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Evidence that the Sigma 1 Protein of Reovirus Serotype 3 Is a Multimer

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In this report, we study the reovirus serotype 3 (strain Dearing) σ1 protein obtained from various sources: from *Escherichia coli* expressing σ1 protein, from reovirus-infected mouse L cells, and from purified reovirions. We demonstrate that the σ1 protein is a multimer in its undisrupted form and present biochemical evidence suggesting that the multimer is made up of four σ1 subunits.

A critical event in viral pathogenesis is the interaction between viral cell attachment proteins and host cell receptors. Viral attachment proteins have been studied to better define the earliest events in virus-cell interactions: cell surface receptor binding and entry into the cell cytoplasm. In general, viral cell attachment proteins have been shown to be located on the viral surface, allowing for interaction with the appropriate host receptor. Although the attachment protein (or proteins) has been identified for a number of viruses, the details of their structure and how this structure relates to function remain poorly understood (30). Recent studies have utilized primary sequence information coupled with ultrastructural and crystallographic analyses to provide important new insights into the structure of certain viral attachment proteins and to define which regions of these proteins are involved in attachment to cells (19, 31, 45).

We have been studying the mammalian reoviruses as a system to understand the molecular and genetic determinants of viral virulence (14). The reovirus virion is icosahedral and possesses a double capsid shell (the core and the outer capsid) (20). The viral genome is enclosed in the core and is made up of 10 segments (genes) of double-stranded RNA. The S1 double-stranded RNA segment encodes the σ1 protein which is an outer capsid (surface) protein. The σ1 protein is the viral hemagglutinin (44) and is responsible for determining various viral properties including cell and tissue tropism (25, 35, 36, 40, 43) and serotype-specific humoral and cellular immunity (15, 16, 39, 41, 42). Most of these properties can be explained by the fact that the σ1 protein is the reovirus cell attachment protein (22, 39).

Since the reovirus σ1 protein plays a central role in viral pathogenesis, we and others have attempted to study the details of its structure and to relate the structure to its functions. As part of this analysis, the S1 gene was sequenced (3, 6, 24). The S1 sequence predicts that the σ1 protein of the type 3 (T3) (Dearing) strain of reovirus consists of 455 amino acids. The sequence is notable for an extensive heptapeptide repeat pattern (a-b-c-d-e-f-g) of primarily hydrophobic residues in the a and d positions that is found in the amino-terminal portion of the protein. This pattern is typical of α-helical portions of proteins that associate to form coiled-coil superhelices. Since prior studies (22, 33) had suggested that a dimer of σ1 is located at each of the virion vertices, we initally proposed that these dimers are stabilized by an extended amino-terminal coiled coil (3).

In this report, we studied the reovirus T3 (Dearing) σ1 protein obtained from various sources: from *Escherichia coli* expressing σ1 protein (from a cloned cDNA S1 gene), from reovirus-infected mouse L cells, and from purified reovirions. We describe techniques by which a high-molecular-weight, apparently multimeric form of σ1 protein was identified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and provide preliminary evidence that this multimer is made up of four σ1 subunits.

MATERIALS AND METHODS

Cells and virus. Mouse L cells were grown in suspension in Joklik modified minimal medium supplemented with 5% fetal calf serum and 2 mM glutamine. Reovirus T3 (strain Dearing) is the laboratory stock. Purified reovirus T3 virions were obtained by growing viral stocks in suspension cultures of mouse L cells at 34°C and extracting virions from infected cells as previously described (12). To label the viral proteins with ^{35}S, the only modification necessary was to add [^{35}S]methionine (6 μCi/ml) to the cell suspension in methionine-depleted medium 16 h after viral infection.

E. coli JM105 harboring the reovirus T3 S1 gene subcloned into an expression vector containing the *tac* promoter was grown in M9 medium supplemented with 0.5% Casamino Acids (Difco Laboratories, Detroit, Mich.) and 50 μg of ampicillin per ml (26).

Purification of σ1 protein from *E. coli* JM105 cells containing reovirus T3 S1 gene subcloned into a *tac* expression vector. The construction of the reovirus S1 expression vector has been previously described (26). Briefly, the S1 cDNA of reovirus T3 (3) was subcloned into the *tac* expression vector pKK233-2 (1), and then the recombinant vector was transfected into *E. coli* JM105. For purification of expressed σ1 protein, a 500-ml culture of transformed *E. coli* JM105 was grown to an optical density at 550 nm of 0.6, and then chloramphenicol was added to a final concentration of 68 μg/ml, and the culture was incubated overnight at 37°C. The cells were harvested and then washed in 40 ml of 1× M9 salts. To induce σ1 protein, we suspended the cell suspension in 50 ml of 1× M9 salts containing a final concentration of 25 μCi of [^{35}S]methionine per ml, 1 mM isopropyl-β-D-

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thiogalactopyranoside, and 0.5× Difco methionine assay medium. The cells then were incubated at 37°C for 1 h with shaking, harvested, washed twice with phosphate-buffered saline (PBS), and sonicated to lyse the cells. The cell extract was centrifuged at 10,000 × *g* for 20 min. The extract was chromatographed by size exclusion on a Sephacryl S-200 column (150 mM NaCl, 10 mM Tris hydrochloride, pH 8). The σ1 protein was purified on an immunoaffinity column as described below. Protein samples eluted from the immunoaffinity column were monitored by SDS-PAGE as described below.

Immunoaffinity chromatography column. Ascites fluid (4 ml) of monoclonal antibody G5 (5) which is directed against the reovirus T3 σ1 protein was fractionated on DEAE and coupled to Sepharose 4BCl by the cyanogen bromide method (27). The immunoaffinity chromatography column had a volume of 10 ml and approximately 16 mg of protein. Cellular extracts containing σ1 protein were passed over the column resin multiple times at 4°C. The column resin then was thoroughly washed with PBSA (PBS and 0.02% sodium azide) until there was no detectable radioactivity and protein (based on optical density at 280 nm) in the eluate. Nonspecific bound material was eluted by washing the column with 0.15 M NaCl containing 0.5% sodium deoxycholate. Material that was specifically bound to monoclonal antibody G5 was eluted in 0.05 M triethylamine (TEA) (pH 11.6) and then neutralized with 1 M Tris hydrochloride (pH 8). The protein in the eluates was monitored by SDS-PAGE.

Purification of σ1 protein from reovirus-infected L-cell extracts. Mouse L cells at a concentration of 10^7 cells per ml were infected with reovirus T3 at a multiplicity of infection of 7. After 1 h of absorption at room temperature, the cells were diluted to 10^6 cells per ml and incubated at 34°C for 16 h. The cells were harvested and suspended at a concentration of 10^7 cells per ml in methionine-free medium containing 50 μCi of [^{35}S]methionine per ml. The cells were incubated at 34°C for 4 h, harvested, and washed with medium. The infected cells were suspended in buffer (0.1 M NaCl, 0.005 M $MgCl_2$, 0.01 M Tris hydrochloride, pH 7.4) with protease inhibitors (10 mM benzamidine, 10 mM phenylmethylsulfonyl fluoride, 5 mM EDTA, 0.02% azide, 1 mM each of the following: leupeptin, bacitracin, soya bean trypsin inhibitor). The cells were disrupted by nitrogen cavitation for 20 min at 400 lb/in^2 in a Parr bomb, and cellular debris was pelleted by low-speed centrifugation. The extract was adjusted to 0.15 M KCl and centrifuged at 160,000 × *g* for 2 h. The supernatant (S100) was collected and chromatographed by G5 immunoaffinity column chromatography as described above, except the final elution buffer included 0.1% sodium deoxycholate.

SDS-PAGE and fluorography. Discontinuous SDS-PAGE was performed by the protocol of Laemmli (21), except for variations in sample preparation technique described in the text. Both 10 and 8.25% acrylamide gels were used. Gradient gels (5 to 15% and 5 to 20%) were made by the method of Walker (37). Gels containing ^{35}S-labeled proteins were treated with Autofluor (National Diagnostics, Inc.) or Enlightning (New England Nuclear Corp., Boston, Mass.), dried under vacuum, and exposed to Kodak XAR-5 film at −70°C. Alternatively, some gels were dried under vacuum without prior treatment with a fluor and exposed to Kodak DEF film at room temperature.

Peptide mapping by limiting proteolysis with SDS-PAGE. The protocol used for peptide mapping was a modification of the procedure described by Cleveland et al. (7). Protein samples were subjected to electrophoresis in a 5 to 20% SDS-polyacrylamide gel after the appropriate sample preparation techniques (as described in the legend to Fig. 2). The gel was fixed in 30% isopropanol–10% acetic acid and then stained in the fix solution containing 0.5% Coomassie brilliant blue R-250 (Sigma Chemical Co., St. Louis, Mo.) and destained in 16.5% methanol–5% acetic acid. Visualized protein bands were excised from the gel and soaked first in 125 mM Tris hydrochloride (pH 6.8)–0.1% SDS–0.1% 2-mercaptoethanol for 30 min and then in 125 mM Tris hydrochloride (pH 6.8)–0.1% SDS for 30 min. Gel slices were boiled for 30 s in the second soak solution and then placed into the wells of a second 5 to 20% polyacrylamide gel. The gel slices were overlaid with Cleveland sample buffers (7) containing 0.4, 2, or 10 μg of α-chymotrypsin (Sigma) before electrophoresis. When the samples had migrated halfway through the stacking gel, electrophoresis was stopped for 30 min, and the samples were subjected to partial proteolysis before resumption of electrophoresis.

SDS-PAGE in a second slab-gel dimension. Samples were incubated under various conditions, as indicated in the text or figure legends, and subjected to electrophoresis in a 5 to 20% gradient slab gel. The polyacrylamide gels were stained and destained as described above. The protein band was excised from the gel, rinsed for 30 min in 125 mM Tris hydrochloride (pH 6.8)–0.5% SDS buffer, and heated to 95°C for 2 min. Gel slices were placed in the wells of a second 5 to 20% gradient gel and overlaid with Laemmli sample buffer (21) before electrophoresis.

Hemagglutination assay. Serial twofold dilutions of reovirus T3 or purified σ1 protein were made in 96-well, V-shaped-bottom microtiter plates in a final volume of 50 μl. Bovine erythrocytes (citrated) (Colorado Serum Co., Denver, Colo.) were washed twice with PBS, and an equal volume was added to the microtiter plate wells to obtain a final concentration of 0.4% (vol/vol). The plates were kept at 4°C, and hemagglutination activity was observed by 4 h.

Cross-linking of [^{35}S]methionine-labeled σ1 protein subunits. The subunits of the multimeric ^{35}S-labeled σ1 protein, purified from bacterial extracts by G5 immunoaffinity chromatography as described above, were cross-linked with dimethyl suberimidate (DMS) by the method of Davies and Stark (11). The ^{35}S-labeled σ1 protein (3 × 10^4 cpm per assay) was incubated in the presence of increasing concentrations of DMS (0 to 500 μg per assay) for 3 h at room temperature. The assay buffer consisted of 15 mM NaCl and 0.2 M triethanolamine hydrochloride (pH 8.5), and the final assay volume was 50 μl. After 3 h the reaction was terminated by the addition of 50 μl of SDS-PAGE sample buffer, and the mixture was heated in a boiling water bath for 5 min before SDS-PAGE.

RESULTS

Isolation and characterization of σ1 protein expressed in *E. coli*. Cell lysates of *E. coli* cells expressing the reovirus T3 (Dearing) σ1 protein (26) were chromatographed by size exclusion on a Sephacryl S-200 column, and the void volume containing the σ1 protein was further chromatographed on an immunoaffinity column containing the anti-σ1 monoclonal antibody G5 (5). The immunoaffinity-bound material was eluted at high pH (TEA, pH 11.6) and then neutralized for storage and analysis.

Samples from pooled TEA fractions were analyzed by SDS-PAGE (Fig. 1). When the sample was boiled before electrophoresis, a single major protein band was observed (Fig. 1A, lane 2). This protein band migrated with the same

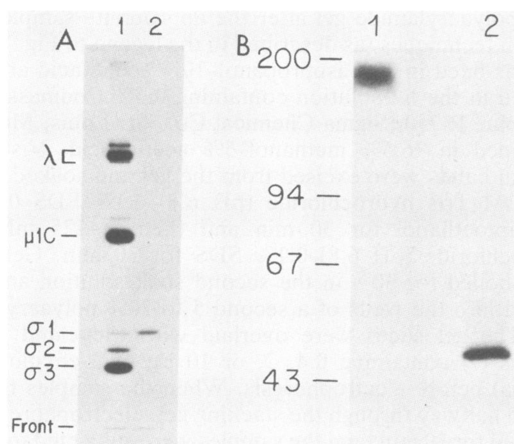

FIG. 1. SDS-PAGE of immunoaffinity-purified expressed σ1 protein. (A) [^{35}S]methionine-labeled virions of reovirus T3 (Dearing) purified from infected mouse L cells (lane 1) and [^{35}S]methionine-labeled σ1 protein purified from expressing *E. coli* (lane 2) were prepared for electrophoresis by boiling for 2 min in Laemmli sample buffer. Samples were analyzed by 8.25% SDS-PAGE. Virion proteins are labeled on the left, as is the dye front. (B) [^{35}S]methionine-labeled, purified, expressed σ1 protein was added to sample buffer and maintained at room temperature (lane 1) or boiled (lane 2) before electrophoresis in a 10% SDS-polyacrylamide gel. Positions of molecular weight markers ($\times 10^3$) are indicated. Gels were prepared for fluorography as described in Materials and Methods.

electrophoretic mobility (approximately 44,000 molecular weight) as virion σ1 protein of reovirus T3 (Dearing) (Fig. 1A, lane 1). The identity of the expressed protein obtained by this purification procedure as authentic, biologically functional σ1 has been thoroughly characterized by Pelletier et al. (26). We confirmed that our purified protein was σ1 by partial chymotrypsin proteolysis (data not shown; see Fig. 2). Our purified protein also displayed functional activity to a dilution of 1:32 in hemagglutinating bovine erythrocytes at 4°C, a property previously described for the T3 (Dearing) σ1 (44).

During our studies, we discovered that if samples from TEA fractions were mixed with sample buffer but not boiled before SDS-PAGE, the electrophoretic mobility of the purified σ1 protein changed dramatically. The 44,000-molecular-weight protein band was absent in this case and was replaced in approximately stoichiometric amounts by a band migrating at about 200,000 molecular weight (Fig. 1B, lane 1). This 200,000-molecular-weight protein band was excised from a gel and compared with protein bands of purified monomer σ1 protein from virions by partial proteolysis with chymotrypsin (Fig. 2). The identity of the proteolytic digestion fragments obtained from these two proteins confirmed that the 200,000-molecular-weight protein represents a form of σ1 protein with alternate mobility. These data also show that identical partial proteolytic patterns were obtained for expressed σ1 protein and virion-associated σ1 protein.

σ1 Protein isolated from infected L-cell extracts. To study σ1 protein made in infected cells, we infected mouse L cells with reovirus T3, and newly synthesized proteins, both viral and cellular, were labeled by the addition of [^{35}S]methionine late in viral infection. The infected cells were disrupted, and preliminary steps were taken to isolate σ1 protein by G5 immunoaffinity chromatography.

Figure 3A shows an autoradiogram of a 10% SDS-polyacrylamide gel displaying designated fractions eluted from the anti-σ1 (G5) immunoaffinity chromatography column. These samples were not boiled before electrophoresis. Fractions 1 to 28 represent unbound proteins removed by the initial PBSA washing of the column resin after application of the infected-cell extract. Fractions 32 to 81 contain proteins that were nonspecifically bound to the column resin and were eluted by a NaCl-deoxycholate wash. We noted a distinct protein band at approximately 65,000 molecular weight in fractions 32 to 34. The identity of this protein is unknown. We speculate that this is a cellular protein, perhaps the reovirus cell surface receptor protein (8) which bound to the σ1 protein and was subsequently eluted by NaCl-deoxycholate. Studies to identify this protein are in progress. Fractions 82 to 86 contain proteins that were eluted with a TEA buffer (pH 11.6) and thus were specifically bound to the G5 immunoaffinity column (i.e., the σ1 protein). The major protein eluted with the high pH TEA buffer had an electrophoretic mobility of approximately 200,000 molecular weight (Fig. 3A). The other proteins eluted in TEA in this experiment were also seen in the other washes and were probably due to overloading the immunoaffinity column with cell extract.

Figure 3B again shows the major 200,000-molecular-weight protein present in the pooled TEA fractions (lane 1, upper arrow) and further shows that upon boiling of the sample before electrophoresis, this protein band disappeared, and a new major protein band of approximately 44,000 molecular weight, the expected location of monomeric σ1 protein, appeared (lane 2, lower arrow). These data indicate that the σ1 protein isolated from reovirus-infected cells can also be identified in SDS-PAGE in a high-molecular-weight form and therefore that the high-molecular-weight form of σ1 protein isolated from bacterial cells is not an artifact of expression in a foreign host. The

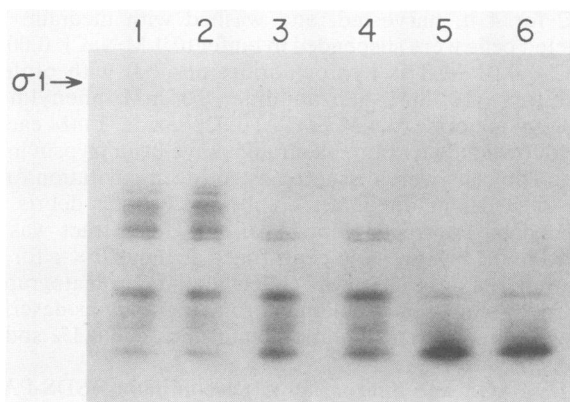

FIG. 2. SDS-PAGE of partial chymotrypsin proteolysis of expressed σ1 protein and virion-associated σ1 protein. [^{35}S]methionine-labeled σ1 protein purified from expressing *E. coli* was added to Laemmli sample buffer at room temperature before electrophoresis in a 5 to 20% SDS-polyacrylamide gel. The σ1 protein band (200,000 molecular weight) was excised from the gel and subjected to partial proteolysis (lanes 2, 4, and 6). [^{35}S]methionine-labeled virions of reovirus T3 (Dearing) purified from infected mouse L cells were added to Laemmli sample buffer and boiled for 2 min before electrophoresis in a 5 to 20% SDS-polyacrylamide gel. The monomer σ1 protein band (44,000 molecular weight) was then excised from the gel and subjected to partial proteolysis (lanes 1, 3, and 5). Partial digestion of the samples was performed with the following amounts of α-chymotrypsin: lanes 1 and 2, 0.4 μg; lanes 3 and 4, 2 μg; lanes 5 and 6, 10 μg. The position of the intact monomer σ1 protein is indicated.

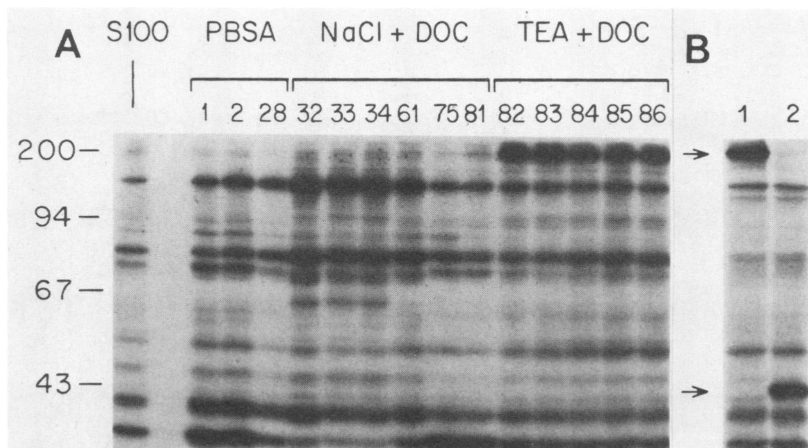

FIG. 3. SDS-PAGE of immunoaffinity-purified σ1 protein from mouse L cells infected with reovirus T3 (Dearing). (A) [^{35}S]methionine-labeled σ1 protein was partially purified by passing an extract from infected cells (S100) through a G5 immunoaffinity column, washing the resin with PBSA (fractions 1 to 28), next washing with 0.15 M NaCl–0.5% deoxycholate (DOC) (fractions 32 to 81), and finally eluting with 50 mM TEA–0.1% deoxycholate (fractions 82 to 86). Samples from indicated fractions were added to sample buffer but not boiled before electrophoresis in a 10% SDS-polyacrylamide gel. Numbers show molecular weight ($\times 10^3$). (B) Fractions eluted in TEA-deoxycholate were pooled, mixed with sample buffer, and either maintained at room temperature (lane 1) or boiled (lane 2) before electrophoresis in a 10% SDS-polyacrylamide gel. Arrows identify eluted σ1 protein (see text).

large difference in electrophoretic mobility observed for σ1 is unlikely to result simply from a variation in conformation of a monomer of this protein. Rather, it is likely that the high-molecular-weight form of σ1 protein seen in SDS-PAGE represents a multimer made up of σ1 subunits.

Virion-associated σ1 protein. To determine whether the putative multimeric form of σ1 protein is found associated with virions, we purified T3 (Dearing) virions from infected mouse L cells and analyzed them by SDS-PAGE (Fig. 4). As previously described, expressed σ1 protein and σ1 protein isolated from infected cells migrated with a high molecular weight (200,000) in SDS-PAGE if mixed with sample buffer but not boiled before electrophoresis (Fig. 1 and 3). When purified virions were similarly incubated in sample buffer (62.5 mM Tris hydrochloride [pH 6.8], 2% SDS, 5% 2-mercaptoethanol, 10% glycerol, 0.001% bromophenol blue) without heating before electrophoresis, only the σ3 protein was released from virion particles and migrated into the gel (Fig. 4, lane 1); other virion proteins (including σ1) presumably remained particle associated and therefore were trapped in the sample wells of the gel (data not shown). Incubation of virions in the same sample buffer at either 60°C (Fig. 4, lane 2) or 95°C (Fig. 4, lane 3) resulted in disruption of virions and migration of σ1 to its expected position at 44,000 molecular weight.

The composition of the sample buffer was altered in an attempt to obtain disruption of virion particles while maintaining the high-molecular-weight form of the virion σ1 protein. Increasing the amount of SDS up to 10% had no effect on particle disruption at room temperature (data not shown). However, increasing the amount of 2-mercaptoethanol in the sample buffer to 15% consistently yielded disruption of virions at room temperature (Fig. 4, lane 4). In this case, the σ1 protein was absent from its expected position (44,000 molecular weight), and a protein band of approximately 200,000 molecular weight was seen (Fig. 4, lane 4). Heating an identical sample to 95°C before electrophoresis resulted in the disappearance of the 200,000-molecular-weight protein and the appearance of σ1 migrating at its expected position of 44,000 molecular weight (Fig. 4, lane 5).

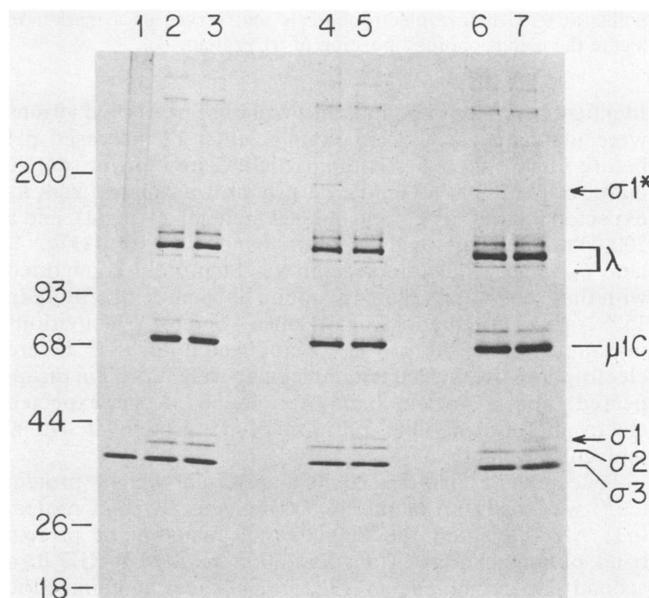

FIG. 4. SDS-PAGE of proteins in purified virions of reovirus T3 (Dearing). [^{35}S]methionine-labeled virions were purified from infected mouse L cells and exposed to various conditions in preparation for electrophoresis in a 5 to 20% SDS-polyacrylamide gradient gel. Samples are grouped in the figure according to the nature of the sample buffer added to virions: lanes 1 to 3, sample buffer (pH 6.8), 5% 2-mercaptoethanol; lanes 4 and 5, sample buffer (pH 6.8), 15% 2-mercaptoethanol; lanes 6 and 7, sample buffer (pH 8.3), 5% 2-mercaptoethanol. (All samples otherwise contained final concentrations of 62.5 mM Tris, 2% SDS, 10% glycerol, and 0.001% bromophenol blue). In each sample buffer group, samples were incubated at different temperatures: lanes 1 and 4, room temperature for 20 min; lanes 2 and 6, 60°C for 10 min; lanes 3, 5, and 7, 95°C for 2 min. Positions of molecular weight markers ($\times 10^3$) are indicated on the left; virion proteins are labeled on the right. σ1* denotes the high-molecular-weight form of the σ1 protein.

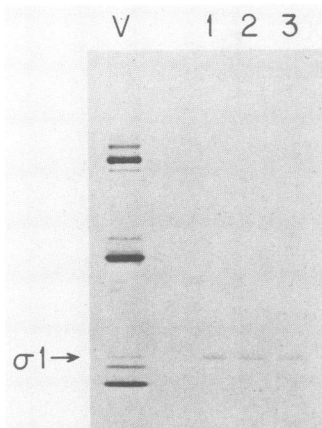

FIG. 5. SDS-PAGE of the multimer form of virion-associated σ1 protein in a second slab-gel dimension. [^{35}S]methionine-labeled virions of reovirus T3 (Dearing) were disrupted under selected conditions and subjected to electrophoresis in a 5 to 20% SDS-polyacrylamide gradient gel. The σ1 protein bands (monomer or multimer) identified by staining with Coomassie blue were excised from the gel and analyzed by electrophoresis in a second 5 to 20% SDS-polyacrylamide gradient gel (pictured here). Disruption conditions for the first dimension: lane 1, sample buffer (pH 6.8), 5% 2-mercaptoethanol, 95°C (σ1 monomer form); lane 2, sample buffer (pH 6.8), 15% 2-mercaptoethanol, 25°C (σ1 multimer form); lane 3, sample buffer (pH 8.3), 5% 2-mercaptoethanol, 60°C (σ1 multimer form). Lane V shows proteins from virions disrupted in sample buffer at 95°C before electrophoresis and serves as a marker to locate the electrophoretic position of σ1 protein.

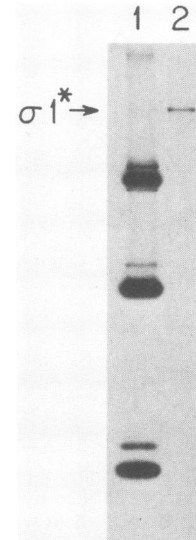

FIG. 6. Comparison of electrophoretic mobilities of virion-associated and expressed σ1 multimers. [^{35}S]methionine-labeled purified T3 reovirions (lane 1) or [^{35}S]methionine-labeled, immunoaffinity-purified, expressed σ1 protein (lane 2) were mixed with sample buffer containing 15% 2-mercaptoethanol and maintained at room temperature before electrophoresis in a 8.25% SDS-polyacrylamide gel. The electrophoretic position of the putative σ1 multimer is denoted by the arrow at σ1*.

Identical behavior of σ1 mobility was observed when virions were incubated at 60°C in sample buffer of increased pH before electrophoresis. After particle disruption in sample buffer of pH 8.3 at 60°C, the σ1 protein was absent from its expected migrating position (44,000 molecular weight), and a 200,000-molecular-weight protein band was seen (Fig. 4, lane 6). This 200,000-molecular-weight protein comigrated with that seen after virion disruption at room temperature in 15% 2-mercaptoethanol (Fig. 4, lanes 4 and 6). When virions in sample buffer at pH 8.3 were heated to 95°C before electrophoresis, the 200,000-molecular-weight protein disappeared, and a protein band was found at the expected electrophoretic mobility position of σ1 protein at 44,000 molecular weight.

To establish that the 200,000-molecular-weight protein (σ1*) was made up of the 44,000-molecular-weight protein (σ1), we subjected the 200,000-molecular-weight protein band obtained after virion disruption to SDS-PAGE in a second slab-gel dimension (Fig. 5). Gel slices containing the high-molecular-weight protein (200,000) from virions disrupted at room temperature in 15% 2-mercaptoethanol (lane 1) and at 60°C at pH 8.3 (lane 2) as well as a gel slice containing the 44,000-molecular-weight virion σ1 protein from normally disrupted virions (lane 3) were heated to 95°C before the second SDS-PAGE to ensure complete denaturation of any protein multimers present in the gel slices. Each of these samples exhibited a single protein band in the second dimension that comigrated with the σ1 protein in a marker lane (Fig. 5, lane V), indicating that each of the original 200,000-molecular-weight protein bands contained only σ1 protein. Partial proteolysis of the high-molecular-weight protein band (200,000) obtained by 15% 2-mercaptoethanol disruption of virions also indicated its identity with virion monomer σ1 protein (data not shown). These data indicate that under the appropriate conditions virion-associated σ1 protein can be identified in multimeric form in SDS-PAGE.

Comparison of virion-associated and expressed σ1 multimers. Virion-associated (Fig. 6, lane 1) and purified expressed σ1 proteins (Fig. 6, lane 2) in their high-molecular-weight forms migrated with the same electrophoretic mobilities in SDS-PAGE. Table 1 summarizes the apparent molecular weights seen for virion and expressed σ1 proteins when various conditions were used to prepare the samples for SDS-PAGE and demonstrates that virion and expressed σ1 behave identically under these different conditions. These data argue for the identity of a multimeric form of σ1 protein from these two sources. The data in Table 1 also emphasize the contributions made by increased concentrations of 2-mercaptoethanol, increased pH, and decreased temperature to observing the σ1 multimer in SDS-PAGE. Note that virion σ1 protein can be visualized in SDS-PAGE after preparation at 25°C only in the presence of increased concentrations of

TABLE 1. Electrophoretic mobility of σ1 protein in SDS-PAGE with different sample preparation techniques

Sample buffer conditions		Electrophoretic mobility of virion-associated (V) and purified expressed (E) σ1 protein[a]:					
% 2-Mercaptoethanol	pH	25°C[b]		60°C		95°C	
		V	E	V	E	V	E
5	6.8	ND	H	L	L	L	L
15	6.8	H	H	H	H	L	L
5	8.3	ND	H	H	H	L	L
1	8.3	ND	H	L	L	L	L

[a] ND, No disruption of virions was obtained under these conditions so that σ1 protein cannot be identified in the gel; H, high-molecular-weight form (multimer) of σ1 protein; L, low-molecular-weight form (monomer) of σ1 protein.

[b] Incubation temperature of sample before electrophoresis.

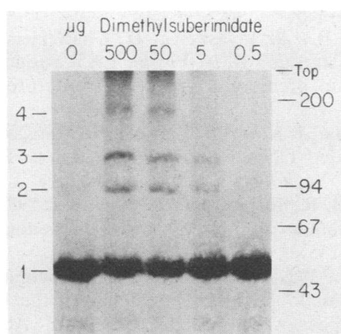

FIG. 7. Cross-linking of σ1 protein multimer. Increasing amounts of DMS were added to purified expressed σ1 protein and incubated for 3 h. Samples were then mixed with sample buffer and boiled for 5 min before electrophoresis in a 5 to 15% gradient polyacrylamide gel. The numbers above the lanes indicate the amount of DMS added to each sample (0 to 500 μg). The numbers on the left-hand side (1 to 4) represent the putative number of σ1 subunits in the observed band. Positions of molecular weight markers ($\times 10^3$) are indicated on the right, as is the location of the top of the gel.

2-mercaptoethanol since under other conditions this σ1 protein apparently remains particle associated.

Cross-linking of the ^{35}S-labeled σ1 protein subunits. To evaluate the number of subunits in the σ1 multimer present in purified preparations of expressed σ1 protein, we performed cross-linking studies. Increasing amounts of the cross-linking reagent DMS were incubated with purified, expressed σ1 protein before boiling in sample buffer and SDS-PAGE. When no cross-linking reagent was added to the protein sample and the sample was boiled before SDS-PAGE, the σ1 protein was dissociated into its monomer form (Fig. 7, band 1). As greater amounts of DMS were added, cross-linking of the σ1 protein subunits was achieved, and three additional higher-molecular-weight bands were seen (bands 2, 3, and 4). Average molecular weight values calculated for those bands from a set of four different cross-linking experiments were 48,000 for band 1, 97,000 for band 2, and 122,000 for band 3. Band 4 appeared outside the linear range in these gels but consistently migrated just under the 200,000-molecular-weight marker protein, as did σ1 protein that had not been boiled before SDS-PAGE. These data suggest that the high-molecular-weight bands (Fig. 7, bands 2 to 4) obtained with cross-linking represent multiples of the monomer σ1 protein. The appearance of a total of four bands in these experiments suggests that the undisrupted form of σ1 protein is a tetramer.

DISCUSSION

In this report, we identified a high-molecular-weight multimeric form of the reovirus T3 (Dearing) σ1 protein in SDS-polyacrylamide gels. In our studies, we used σ1 protein isolated from *E. coli* containing the cDNA of the S1 gene, from reovirus-infected L cells, and from purified virions. We demonstrated the identity of the σ1 multimer obtained from these different sources and presented evidence that this multimer is made up of four σ1 subunits.

The mobility of reovirus T3 (Dearing) σ1 in SDS-PAGE, whether as a multimer (~200,000 molecular weight) or a monomer (~44,000 molecular weight), depends on the method used to prepare samples before electrophoresis. We discovered that increased concentration of 2-mercaptoethanol and increased pH in the sample buffer, as well as decreased temperature at which samples were heated before SDS-PAGE, each contributed independently to the isolation of σ1 protein in its multimer form. These variables presumably operate through effects on specific regions of σ1 that provide interactions between σ1 subunits, even under conditions that are highly disruptive (2% SDS, 60°C). An effect of 2-mercaptoethanol on protein multimers, apparently through unique solvent properties of this molecule rather than its reducing capability, has been described previously (32). Potential effects of pH and temperature on a protein multimer are obvious.

To evaluate the number of subunits in the σ1 multimer, we performed cross-linking studies with purified expressed σ1 protein. The observation of four bands of increasing molecular weight upon cross-linking with DMS, the highest cross-linked protein band migrating with the same electrophoretic mobility as the identified σ1 multimer, is most consistent with the interpretation that the multimer is made up of four σ1 subunits, i.e., that it is a tetramer. We are currently engaged in additional studies to define more definitively the nature of the σ1 multimer and the regions of the protein that participate in the formation of this multimer.

Previous investigators had speculated that virion-associated σ1 protein is a multimer. On the basis of densitometric scanning of autoradiograms of ^{35}S-labeled virion-associated proteins, Smith et al. (33) estimated that σ1 accounts for only about 1% of virion protein and that there are approximately 31 molecules of σ1 per virion particle. Lee et al. (22) used monoclonal antibodies to determine that the σ1 protein is located at the virion vertices in association with the λ2 protein and suggested that there are actually 24 molecules of σ1 per virion particle, a dimer at each of the 12 vertices. With consideration for these previous findings, the data presented in this report would suggest that there are maximally 48 molecules of σ1 per virion (a tetramer at the 12 vertices) and thus that σ1 more nearly accounts for 2% of total virion protein. This value does not seem to us to be significantly different from the previous 1% estimation. It also is possible that a percentage of σ1 protein is lost from T3 (Dearing) virions during the virus purification procedure, leading to an underestimation of the number of σ1 molecules per virion by the method of Smith et al. (33). In fact, Drayna and Fields (13) have shown that the association of σ1 protein with virions is unstable under certain conditions. Consistent with this interpretation, we routinely find that purified virions of most other reovirus strains contain larger amounts of σ1 than those of T3 (Dearing) (data not shown).

Considering the predicted amino acid sequence (3, 6, 24) and electron microscopy work performed in our laboratory (D. B. Furlong, M. L. Nibert, and B. N. Fields, submitted for publication), we propose that the σ1 protein is made up of an extended amino-terminal α-helical tail topped with a carboxy-terminal globular domain containing the cell attachment site. The similarity of a region of amino acid sequence in the amino-terminal third of σ1 to the coiled-coil dimer region of myosin initially led us to predict that this region of σ1 participates in forming a coiled-coil dimer. Our current observation that the σ1 protein associates to form tetramers may reflect that four rather than two σ1 monomers interact via this region of primary sequence. Alternatively, it is possible that the hydrophobic interactions provided by regular heptapeptide repeats in the myosinlike region of σ1 occur primarily between two subunits, while additional, distinct interactions stabilize the higher-order σ1 tetramer. Such a model has been proposed to explain the structure of

the intermediate filament four-chain complex (34) and the filaments of myosin (23). The four-α-helical bundle is a commonly observed structural motif (38). A more definitive description of the three-dimensional structure of the σ1 multimer awaits further study.

The multimeric nature of σ1 may contribute to the function of σ1 as the reovirus cell attachment protein (22, 39). The identification of the receptor-binding domain of σ1 and its positioning in the σ1 multimer coupled with further studies of reovirus-host cell interactions will give insight into the importance of σ1 multimeric structure for cell attachment. It is of interest that other microorganisms have used coiled-coil α-helices for stabilizing multimers of surface proteins that function in binding to cells (9). The influenza viral hemagglutinin is the best described of these proteins (45). Other microbial surface multimers include the trypanosomal variable surface glycoprotein (10, 17) and the streptococcal M protein (28).

An unusual aspect of the proposed tetrameric nature of the reovirus σ1 protein is indicated by the association of σ1 with the λ2 protein at the capsid vertices of assembled virions (22). Since the λ2 vertex structure is pentameric (29), our findings suggest that there is a four to five symmetry mismatch between the σ1 and λ2 proteins. Symmetry mismatches have been identified at the head-tail junctions of all double-stranded DNA bacteriophages studied to date and have been proposed to allow the movement of protein assemblies relative to one another during the process of DNA translocation at this phage-head vertex (4, 18). It is interesting to speculate that a symmetry mismatch at reovirus vertices reflects dynamic processes that occur at these sites such as the extrusion of nascent mRNA from the λ2 spike (2).

In summary, we have presented evidence that the σ1 protein of reovirus T3 (Dearing) is a multimer in its undisrupted form and have shown biochemical analysis that suggests that the multimer is in a tetrameric form. Our future studies include crystallographic analysis of the σ1 protein to evaluate definitively the multimeric form of the protein. Elucidation of the ultrastructure of the σ1 protein will provide important insights into the structural organization of virion vertices and the various aspects of σ1 function as the reovirus cell attachment protein.

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