographs were taken at a magnification of $\times 50,000$ on a JEOL 100B electron microscope operated at 80 kV with a 50- μ m condensor and objective apertures.

STEM. Micrographs were obtained on the scanning transmission electron microscope (STEM) at the Brookhaven National Laboratories (36). Viral specimens were adsorbed to very thin carbon film over a holey carbon support on a titanium grid. After being washed several times with ammonium acetate, specimens were dehydrated by freeze-drying and transferred to the microscope under vacuum. The microscope was operated at 40 kV with a 10^{-10} to 10^{-12} A beam current. Elastically scattered electrons were collected to form the images shown. Tobacco mosaic virus was included as a size marker.

Purification of $\sigma 1$ protein. 1×10^{13} to 1.5×10^{13} CsCl-isolated, dialyzed T2 Jones ISVPs were heated to 52°C for 30 min in virion storage buffer. The treatment mixture was then cooled to 4°C, and the viral particles were pelleted by centrifugation at 5°C in a 50.1 rotor spun at 35,000 rpm for 90 min. The decanted ultracentrifugation supernatant (10 ml) was passed in serial aliquots through a single Centricon-30 microconcentration unit (Amicon Corp., Danvers, Mass.) by centrifugation at 5°C in an SS-34 rotor (Sorvall, Newtown, Conn.) spun at 5,000 rpm for 5- to 10-min intervals. $\sigma 1$ protein present in the supernatant was obtained in a final volume of approximately 80 μ l (deadstop plus wash volumes). Purified, concentrated $\sigma 1$ protein was stored at 4°C until use.

RESULTS

Electron microscopy of purified virions of reovirus T2 Jones. Stocks of reovirus T2 Jones were used to infect L

cells in Spinner cultures and after 2.5 to 4 days of growth, purified virions were obtained by Freon extraction and CsCl density gradient centrifugation. Representative preparations of T2 Jones virions were found to have a buoyant density in CsCl of approximately 1.36 g/cm^3 ($n = 2$) and an average particle-to-PFU ratio of 135 ± 11 ($n = 5$), as determined by optical density measurements at 260 nm (by using the conversion factor of Smith et al. [29] to obtain an estimate of particle concentration) and a plaque assay on L-cell monolayers. The protein and genomic ds RNA profiles of these virions in a silver-stained SDS-polyacrylamide gel are shown (Fig. 1, lane 2).

When negatively stained T2 Jones virions were examined by CTEM, an interesting feature was observed. Structures consisting of long fibers topped with knobs were frequently seen extending from the surfaces of the virion particles (Fig. 2A to D and F to I, arrows). These structures (henceforward referred to as fibers) were observed by using either 2% ammonium molybdate or 0.5% uranyl acetate as stains but were best seen when particles were lightly stained with 0.5% uranyl formate. The numbers of fibers per particle ranged from zero to seven, and when several fibers were present on a single particle, they usually appeared to be regularly spaced around the capsid perimeter (Fig. 2C and I). The

FIG. 1. SDS-polyacrylamide gel of virions and subviral particles of reovirus T2 Jones. Equal amounts (6×10^{10} particles each) of CsCl-isolated top component particles (lane 1), virions (lane 2), ISVPs (lane 3), and cores (lane 4) of reovirus T2 Jones were disrupted by 2 min of boiling in sample buffer. Viral proteins and ds RNA segments were separated in an SDS-polyacrylamide gel (5 to 10% gradient) and visualized by silver staining. Viral proteins are labeled on the left side; the three size groups of ds RNA genome segments are labeled on the right. Unlabeled bands are as follows: the band migrating above the λ proteins in the top component and virions (lanes 1 and 2) is a small amount of undisrupted μ 1C dimer (M. Nibert, manuscript in preparation); the band migrating below the undigested μ 1C remaining in ISVPs (lane 3) is the μ 1-derived equivalent of the μ 1C-derived δ protein (5; Jayasuriya et al., submitted); the band migrating in the μ region in cores (lane 4) is the minor core protein μ 2. Artifactual doubling of some genome segment bands occurred during the drying of this gel.

fibers seemed to be somewhat variable in length. Purified virions derived from three independent clones of reovirus T2 Jones exhibited these surface projections.

We also examined identically prepared purified virions of reovirus T1 Lang and T3 Dearing with the electron microscope. In addition to T2 Jones, these viruses represent the prototype strains of the three identified serotypes of mammalian reoviruses (25, 26). Fiber structures were seen extending from the surfaces of T1 Lang and T3 Dearing virions but at a frequency that was reduced compared with that of T2 Jones (data not shown). We therefore initially concentrated on characterizing the T2 Jones fiber.

Structural predictions for the T2 Jones σ 1 protein. The morphology of the fibers extending from T2 Jones virions reminded us of the structure that has been proposed for the T3 Dearing σ 1 protein from an analysis of its predicted amino acid sequence (2). The predicted amino acid sequence of the T2 Jones σ 1 protein is also available (6). Like the T3 Dearing σ 1 protein, the T2 Jones σ 1 protein contains an extensive region of sequence in its amino-terminal half in which hydrophobic residues occupy regular positions in a series of heptapeptide repeats (Fig. 3). As previously discussed in regard to the T3 Dearing σ 1 protein (2), this repeat pattern has been observed in a number of fibrous proteins, such as myosin, and appears to contribute to the formation of coiled-coil α -helical multimers of these proteins. Regularly positioned pairs of acidic and basic residues (16, 17), as well as skip sites at which a switch in the positions regularly occupied by hydrophobic residues occurs (16, 31), are other features relevant to the formation of stable coiled coils that we found in the heptad repeat region of the T2 Jones σ 1 protein (Fig. 3). We have also observed similar features in the predicted amino acid sequences of the T1 Lang σ 1 protein (6) and the σ 1 proteins of a set of seven electrophoretically distinct field isolates of reovirus T3 (T. Dermody and B. Fields, unpublished data) (data not shown). This analysis suggests that an amino-terminal fibrous domain is common to all reovirus σ 1 proteins.

Electron microscopy of subviral particles of reovirus T2 Jones. In an attempt to determine whether the σ 1 protein makes up the fibers seen on T2 Jones virions, different types of subviral particles with unique RNA and protein compositions (top component, ISVPs, and cores) were isolated and examined with the electron microscope.

(i) **Top component particles.** Reovirus top component particles containing the viral structural proteins but lacking genomic ds RNA are obtained as a second product of the virion purification procedure (for a review, see references 12 and 12a). We obtained preparations of top component particles of reovirus T2 Jones and found them to have a buoyant density in CsCl of approximately 1.31 g/cm^3 ($n = 2$) and a protein profile very similar to that of virions (Fig. 1, lane 1). Top component preparations often appeared to contain an amount of σ 1 protein that was reduced relative to that of virions, but σ 1 protein was always detectable in these preparations. When the top component was compared with decreasing amounts of virions in silver-stained SDS-polyacrylamide gels, we found that the top component particles contained only 1 to 2% of the amount of genomic ds RNA present in virions (relative to the amounts of the major viral proteins present in each). The ds RNA profiles of samples of top component particles and virions containing approximately equal amounts of viral proteins are shown (Fig. 1, lanes 1 and 2).

When negatively stained top component was examined with the electron microscope, most particles were found to

FIG. 2. Electron micrographs of negatively stained virions and top component particles of reovirus T2 Jones. CsCl-isolated virions (panels A to D and F to I) and top component particles (panels E and J) were stained with 0.5% uranyl formate and examined by CTEM. Arrows indicate some of the fiber structures that extend from the particles. Magnification, $\times 200,000$.

be penetrated with stain, as has been previously observed (29) (Fig. 2E and J). This penetration by stain presumably reflects the absence of genomic ds RNA in the interior of these top component particles. Nonetheless, fiber structures indistinguishable from those seen on virions were observed extending from the penetrated particles (Fig. 2E and J). In the top component particles shown in Fig. 2, the regular spacing of fibers around the capsid perimeter is especially evident. As many as six fibers were seen extending from a single top component particle. Since the top component preparations contained greatly reduced amounts of genomic ds RNA, this analysis suggests that the fiber structures consist of one or more of the viral structural proteins.

(ii) ISVPs and cores. Depending on the reaction conditions used, treatment of reovirus virions with proteolytic enzymes in vitro can result in the generation of two other distinct forms of subviral particles, ISVPs and cores (5, 11, 28). These particle types each differ from virions by lacking certain of the viral structural proteins. Virions of reovirus T2 Jones were treated with chymotrypsin under the appropriate conditions, and ISVPs or cores were purified from their respective reaction mixtures by CsCl density gradient centrifugation. T2 Jones ISVPs were found to have a buoyant density in CsCl of approximately 1.38 g/cm^3 ($n = 1$) and an average estimated particle-to-PFU ratio of 68 ± 7 ($n = 2$). Similar to the ISVPs of strains T3 Dearing (11) and T1 Lang (32), T2 Jones ISVPs lacked $\sigma 3$ protein and contained the δ cleavage product of $\mu 1C$ (Fig. 1, lane 3). T2 Jones ISVPs also contained an amount of $\sigma 1$ protein approximately equal to that in virions. Visual inspection of an SDS-polyacrylamide gel of a time course of T2 Jones digestion with chymotrypsin revealed no decrease in the intensity of the $\sigma 1$ protein band even at the latest time analyzed (60 min) (data not shown), indicating that the T2 Jones $\sigma 1$ protein was resistant to chymotrypsin digestion under the conditions used. Cores of strain T2 Jones were found to have a buoyant density in CsCl of approximately 1.43 g/cm^3 ($n = 1$). The purified cores, like those of T3 Dearing (11), lacked all three of the outer capsid proteins $\sigma 1$, $\sigma 3$, and $\mu 1C$ (or δ) (Fig. 1, lane 4).

The CsCl-purified ISVPs and cores of reovirus T2 Jones were analyzed with the electron microscope. Negatively stained ISVPs were found to be distinguishable from virions since the capsomeric subunits making up the viral outer capsid were much more apparent in ISVPs (Fig. 4A and B). Fiber structures like those seen on virions and top component particles were seen extending from the surfaces of the ISVPs (Fig. 4A and B). As many as eight fibers were observed on a single particle. Cores, on the other hand, did not exhibit the fiber structures. These core particles other-

	a	b	c	d	e	f	g
21	ser	ala	asn	ser	lys	his	glu
	ILE	glu	glu	ILE	lys	lys	gln
	ILE	lys	asp	ILE	ser	ala	asp
	VAL	asn	arg	ILE	ser	asn	ile
	VAL	asp	ser	ILE	gln	gly	gln
	LEU	gly	gly	LEU	ser	val	arg
	VAL	ser	ala	ILE	glu	ser	gly
	VAL	ser	glu	asn	gly	asn	arg
	ILE	asp	arg	LEU	glu	arg	asp
	VAL	ser	gly	ILE	ser	ala	ser
	VAL	ser	gly	ILE	asp	ser	arg
	LEU	ser	glu	LEU	gly	asp	arg
	VAL	asn	val	ALA	glu	gln	arg
	ILE	gly	gln	LEU	asp	thr	val
	thr	asp	asn	LEU	leu	glu	arg
	ALA	ser	arg	LEU	glu	thr	glu
	VAL	ser	ala	ILE	thr	asn	asp
	LEU	gly	ser	LEU	asn	thr	arg
	VAL	thr	thr	glu	LEU	asn	asp
	VAL	arg	gln	thr	ILE	ala	ala
	ILE	asp	thr	arg	LEU	thr	thr
	LEU	glu	thr	asp	ALA	val	thr
	ser	val	gly	gln	gly	leu	gln
							181

FIG. 3. Heptapeptide repeat region of the T2 Jones $\sigma 1$ protein. The predicted amino acid sequence of the T2 Jones $\sigma 1$ protein (6) is written in heptads for the region in which a regular heptapeptide repeat pattern of hydrophobic residues occurs. Letters a, b, c, d, e, f, and g denote the seven amino acid positions in the repeat. Hydrophobic residues (alanine, valine, leucine, isoleucine, and methionine) in the a, d, and e positions are capitalized, and the predominantly hydrophobic nature of these positions is emphasized with enclosing lines. A skip where paired hydrophobic residues switch from the a and d to the a and e positions is apparent. Acid-base pairs in the e and g and the b and d positions of consecutive heptads are connected with lines.

FIG. 4. Electron micrographs of negatively stained ISVPs and cores of reovirus T2 Jones. CsCl-isolated ISVPs (panels A and B) and cores (panels C to E) were stained with 0.5% uranyl formate and examined by CTEM. Arrows indicate some of the fibers extending from ISVPs. Cores are shown in various orientations and have no associated fibers. Magnification, $\times 200,000$.

wise appeared as previously described, with their appearance including the presence of the $\lambda 2$ vertex spikes (39) (Fig. 4C to E). The finding that ISVPs but not cores contained extended fibers is consistent with the conclusion that the fibers are composed of one of the two outer capsid proteins present in ISVPs, the $\sigma 1$ and $\mu 1C$ proteins. Since $\mu 1C$ is a major component of the reovirus outer capsid, the small number of fibers seen per particle makes it unlikely that they are made up of $\mu 1C$.

Release of the $\sigma 1$ protein from virions and ISVPs of reovirus T2 Jones. In an attempt to show conclusively that the fibers consist of $\sigma 1$ protein, we developed a technique for isolating the $\sigma 1$ protein from reovirus particles. Drayna and Fields (9) observed that heating virions of reovirus T2 Jones to 55°C resulted in the loss of $\sigma 1$ protein from the particles. To confirm the heating conditions required to obtain loss of $\sigma 1$ protein from reovirus T2 Jones, virions and ISVPs were heated to various temperatures between 37 and 60°C. The particles were subsequently pelleted in an ultracentrifuge, and their proteins were analyzed in SDS-polyacrylamide gels. The results suggested that the $\sigma 1$ proteins of both virions and ISVPs were released from viral particles by mild heating (data not shown).

The $\sigma 1$ protein released from ISVPs of reovirus T2 Jones was isolated for an analysis of its morphology with the electron microscope. T2 Jones ISVPs were heated to 52°C, and the viral particles were then pelleted in an ultracentrifuge. The decanted supernatant was passed through a Centricon-30 microconcentration unit (Amicon) so that a greater than 100-fold concentration of the retained material was obtained. When this retained material was examined in an SDS-polyacrylamide gel, it was found to contain a single protein having the electrophoretic mobility of the T2 Jones $\sigma 1$ protein (Fig. 5, lanes 1 and 3). The heated and pelleted ISVPs, conversely, were found to have lost $\sigma 1$ (Fig. 5, lane 2). When the isolated $\sigma 1$ protein was negatively stained and analyzed with the electron microscope, structures consisting

of long fibers topped with knobs were observed in large numbers (Fig. 6). The appearance of these structures was strikingly uniform and similar, if not identical, to that of the fibers seen extending from viral particles. These observations suggest that the fibers are released from viral particles with mild heat but otherwise maintain a large part of their native morphology. They also indicate that the fibers are made up of the $\sigma 1$ protein.

A study was done to compare the lengths of negatively stained fibers seen extending from T2 Jones ISVPs with those observed in the preparation of $\sigma 1$ protein isolated after release from ISVPs at 52°C (Fig. 7). A total of 214 fibers attached to ISVPs were measured and had an average length of 27.8 ± 7.6 nm (278 ± 76 Å). The distribution of lengths was skewed toward values lower than the median length of 30.4 nm. The lengths of attached fibers appeared to approach a maximum value of between 36 and 40 nm. A total of 157 released fibers were measured and had an average length of 41.4 ± 3.4 nm. The distribution of lengths of released $\sigma 1$ was approximately normal (median, 42.2 nm). These findings are consistent with the identities of the released $\sigma 1$ fibers and the fibers extending from ISVPs and suggest that some portion of the fibrous domain of $\sigma 1$ is buried in the particle surface and not seen (therefore not included in measurements) when $\sigma 1$ protein is particle associated.

Two states of the $\sigma 1$ protein in reovirus virions. Additional electron micrographs of reovirus virions (Fig. 8 and 9) illustrate another interesting observation made during the course of these studies. The perimeters of T2 Jones virions were usually marked with shallow divots (Fig. 8; also refer to Fig. 2), making most of these particles appear faceted. These divots often appeared to be regularly spaced around the capsid perimeter. Many of the divots observed on T2 Jones virions were located at the base of an extended $\sigma 1$ fiber (Fig. 8; also refer to Fig. 2); however, divots without fibers were also seen. On the other hand, when a fiber could be traced down its stem to the capsid surface, a divot was usually found at its base. In the case of some T2 Jones virions, different regions of the particle perimeter appeared to have two distinct contours. Regions marked with divots often had fibers extending from them and had a faceted appearance, as described above. Other regions, however, lacked divots and had no associated fibers (Fig. 8). As a consequence, these latter regions of the particle perimeter had a rounded rather than a faceted contour. T2 Jones virions exhibiting both types of surfaces were commonly seen.

Virions that appeared completely round and lacked extended $\sigma 1$ fibers were sometimes observed in T2 Jones preparations but were seen much more frequently in purified preparations of other reovirus strains. T3 Dearing virions (Fig. 9) and T1 Lang virions (data not shown) were predominantly round and, as has already been stated, possessed extended fibers less frequently than T2 Jones did. A conventional electron micrograph of a T3 Dearing virion, as seen down its fivefold axis, is shown (Fig. 9A). Its entirely circular perimeter contrasted with faceted T2 Jones particles viewed in the same orientation (Fig. 8A, particle in upper right corner of lower panel). In freeze-dried, unstained preparations of reovirus T3 Dearing examined at the Brookhaven National Laboratories by STEM (36), round virions without fibers (Fig. 9B) were often seen, as well as occasional faceted virions with extended fibers (Fig. 9C). Using either CTM or STEM, we found that the variation observed in the contour of the virion capsid perimeter was independent of the axis on which the particle was sitting on the microscope grid. In addition, these variations were not

FIG. 5. SDS-polyacrylamide gel of ISVPs, heat-treated ISVPs, and heat-released $\sigma 1$ protein of reovirus T2 Jones. CsCl-isolated ISVPs (lane 1) were heated to 52°C. The heat-treated ISVPs were recovered by pelleting in an ultracentrifuge (lane 2). Material released from ISVPs by heating was concentrated by passing the ultracentrifugation supernatant through a Centricon-30 microconcentration unit (Amicon) (lane 3). Aliquots of each sample were disrupted by 2 min of boiling in sample buffer, and proteins were resolved in an SDS-8.25% polyacrylamide gel. Proteins were visualized by Coomassie staining.

FIG. 6. Electron micrographs of negatively stained $\sigma 1$ protein isolated from reovirus T2 Jones ISVPs. Isolated $\sigma 1$ protein was stained with 0.5% uranyl formate and examined by CTEM. For each structure, the fiber region and terminal knob are easily seen. Magnification, $\times 200,000$.

seen with ISVPs, which appeared to be uniformly round (Fig. 3). The observation of a variation in the contour of the reovirus outer capsid correlating with the presence or absence of fibers suggested to us that the $\sigma 1$ protein can occur in reovirus virions in both an extended and an unextended state. It also seems that in purified virions of different reovirus strains, the distribution of $\sigma 1$ protein between these two states may vary.

DISCUSSION

The discovery of the $\sigma 1$ fiber extending from the surfaces of reovirus particles has important implications for $\sigma 1$ function as the viral cell attachment protein (14, 37). The terminal knob of $\sigma 1$ protein, which was seen to be projected as far as 40 nm away from the particle surface by the fiber domain, is likely to contain the receptor-binding site of the $\sigma 1$ protein

(4). Displaying attachment sites at a distance from a particle surface by way of extended protein structures seems to be a general strategy that allows viruses and microorganisms to interact efficiently with target-cell receptors (8). The influenza virus hemagglutinin (40) is one well-characterized example of an animal virus cell attachment protein in which this strategy has been applied. The tail fibers of T-even bacteriophages (19) and bacterial adhesion pili (15) are examples of multi-protein assemblies that represent more complex applications of the strategy. It is not obvious whether displaying an attachment site at the end of a fiber simply allows a larger area to be traversed by the attachment site in its search for a ligand or whether it might in some cases allow the attachment site to reach a ligand that is sterically shielded. The extended domain of an attachment protein may also be important for reducing the relative speed and torque at the contact point between a virus or microorganism and its cell-bound receptor. One animal virus cell

FIG. 7. Histograms of lengths measured for the T2 Jones $\sigma 1$ fibers. T2 Jones $\sigma 1$ fibers extending from ISVPs ($n = 214$) (A) and heat-released, isolated T2 Jones $\sigma 1$ fibers ($n = 157$) (B) were measured from micrograph negatives (magnification, $\times 50,000$) with a Nikon V16 Profile Projector. The numbers of fibers counted to have lengths falling in consecutive 2.0-nm (20-Å) intervals are graphed. No fibers were measured to have lengths outside the illustrated range. The arrow in panel A indicates the value of the mean length of heat-released $\sigma 1$ fibers (41.4 nm) determined from the data shown in panel B.

attachment protein with an especially striking morphologic resemblance to the extended reovirus $\sigma 1$ protein is the fiber protein of adenovirus (35). This similarity raises possibilities for other similarities in structure, assembly, and mechanisms of uptake and infection between these two nonenveloped viruses. The possibility of structures analogous to the $\sigma 1$ fiber in other members of the family *Reoviridae* (12, 12a) is certainly also raised.

A prediction for the structural motif used to build the fibrous domain of $\sigma 1$ protein has been presented previously (2). This model includes a fiber constructed from a region of primary sequence in the amino-terminal third of $\sigma 1$ that shows strong sequence homology to myosin and contains a pattern of regular heptapeptide repeats of hydrophobic residues capable of forming an α -helical coiled coil (Fig. 3). A modification of the model that is indicated by the current findings concerns the length of the fibrous domain. It is evident from the pictures of isolated T2 Jones $\sigma 1$ protein that a relatively small amount of the visual area of the $\sigma 1$ protein complex is contributed by the terminal knob. This suggests that a larger portion of the $\sigma 1$ molecule than previously postulated participates in forming the fiber. The visual interpretation is supported by calculation. Assuming a rise of 0.149 nm (1.49 Å) per residue in a perfect α helix (17), we calculate that the region of sequence in the T2 Jones $\sigma 1$

protein that contains regular repeats of hydrophobic residues (approximately 150 amino acids) could occur as an extended α helix that is about 22 nm in length. This value differs substantially from the length of 41.4 nm measured for isolated T2 Jones $\sigma 1$ protein; calculation shows that a perfect α helix of this measured length would comprise about 280 amino acids. To account for the greater than expected length of the $\sigma 1$ fiber observed in this study, we predict that an additional region of primary sequence immediately carboxy

FIG. 8. Electron micrographs of negatively stained reovirus T2 Jones virions. Long arrows indicate some of the extended $\sigma 1$ fibers. Short arrows indicate divots found at the bases of extended fibers and at other locations on the perimeters of virions. Arcs on two virions highlight long regions of the capsid perimeter that have no extended fibers and exhibit rounded contours. Magnification, $\times 175,000$.

terminal to the region of regular heptapeptide repeats forms part of the fibrous domain. An accurate description of the regions of the $\sigma 1$ protein sequence that make up the fiber and their structural organization requires other biochemical and ultrastructural studies.

A correlate of the coiled-coil model for $\sigma 1$ structure is that the $\sigma 1$ protein normally occurs in multimeric complexes. This possibility is supported by biochemical data; however, these data currently conflict on whether the $\sigma 1$ protein exists as a dimer (14, 41) or tetramer (3) in viral particles. The morphologic uniformity of the $\sigma 1$ fibers isolated after heat release in this study and the consistency of these fibers with those seen extending from viral particles suggest that a large

portion of the $\sigma 1$ protein complex maintains its native conformation under our isolation conditions. Thus, we expect that the $\sigma 1$ protein released from viral particles by mild heat will be useful in confirming the order of the $\sigma 1$ multimer and in studying other aspects of $\sigma 1$ protein structure and function.

Other observations described in this study raise the interesting possibility that the $\sigma 1$ protein occurs in viral particles in two distinct states: extended and unextended. This suggestion is based in part on a variation in the frequency with which $\sigma 1$ fibers were seen extending from the virions of different reovirus strains. It is otherwise based on a variation observed in the contour of the capsid perimeters of different virion particles and a correlation of the contour type with the

FIG. 9. (A) CTEM micrograph of a negatively stained reovirus T3 Dearing virion. The particle is aligned with a fivefold symmetry axis nearly parallel to the electron beam. The capsid perimeter is completely rounded. Magnification, $\times 350,000$. (B and C) STEM (36) micrographs of unstained, freeze-dried reovirus T3 Dearing virions. Arrows indicate fibers extending from the virion in panel C. The capsid perimeter of the particle in panel B is rounded; that of the particle in panel C is faceted. Thick rods seen in the micrographs are tobacco mosaic virions which were included as a size markers. Magnification, $\times 250,000$.

presence or absence of extended σ 1 fibers. Other investigators (23) have described two forms of reovirus particles, i.e., circular and angular, but have attributed the variation to the axial projection of the capsid during electron microscopy. Numerous published micrographs reveal reovirus particles with two distinct contours (e.g., see reference 1). Our interpretation of all these observations is that, rather than being a consequence of axial projection, a divot in the virion surface represents a space revealed where a σ 1 fiber has been extended. Because of these divots, some virions appear faceted. When the σ 1 fibers of a virion remain mostly unextended, that particle appears round. Protruding fibers were not previously described in association with either round or angular reovirus particles; however, it is possible that fibers were present in the angular particles but not recognized. Consistent with this suggestion is our finding that the visualization of σ 1 fibers is sensitive to the thickness of the stain and support film used in electron microscopy.

An interesting question suggested by the occurrence of an unextended state of σ 1 protein is what form the unextended σ 1 protein complex assumes within the virion particle. It has been postulated that the fibrous domain of σ 1 sticks down into the λ 2 protein spikes at the vertices of the virion icosahedron (2). The regular spacing of extended σ 1 fibers observed around perimeters of particles is consistent with the vertex location of σ 1 (10); however, the current findings also show that a large portion of the fibrous domain and the globular head region of the σ 1 protein are not always buried in the λ 2 spike but can extend free of the particle surface. We are currently attempting to determine whether local dissociation of the σ 1 multimer or sliding of the σ 1 fibrous domain within the λ 2 core spike plays a role in the packaging of σ 1 in virions and the extension of the σ 1 fiber. The observed maximum length of 36 to 40 nm for ISVP-attached fibers, in comparison with the average length of 41.4 nm for released fibers, probably indicates that some minimum length of the extended σ 1 is always buried in the viral capsid and serves to keep the protein attached to its viral particle. This implies a particle-proximal virus attachment site in the extended σ 1 protein that is distinct from the previously discussed distal cell attachment site.

In summary, we obtained electron micrographs of the reovirus cell attachment protein σ 1 and observed the σ 1 protein complex extending from the surfaces of viral particles. Our observations may suggest a linearly oriented model of σ 1 protein structure that includes an amino-terminal virus attachment site, a long fiber domain constructed from sequences in the amino-terminal two-thirds of the σ 1 sequence, and a carboxy-terminal cell attachment domain that sits as a knob at the end of the σ 1 fiber. Conformational changes in the σ 1 protein complex and in other components of the viral outer capsid are likely to be important for the transition of σ 1 between its unextended and extended states. The unextended state of σ 1 may correspond to a form of the protein that provides a maximal amount of environmental stability to the virus in its movement between hosts in its natural cycle of infection. In its extended state, σ 1 is probably most efficient at accessing and binding to receptors on the surfaces of target cells and thereby carrying out its primary function as the cell attachment protein. We are currently investigating the mechanism of σ 1 extension and its biologic consequences.

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