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The N-Terminal Heptad Repeat Region of Reovirus Cell Attachment Protein $\sigma 1$ Is Responsible for $\sigma 1$ Oligomer Stability and Possesses Intrinsic Oligomerization Function

GUSTAVO LEONE, ROY DUNCAN, DAVID C. W. MAH,¹ ANGELA PRICE,
L. WILLIAM CASHDOLLAR,* AND PATRICK W. K. LEE²

Department of Microbiology and Infectious Diseases, University of Calgary Health Sciences Centre, Calgary, Alberta, Canada T2N 4N1; and *Department of Microbiology, Medical College of Wisconsin, Milwaukee, Wisconsin 53286

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The oligomerization domain of the reovirus cell attachment protein ($\sigma 1$) was probed using the type 3 reovirus $\sigma 1$ synthesized *in vitro*. Trypsin cleaved the $\sigma 1$ protein (49K molecular weight) approximately in the middle and yielded a 26K N-terminal fragment and a 23K C-terminal fragment. Under conditions which allowed for the identification of intact $\sigma 1$ in the oligomeric form (~ 200 K) by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, the N-terminal 26K fragment was found to exist as stable trimers (80K) and, to a less extent, as dimers (54K), whereas the C-terminal fragment remained in the monomeric form. A polypeptide (161 amino acids) containing the N-terminal heptad repeat region synthesized *in vitro* was capable of forming stable dimers and trimers. Using various criteria, we demonstrated that the stability of the intact $\sigma 1$ oligomer is conferred mainly by the N-terminal heptad repeat region. Our results are summarized in a model in which individual heptad repeats are held together in a three-stranded α -helical coiled-coil structure via both hydrophobic and electrostatic interactions. © 1991 Academic Press, Inc.

INTRODUCTION

The reovirus cell attachment protein (protein $\sigma 1$) is strategically located at the twelve vertices of the outer capsid of the viral icosahedron (Lee *et al.*, 1981; Furlong *et al.*, 1988) and plays a pivotal role in viral infectivity and tissue tropism (Sharpe and Fields, 1985). In electron microscopy, this protein can sometimes be seen as lollipop-shaped structures with proximal fibrous tails and distal globular heads projecting from the surfaces of viral particles (Furlong *et al.*, 1988). Protein $\sigma 1$ purified from reovirions or from a vaccinia expression system also has a similar morphology (Furlong *et al.*, 1988; Banerjea *et al.*, 1988; Fraser *et al.*, 1990). The observations that the C-terminal half of $\sigma 1$ contains the receptor-binding domain (Nagata *et al.*, 1987; Yeung *et al.*, 1989) and the N-terminal one-quarter possesses intrinsic virion-anchoring property (Mah *et al.*, 1990) suggest that the fibrous tail and the globular head represent the N- and C-terminal portions, respectively, of this protein. These findings concur with sequence analysis of the reovirus S1 gene (encoding $\sigma 1$), which predicts the existence of distinct structural domains in the $\sigma 1$ protein (Bassel-Duby *et al.*, 1985; Duncan *et al.*, 1990; Nibert *et al.*, 1990). The

N-terminal one-third of $\sigma 1$ is highly α -helical and contains a heptapeptide repeat of hydrophobic residues, suggestive of a coiled-coil structure. This is followed by a middle region composed largely of β -sheets. The C-terminal one-third of $\sigma 1$ does not possess any distinct patterns and is therefore predicted to assume a complex globular structure.

The oligomeric nature of $\sigma 1$ has also been examined (Bassel-Duby *et al.*, 1987; Banerjea *et al.*, 1988). When subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) under dissociating conditions (boiled in SDS-containing sample buffer), the 455 amino acid long $\sigma 1$ migrates as a monomeric 44K molecular weight protein. However, if the boiling step is omitted, $\sigma 1$ migrates as an oligomer (~ 200 K molecular weight). This observation, coupled with the identification of a total of four bands upon chemical cross-linking of purified $\sigma 1$ (with the largest species migrating at a position corresponding to approximately 200K), has led to the suggestion that $\sigma 1$ is a tetramer (Bassel-Duby *et al.*, 1987). The oligomerization state of $\sigma 1$ is apparently closely linked to its function since of the two $\sigma 1$ species (monomeric and oligomeric) synthesized in an *in vitro* transcription and translation system, only the oligomeric form is capable of binding to cell receptors (G. Leone, R. Duncan, and P. W. K. Lee, unpublished data). This observation prompted us to examine the nature of $\sigma 1$ oligomerization, an understanding of which should lead to better definitions of structure–function relationships of this protein.

¹ Present address: McIntyre Medical Sciences Building, McGill Cancer Centre, 7/F, 3655 Drummond Street, Montreal, Quebec, Canada H3G 1Y6.

² To whom requests for reprints should be addressed.

In this report, we used trypsin treatment as an initial step to identify regions that are important for maintaining and stabilizing the $\sigma 1$ oligomeric structure. Of the two fragments generated by such treatment (Yeung *et al.*, 1989), only the N-terminal fragment, but not the C-terminal fragment, was found to exist as stable trimers (and dimers to a less extent) upon SDS-PAGE analysis. Subsequent *in vitro* transcription and translation experiments revealed that the N-terminal one-third of $\sigma 1$, which harbors the heptapeptide repeat region, possesses intrinsic dimerization and trimerization function. We further demonstrated that ionic interactions, in addition to hydrophobic interactions, within the heptapeptide repeat region are also responsible for stabilizing the $\sigma 1$ oligomer. These observations have led to a structural model of the oligomerization domain of protein $\sigma 1$.

MATERIALS AND METHODS

Plasmid construction

The plasmid (pG4T3) used in the present study was derived from our prokaryotic S1-gene expression vector pSP4 (Masri *et al.*, 1986) in which the S1 gene contained additional sequences at the 3'-end derived from pBR322 (*Pst*I-*Eco*RI) during the original subcloning procedure. This plasmid was cleaved with *Sst*II, which cuts the S1 gene at position 1397, and the synthetic linker

5'-GGCACTGGGGCATTTCATCGGTAC-3'

3'-CGCCGTGACCCCGTAAAGTAGC-5'

was added which contained the authentic 3'-terminal S1 gene sequence from the *Sst*II site to the 3'-end of the gene where a unique *Kpn*I site was added. A *Bam*HI-*Kpn*I fragment was isolated (from the *Bam*HI site at position 15, which cuts immediately after the ATG initiation codon of $\sigma 1$ to the newly introduced *Kpn*I site at the 3'-end of the gene) and ligated to an *Eco*RI-*Bam*HI translation initiation linker (Pharmacia):

5'-AATTCGAGGAAAAATTATG-3'

3'-GCCTCCTTTTTTAATACCTAG-5'.

The resultant *Eco*RI-*Kpn*I fragment was cloned into the *Eco*RI-*Kpn*I site of pGEM-4 (Promega Biotec) to produce pG4T3.

In vitro transcription-translation

The plasmid pG4T3 was linearized with either *Hind*III or *Eco*RV restriction endonucleases and used in *in vitro* transcription reactions to generate full-length or truncated S1 mRNAs. The mRNAs were then translated *in*

vitro in rabbit reticulocyte lysates according to the manufacturer's specifications (Promega). A typical translation mixture contained 50–100 ng of RNA and 20 μ Ci of [³⁵S]methionine in a total volume of 25 μ l. After incubation at 37° for 45 min, reactions were stopped by the addition of 200 μ l phosphate-buffered saline (PBS). Protein sample buffer (5 \times) was then added (final 1 \times concentration was 50 mM Tris, pH 6.8, 1% SDS, 2% β -mercaptoethanol, 10% glycerol, and 0.01% bromophenol blue) and the mixtures were further incubated at 37° for 30 min (nondissociating condition) or boiled for 5 min (dissociating condition) prior to SDS-PAGE.

Trypsin digestion

Upon completion of the *in vitro* translation reaction, TLCK-treated trypsin (Sigma) dissolved in PBS (0.05 mg/ml) was added to the translation mixtures to a final concentration of 0.005 mg/ml (unless otherwise stated). After incubation at 37° for 30 min, trypsin inhibitors (soybean and egg white trypsin inhibitors, Sigma) were added and the mixtures were further incubated in protein sample buffer prior to SDS-PAGE (see above).

Immunoprecipitation

The monoclonal anti- $\sigma 1$ antibody G5 has been previously described (Burstin *et al.*, 1982) and shown to interact with the C-terminal tryptic fragment of $\sigma 1$ (Yeung *et al.*, 1989). The N-terminal specific polyclonal anti- $\sigma 1$ antiserum was prepared in rabbits using the SDS-PAGE-purified trpE- $\sigma 1$ fusion protein (containing trpE and residues 1–158 of $\sigma 1$) expressed in *Escherichia coli* using the pATH3 vector (Cashdollar *et al.*, 1989). Aliquots of lysates or trypsin-treated lysates were mixed with an equal volume of appropriate dilutions of the antibodies and incubated at room temperature for 1 hr. Fixed *Staphylococcus aureus* (10% suspension) that had been preadsorbed with BSA (5 mg/ml) was then added to the mixture and incubated for an additional 30 min. Immunoprecipitates were washed three times with wash buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 0.1% SDS, 1% Triton X-100), resuspended in 140 μ l high-pH buffer (50 mM Tris, 0.1 M H₃PO₄, 2 mM DTT, 0.1% SDS, 6 M urea, pH to 11.6 with NaOH), and incubated at 37° for 45 min. Suspensions were then pelleted and supernatants neutralized with 7 μ l neutralizing solution (0.1 M H₃PO₄, 1 M Tris, pH 7.4). Protein sample buffer was then added to the samples and either incubated at 37° for 30 min (nondissociating condition) or boiled for 5 min (dissociating condition) prior to SDS-PAGE.

SDS-PAGE

Discontinuous SDS-PAGE was performed using the protocol of Laemmli (1970). Both 10 and 12.5% acryl-

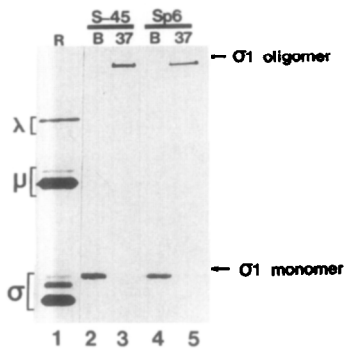


FIG. 1. Detection of oligomeric forms of *in vitro*-translated σ 1 and soluble σ 1 from reovirus-infected cells. [35 S]Methionine-labeled reovirus-infected cell lysates (S-45) prepared as previously described (Lee *et al.*, 1981), and [35 S]methionine-labeled *in vitro* translation products of the reovirus S1 mRNA in rabbit reticulocyte lysates (Sp6), were precipitated with an anti- σ 1 monoclonal antibody (G5). After being released from the immunoadsorbent, precipitated proteins were mixed with protein sample buffer and were either boiled for 5 min (B) or incubated at 37° for 30 min (37) prior to SDS-PAGE. R (lane 1) represents purified [35 S]methionine-labeled reovirus.

amide gels were used. Gels containing 35 S-labeled proteins were fixed and then treated with DMSO-PPO, dried under vacuum, and exposed to Kodak XAR-5 film at -70°.

Gels to be used for band excision were dried under vacuum without prior treatment, and then exposed to film. Developed x-ray film was superimposed on dried gels and bands to be excised were marked with a pouncer. Marked protein bands were excised from gels and rehydrated in Laemmli running buffer. Proteins from excised bands were electroeluted into electroelution cups in a volume of 200 μ l. Protein sample buffer was added to electroeluted proteins and boiled for 5 min prior to SDS-PAGE.

RESULTS

Generation of σ 1 oligomers *in vitro*

Since some of the following studies on σ 1 oligomerization involved the use of genetically truncated protein σ 1, it was necessary to express σ 1 in an *in vitro* system and to ascertain that oligomeric σ 1 was indeed generated in such a system. To this end, T3 reovirus S1 mRNA was prepared *in vitro* using Sp6 RNA polymerase and translated in a rabbit reticulocyte lysate. Translation products were then immunoprecipitated with an anti- σ 1 antibody (G5) and analyzed by SDS-PAGE under conditions that would not cause the disruption of σ 1 oligomers (see Materials and Methods). The results, illustrated in Fig. 1, show that stable σ 1 oligomers were indeed produced *in vitro* (lanes 4 and 5) and their migration rate was identical to that of

authentic σ 1 from T3 reovirus-infected cells (lanes 2 and 3). Like the authentic protein, σ 1 oligomers synthesized *in vitro* were capable of binding to cell receptors (R. Duncan, G. Leone, and P. W. K. Lee, unpublished observations), and were cleaved by trypsin to yield a well-defined pattern (see below). Some σ 1 monomers (44K) were also produced in this system, but they were not precipitable by the anti- σ 1 antibody, did not manifest cell-binding function, and were highly susceptible to degradation by trypsin or chymotrypsin even when these proteases were used at very low concentrations (data not shown). Subsequent analysis exclusively of the oligomeric form of σ 1 was therefore possible.

Trypsin digestion of σ 1 oligomers

Previously it was found that trypsin cleaves the σ 1 oligomer near the middle to generate two fragments of approximately equal size (Banerjee *et al.*, 1988; Yeung *et al.*, 1989). The cleavage site has now been determined to be between Arg245 and Ile246 (Duncan and Lee, unpublished data). Thus the monomeric forms of the N-terminal fragment (245 amino acids) and the C-terminal fragment (210 amino acids) have molecular weights of approximately 26K and 23K, respectively, corresponding to their migration rates in SDS-PAGE under dissociating conditions (Yeung *et al.*, 1989). Such a cleavage pattern was also obtained with σ 1 synthesized *in vitro* (Fig. 2, lane 3). An additional minor band of approximately 21K molecular weight was also observed sometimes. Using N- and C-terminal-specific

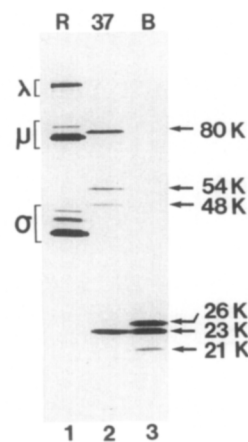


FIG. 2. Trypsin digestion of protein σ 1. *In vitro*-translated [35 S]methionine-labeled σ 1 protein was digested with 0.005 mg/ml of trypsin as described under Materials and Methods. After the addition of trypsin inhibitors and protein sample buffer, samples were either incubated at 37° for 15 min (37) or boiled for 5 min (B) prior to SDS-PAGE. R (lane 1) represents purified [35 S]methionine-labeled reovirus. Molecular weights of individual protein bands are indicated at right.

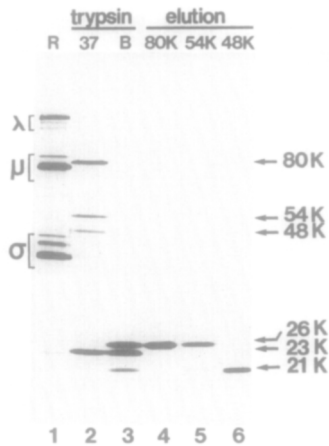


Fig. 3. SDS-PAGE of oligomeric tryptic fragments of $\sigma 1$. Protein $\sigma 1$ tryptic digests prepared as described under Materials and Methods were either incubated at 37° for 15 min (37) or boiled for 5 min (B) in protein sample buffer and then subjected to SDS-PAGE. The 80K, 54K, and 48K fragments from the unboiled (37) sample were excised from the gel, eluted, boiled in protein sample buffer, and then reanalyzed by SDS-PAGE (lanes 4, 5, and 6, respectively). R (lane 1) represents purified [³⁵S]methionine-labeled reovirus. Molecular weights of individual protein bands are indicated at right.

sera, we have identified the 26K and 21K tryptic fragments to be of N-terminus origin, and the 23K fragment to be of C-terminus origin (Fig. 4).

N-terminal half of $\sigma 1$ is a trimer

To see whether any of the tryptic fragments could be identified in the oligomeric state, trypsin-treated $\sigma 1$ in SDS-containing sample buffer was incubated at 37° (rather than boiled) prior to SDS-PAGE. It was found that the 26K N-terminal fragment was replaced quantitatively by a band migrating at approximately 80K molecular weight (Fig. 2, lane 2). The migration rate of the 23K C-terminal fragment remained unchanged. The 21K N-terminal fragment was absent; instead two faint bands of approximately 48K and 54K molecular weight appeared. Protein bands of 80K, 54K, and 48K molecular weight were excised from a gel, eluted, boiled in sample buffer, and subjected to SDS-PAGE (Fig. 3). Both the 80K and 54K proteins were found to be converted to the 26K N-terminal fragment, whereas the 48K protein was converted to the 21K N-terminal fragment.

The most reasonable interpretation of these results would be that the 80K and 54K bands represent the trimer and dimer forms, respectively, of the 26K N-terminal fragment, and the 48K band represents the dimer of the 21K N-terminal fragment. The region in the 26K N-terminal fragment that is absent in the 21K N-terminal fragment may therefore be involved in stabiliz-

ing the third subunit of the 80K N-terminal trimer. The identity of this region is presently unknown.

Titration of trypsin digestion of $\sigma 1$ oligomers

It is important to note that under conditions where the N-terminal tryptic fragment exists as stable trimers (and dimers), the C-terminal fragment was consistently found to be in the monomeric state. This is in contradiction to the findings of Banerjee *et al.* (1988) who reported that both the N- and C-terminal tryptic fragments exist as stable oligomers (tetramers) in SDS at 37°. To reconcile such differences, we decided to examine the effects of varying the trypsin concentration on $\sigma 1$ cleavage pattern. At the lowest concentration (0.005 mg/ml) of trypsin that cleaves $\sigma 1$ oligomers completely, an identical pattern to that shown in Fig. 2 was observed (Fig. 4A, lane 2). Increasing the concentration of trypsin to 0.05, 0.1, and 0.5 mg/ml, and subsequently boiling the samples in sample buffer, resulted in the gradual disappearance of the 26K N-terminal fragment with the concomitant appearance, in almost stoichiometric amounts, of a 25K molecular weight fragment (Fig. 4A, lanes 3–5). Thus the 25K band is a cleavage product of the 26K band. The 23K C-terminal fragment remained unchanged.

When identical samples were instead incubated at 37° in sample buffer prior to SDS-PAGE, both the 26K and 25K bands were replaced by bands with molecular weights of approximately 80K and 75K, respectively (Fig. 4A, lanes 6–9). Again, the mobility of the 23K C-terminal fragment remained unchanged. Radioimmunoprecipitation of the samples with the N- and C-terminus-specific antibodies confirmed that the 25K and 75K proteins, like the 26K and 80K proteins, were of N-terminus origin (Fig. 4B), and that the 23K protein was of C-terminal origin (Fig. 4C).

It is possible to explain the discrepancies between our present findings and those reported by Banerjee *et al.* (1988) on the basis of the methods used for protein $\sigma 1$ detection. A polyclonal anti-T3 reovirus serum was used by the aforementioned investigators to identify $\sigma 1$ tryptic fragments on Western blots as opposed to radioimmunoprecipitation with anti- $\sigma 1$ N- and C-terminal-specific antibodies used in our studies. In our hands, polyclonal anti-native $\sigma 1$ serum is incapable of recognizing the presumably denatured C-terminal tryptic fragment on a blot, although the N-terminal fragment is easily detectable. These observations, together with the fact that the trypsin concentration (1 mg/ml) used by Banerjee *et al.* was within the concentration range where we found both the 26K and the 25K N-terminal cleavage products, have led us to conclude that the two bands previously identified by these inves-

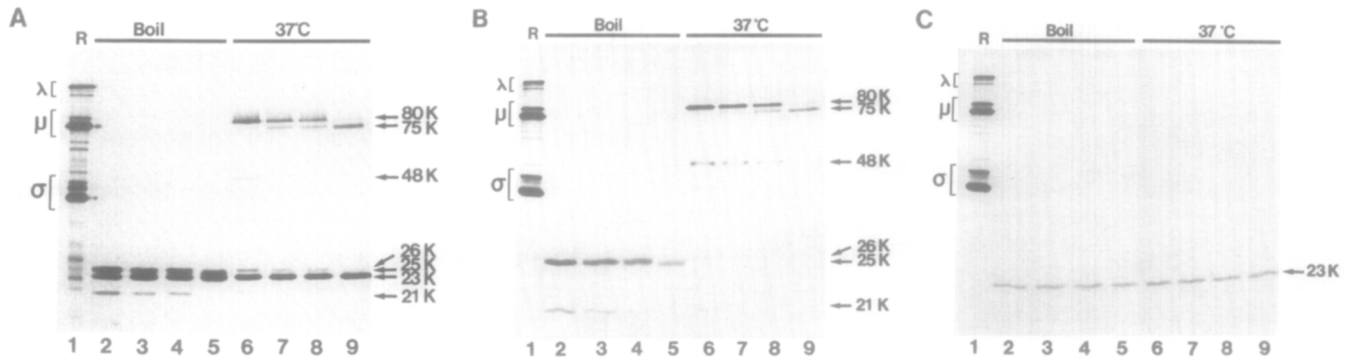


FIG. 4. Titration of trypsin digestion of $\sigma 1$ oligomers. *In vitro*-translated [^{35}S]methionine-labeled $\sigma 1$ was digested with various concentrations [0.005 mg/ml (lanes 2 and 6), 0.05 mg/ml (lanes 3 and 7), 0.1 mg/ml (lanes 4 and 8), and 0.5 mg/ml (lanes 5 and 9)] of trypsin. Tryptic digests were either subjected to SDS-PAGE directly (A), or immunoprecipitated with the anti- $\sigma 1$ N-terminal-specific serum (B), or with the anti- $\sigma 1$ C-terminal-specific antibody (C) prior to SDS-PAGE. All samples were either boiled for 5 min (Boil) or incubated at 37° for 15 min (37°) in protein sample buffer before electrophoresis. R (lane 1) represents purified reovirus. Molecular weights of individual bands are indicated at right.

tigators as N-terminal and C-terminal oligomers correspond to our 80K and 75K bands, and are therefore in fact both N-terminal oligomers.

Role of the N-terminal portion in stabilizing $\sigma 1$ oligomers

To determine the extent of involvement of the N-terminal portion in stabilizing the $\sigma 1$ oligomer, native $\sigma 1$ and trypsin-treated $\sigma 1$ were subjected to various treatments prior to SDS-PAGE and the relative stability of native $\sigma 1$ oligomers and N-terminal trimers (80K protein) was compared. Whereas dissociation of oligomers by heat was used as a general measure of oligomer stability, the effects of pH variation and presence or absence of urea and β -mercaptoethanol were also examined.

Under all conditions tested, native $\sigma 1$ oligomers and N-terminal trimers behaved identically (Fig. 5). Both $\sigma 1$ oligomers and N-terminal trimers were stable at 50° in sample buffer but dissociated at 60° (Fig. 5A). Both were stable under alkaline (pH 11.6) to mild acidic (pH 6.0) conditions, but dissociated at pH 5.5 (Fig. 5B). Urea (6 M) apparently had no effect on either oligomeric species (Fig. 5C). It was previously reported that $\sigma 1$ oligomers were rendered less stable when the concentration of β -mercaptoethanol in the sample buffer was reduced (Bassel-Duby *et al.*, 1987). Consistent with these findings, we observed that both native $\sigma 1$ oligomers and N-terminal trimers were less stable in the total absence of β -mercaptoethanol (Fig. 5C).

In summary, the stability of the N-terminal trimer was found to be very similar to that of the native $\sigma 1$ oligomer. These data suggest that interactions between $\sigma 1$ subunits responsible for stabilizing the oligomeric

structure occur mainly, although by no means solely, within the N-terminal half of the $\sigma 1$ protein.

Involvement of ionic interactions within the N-terminal half of $\sigma 1$

The absence of cysteine residues within the N-terminal tryptic fragment of $\sigma 1$ suggests that the stabilizing effect of β -mercaptoethanol could not be directly due to its reducing properties. Indeed, enhanced oligomer stability was not observed when another reducing agent, dithiothreitol, was used in place of β -mercaptoethanol (Fig. 6A).

A less well-characterized property of β -mercaptoethanol is that of chelation (McMichael and Ou, 1977). If β -mercaptoethanol indeed stabilizes oligomers by chelating divalent cations, addition of divalent cations should destabilize oligomers. Indeed, MgCl_2 at concentrations above 1 mM was found to destabilize N-terminal trimers (Fig. 6B) as well as native $\sigma 1$ oligomers (data not shown). Other divalent cations (zinc and calcium) had a similar effect (data not shown). The effects of divalent cations and β -mercaptoethanol on oligomer stability are clearly antagonistic. Thus the destabilizing effect of 5 mM and 20 mM MgCl_2 could be reversed by the inclusion of 1 and 5% β -mercaptoethanol, respectively, in the sample buffer (Fig. 6B). Similar results were obtained using EDTA as the chelator in place of β -mercaptoethanol, except that at EDTA concentrations above 5 mM, oligomer stability was inconsistent (data not shown).

The disruption of $\sigma 1$ oligomers by divalent cations suggests that ionic interactions are involved in stabilizing the N-terminus trimer (and hence the $\sigma 1$ oligomer). Indeed, the distribution of charged residues in the N-terminal coiled-coil region was found to highly favor the

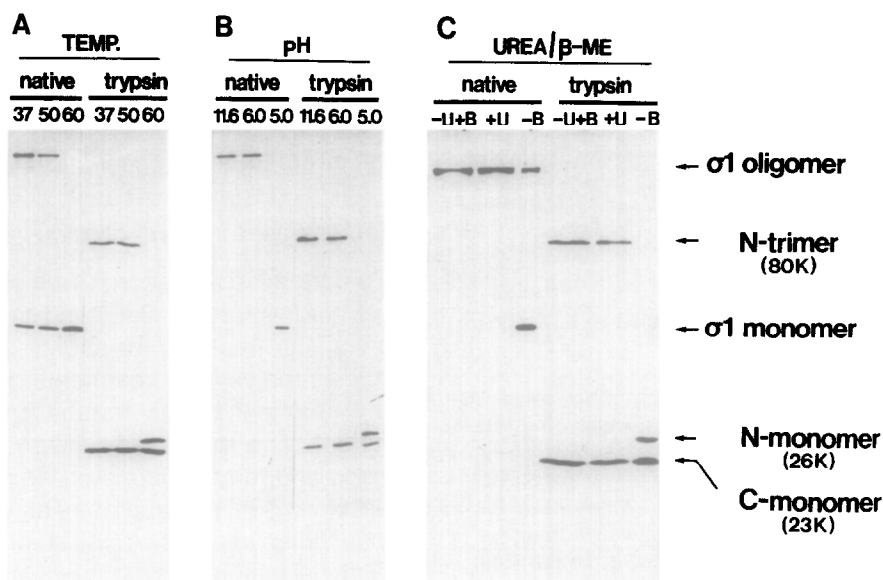


Fig. 5. Oligomer stability of intact $\sigma 1$ and of $\sigma 1$ tryptic fragments under various conditions. (A) Trypsin-treated (trypsin) and untreated (native) $\sigma 1$ were incubated in protein sample buffer at 37° (37), 50° (50), or 60° (60) for 30 min prior to SDS-PAGE. (B) Samples were diluted threefold with 50 mM Tris buffer at the pHs (11.6, 6.0, and 5.0) indicated and incubated at room temperature for 15 min. Protein sample buffer was then added to the samples which were then further incubated at 37° for 30 min prior to electrophoresis. (C) Samples were incubated in regular protein sample buffer which contained 2% β -mercaptoethanol (-U+ β , control), or in protein sample buffer containing 6 M urea in addition to 2% β -mercaptoethanol (+U), or in protein sample buffer lacking both urea and β -mercaptoethanol (- β), at 37° for 30 min prior to electrophoresis. Native and trypsin lanes in each experiment originate from the same gel.

formation of salt bridges between $\sigma 1$ subunits (see Discussion). However, the presence of the extended heptad repeat in the same region suggests that hydrophobic interactions must play a major role in oligomer stabilization. In this regard, the aforementioned ionic interactions are presumed to serve an augmentative function. In agreement with the above hypothesis, temperature stability experiments indicated that in the presence of divalent cations (abolishing ionic interac-

tions), oligomers were stable up to a temperature of 20°, but in the absence of divalent cations (maximizing ionic interactions), oligomers were stable up to a temperature of 50° (data not shown).

Intrinsic dimerization and trimerization properties of the heptad repeat region of $\sigma 1$

It is believed that sequences with heptapeptide repeats of apolar residues are involved in stabilizing

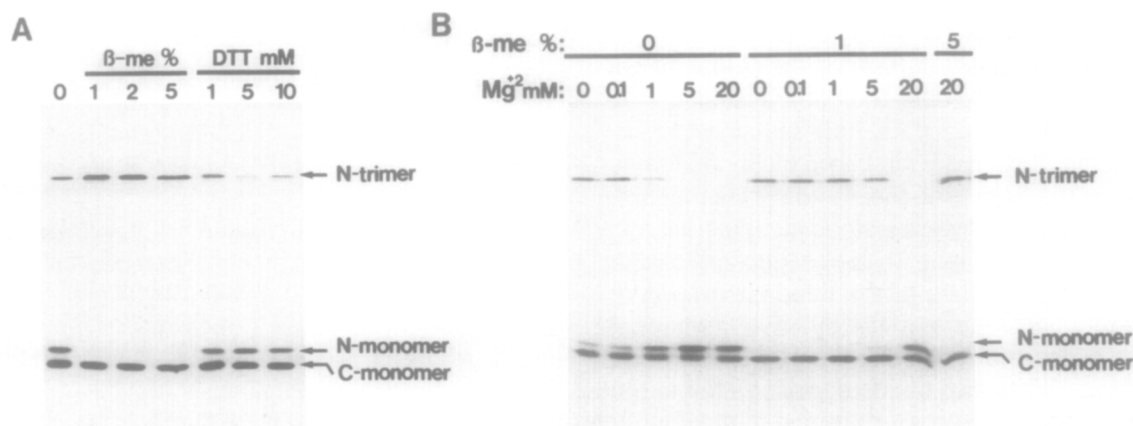


Fig. 6. Stability of the N-terminal tryptic trimer in the presence of β -mercaptoethanol, dithiothreitol (DTT), and Mg^{2+} . (A) Trypsin-treated $\sigma 1$ was incubated in protein sample buffer containing either β -mercaptoethanol or DTT at concentrations indicated for 30 min at 37° prior to SDS-PAGE. (B) $MgCl_2$ was added to trypsin-treated $\sigma 1$ to the final concentrations indicated. Samples were then immediately incubated in protein sample buffer containing 0, 1, and 5% β -mercaptoethanol for 30 min at 37° prior to electrophoresis.

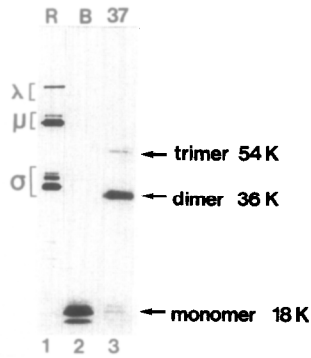


Fig. 7. SDS-PAGE of an *in vitro*-translated $\sigma 1$ polypeptide containing the N-terminal heptapeptide repeat. mRNA encoding the first 161 amino acids of $\sigma 1$ was made and translated as described under Materials and Methods. After the addition of protein sample buffer, the samples were either boiled for 5 min (B) or incubated at 37° for 30 min (37) prior to electrophoresis. R represents the reovirus marker. Molecular weights of individual protein bands are indicated at right.

coiled-coil structures through hydrophobic interactions between the α -helices. In the case of $\sigma 1$, the heptad repeat region spans the N-terminal one-third of the protein (from residue 28 to 164). It was then of interest to see whether this region possesses intrinsic dimerization and, in view of the trimeric nature of the N-terminal tryptic fragment of $\sigma 1$, trimerization functions.

To this end, the plasmid pG4T3 was treated with the restriction endonuclease *EcoRV*, which cuts the S1 gene at nucleotide 497 (encoding the N-terminal 161 amino acids). Run-off mRNA transcripts were then prepared and translated *in vitro*, and the ability of the translational products to oligomerize was then determined by SDS-PAGE. The results are shown in Fig. 7. When the samples were boiled in sample buffer prior to SDS-PAGE, closely migrating bands of approximately 18K molecular weight were found (Fig. 7, lane 2), as was predicted from the amino acid sequence. The lack of absolute homogeneity in size of the translational products was due to the absence of a translation stop codon in the mRNA transcripts, necessitating translation termination to occur by the falling off of ribosomes close to, but not precisely at, the 3' ends of the mRNAs. When identical samples were instead incubated at 37° in sample buffer (Fig. 7, lane 3), there was a noticeable decrease in the intensities of the bands migrating at approximately 18K. Concomitantly, two additional bands with estimated molecular weights of 36K and 54K appeared. These two bands corresponded to the dimeric (36K) and trimeric (54K) forms of the *EcoRV* translation products. Clearly, the dimer was the predominant oligomeric form identified. Whether trimer formation was inefficient or whether trimers were less stable in our SDS-PAGE system is not known. None-

theless, for the first time, direct evidence is presented that demonstrates the intrinsic ability of a heptad repeat (18 repeats in this case) to form dimers and trimers, an ability that may very well depend on the number of repeats present in the polypeptide.

Heptad repeat imparts oligomer stability

To determine the extent to which the heptad repeat region is involved in stabilizing the N-terminal tryptic trimers, the *EcoRV* translational products were subjected to the various treatments previously applied to the N-terminal tryptic fragment and the native $\sigma 1$ oligomer. The results are shown in Fig. 8. Both the dimeric and trimeric forms of the heptad repeat were unstable at 50°, a temperature at which the N-terminal tryptic trimer was found to be stable. However, like the N-terminal trimer, heptad dimers and trimers were both stable under alkaline (pH 11.6) to mild acidic (pH 6.0) conditions (but dissociated at pH 5.5), and in 6 M urea, and were destabilized by the absence of β -mercaptoethanol or by the presence of divalent cations in the sample buffer. Again, as observed for the N-terminal tryptic trimer, the destabilizing effect of divalent cations could be neutralized by β -mercaptoethanol.

DISCUSSION

The reovirus cell attachment protein, $\sigma 1$, is a homooligomer and has a lollipop morphology. The receptor-binding domain of $\sigma 1$ resides in the C-terminal globular head and the virion-anchoring domain resides in the N-terminal fibrous tail. Sequence analysis of the S1 genes of all three reovirus serotypes concur with such structural and functional domain assignments. The ob-

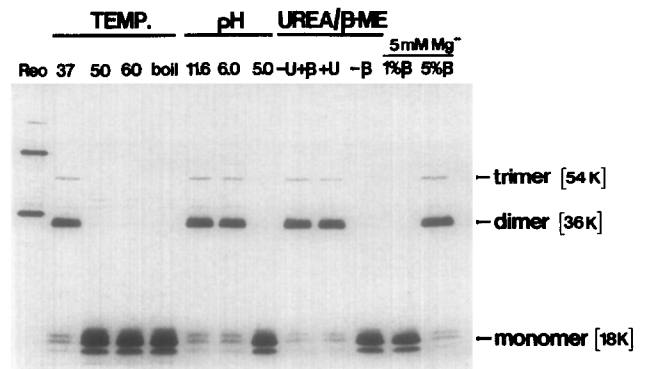


Fig. 8. Oligomer stability of the N-terminal 161 amino acid long polypeptide. The polypeptide synthesized was subjected to various treatments as described in the legends to Fig. 5 and 6. Protein sample buffer was then added and the samples were incubated at 37° for 30 min prior to SDS-PAGE. Reo represents the reovirus marker. Molecular weights are indicated at right.

ervation that receptor-binding function is manifested by the oligomeric form, but not by the monomeric form of $\sigma 1$, prompted us to examine the region(s) on $\sigma 1$ that is responsible for the stability and the formation of the $\sigma 1$ oligomer. Our present analysis of the two tryptic fragments of $\sigma 1$ has revealed that the stability of the $\sigma 1$ oligomer is maintained mainly via the N-terminal fibrous portion, rather than the C-terminal globular portion of $\sigma 1$. Under nondissociating conditions (preincubation in SDS-containing sample buffer at 37° for 30 min), the N-terminal half of $\sigma 1$, like intact $\sigma 1$, migrates in SDS-PAGE as an oligomer (trimer), whereas the C-terminal half migrates as a monomer. We have evidence, however, that the latter tryptic fragment is also a trimer (albeit a less stable one) which dissociates under the assay conditions used in the present study. The additional observation that both the N-terminal tryptic fragment and the full-length $\sigma 1$ oligomer respond to temperature and pH changes, and to β -mercaptoethanol in a similar manner, further suggests that the N-terminal half of $\sigma 1$ plays a major role in stabilizing the $\sigma 1$ oligomer.

That the N-terminal portion of $\sigma 1$ possesses oligomerization potential was first suggested by Bassel-Duby *et al.* (1985) from sequence analysis of the type 3 reovirus S1 gene. First of all, the N-terminal one-third of $\sigma 1$ was found to be highly α -helical. The additional presence, in the same region, of an extended heptad repeat (a-b-c-d-e-f-g)*n*, where a and d are characteristically apolar residues, further indicates the propensity of this region to adopt a coiled-coil rope-like structure. Such theoretical considerations are clearly compatible with the fibrous morphology of $\sigma 1$ as revealed by electron microscopy (Furlong *et al.*, 1988; Banerjee *et al.*, 1988; Fraser *et al.*, 1990), and with the present demonstration that the heptad repeat region alone, when synthesized in an *in vitro* system, is capable of forming dimeric and trimeric structures whose stability is remarkably similar to that of the trimeric N-terminal tryptic fragment or of the native $\sigma 1$ oligomer. In view of the recent speculations on the structural and functional aspects of the "leucine zipper," we consider our direct demonstration that the heptad repeat possesses intrinsic dimerization and trimerization function to be significant (see also note added in proof). There is little doubt that, in the case of the trimer, apolar residues at positions a and d contribute to hydrophobic interactions that are responsible for holding the three strands together in a coiled-coil configuration (Figs. 9A and 9B). In addition, ionic interactions were also found to play a role since the dimers and trimers were destabilized by divalent cations but were restabilized by the addition of a chelator. Indeed, an examination of the distribution of charged residues

in positions e and g reveals that when the three α -helices are placed in parallel and in register to each other, residues of opposite charge on adjacent α -helices invariably lie in juxtaposition (Fig. 9C) (see also Nibert *et al.*, 1990). At locations where a corresponding residue of opposite charge is not present on an adjacent α -helix, a spatially close residue on the same helix is present to neutralize the charge. Analysis of analogous regions on the $\sigma 1$ proteins of the other two serotypes reveals a similar, albeit less perfect, distribution pattern of charge residues (data not shown).

It is important to point out that whereas the present study clearly indicates that the $\sigma 1$ oligomer is stabilized mainly via the N-terminal half of the protein, the implications of the intrinsic oligomerizing function of the heptad repeat region on the oligomerization process of the intact $\sigma 1$ oligomer need to be viewed with caution. Recent evidence suggests that intracellular protein folding and oligomerization are mediated by chaperones and that these events are ATP-dependent. In the case of $\sigma 1$, we have recently observed that whereas the oligomerization of the N-terminal one-third of $\sigma 1$ occurs spontaneously, that of full-length $\sigma 1$ is an ATP-dependent event (unpublished data), which further suggests that, as has been reported for a number of oligomeric proteins, the formation of the $\sigma 1$ oligomer is chaperone-mediated. This would in turn imply that the intrinsic oligomerizing function of the heptad repeat region is necessary, but not sufficient, for intact $\sigma 1$ oligomerization, and that a domain(s) downstream of this region must also be involved. We are currently probing the interactions between the three governing factors, namely, the heptad repeat region, a downstream domain(s), and chaperones, in the $\sigma 1$ oligomerization process, the revelation of which may have general and fundamental implications.

Our demonstration that the N-terminal tryptic fragment is a trimer and the N-terminal heptad repeat region possesses intrinsic trimerization function suggests that native protein $\sigma 1$ is most likely also a trimer, although the possibility of $\sigma 1$ being a multiple of a trimer (e.g., dimer of a trimer) cannot be ruled out based on the present data alone. Either model contradicts a recent suggestion, based on sequence analysis and computer-processed electron microscopy, that $\sigma 1$ is a tetramer (Fraser *et al.*, 1990). Although the precise reasons for this discrepancy remain to be revealed, we have recently obtained data from biophysical studies and *in vitro* $\sigma 1$ assembly experiments that are compatible with a trimeric, but not a tetrameric (or a hexameric), model of $\sigma 1$ (unpublished data). A trimeric $\sigma 1$ would theoretically migrate at a position corresponding to 150K in SDS-PAGE, rather than at \sim 200K. How-

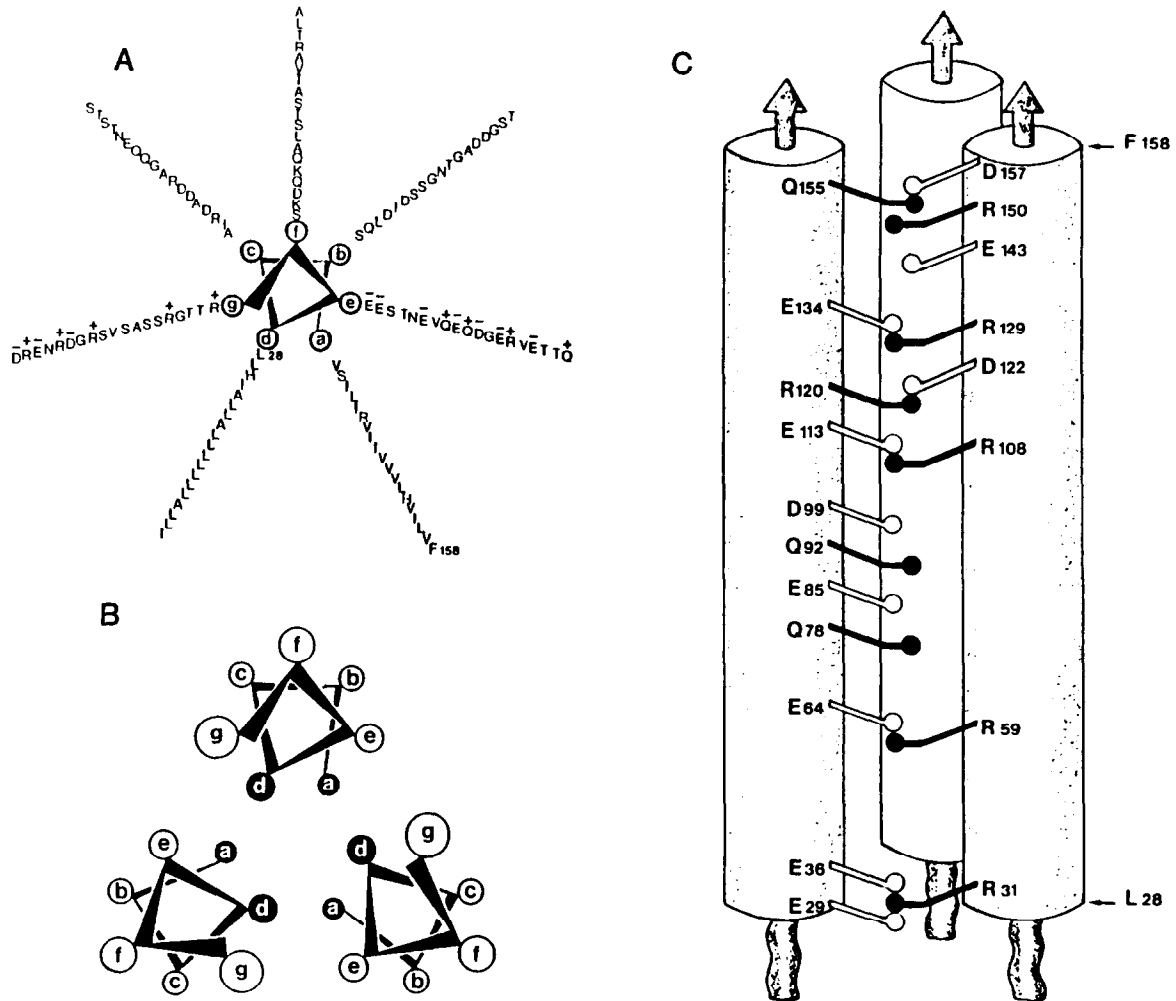


FIG. 9. Analysis of sequences involved in $\sigma 1$ trimerization. (A) α -helical wheel depiction of the heptad repeat region (amino acids 28 to 158) of $\sigma 1$. Note the predominant presence of hydrophobic residues at positions a and d (see also Nibert *et al.*, 1990). Charged residues at positions e and g are also indicated. (B) Cross-section of three parallel α -helices depicting plausible hydrophobic interactions between amino acids at positions a and d of adjacent helices. (C) Schematic diagram showing interactions between negatively charged (white) and positively charged (black) residues at positions e (helix at left) and g (helix at right) on two adjacent helices (see also Nibert *et al.*, 1990).

ever, our present studies suggest that under the conditions used for its detection (incubation at 37° in SDS), the N-terminal fibrous tail of the $\sigma 1$ trimer would still be intact, whereas the C-terminal globular head would be totally unfolded. The resulting $\sigma 1$ structure would accordingly resemble a "hydra," rather than a "lollipop," and would therefore manifest a somewhat retarded migration rate. Results from more refined experiments correlating incubation temperatures (in SDS) with migration rates of intact $\sigma 1$ and $\sigma 1$ tryptic fragments concur with such a rationale (unpublished observation).

It is noteworthy that, in terms of oligomerization status, $\sigma 1$ is not unlike other viral cell-attachment proteins such as the influenza hemagglutinin (Wilson *et al.*, 1981; Wiley *et al.*, 1981), the VSV G protein (Doms *et*

al., 1987; Kreis and Lodish, 1986), the adenovirus fiber (van Oostrum *et al.*, 1987; Devaux *et al.*, 1990), the envelope glycoprotein (gp120) of the human immunodeficiency virus (Weiss *et al.*, 1990), and the coronavirus spike protein (Delmas and Laude, 1990), all of which appear to be trimeric, and the trimeric state may reflect a unifying concept, hitherto unidentified and perhaps pertinent to structural and/or functional requirements, in the virus attachment process.

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Note added in proof. Shortly after submission of our manuscript, Banerjee and Joklik (1990) reported the intrinsic oligomerizing property of the N-terminal heptad repeat region of $\sigma 1$.

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