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## Biochemical and Biophysical Characterization of the Reovirus Cell Attachment Protein $\sigma$ 1: Evidence That It Is a Homotrimer

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Received March 29, 1991; accepted May 22, 1991

The oligomerization state of the reovirus cell attachment protein  $\sigma$ 1 (49K monomeric molecular weight) was determined by biochemical and biophysical means. Full-length (protein product designated A) and C-terminal truncated (protein product designated B) serotype 3 reovirus S1 mRNA transcripts synthesized *in vitro* were cotranslated in a rabbit reticulocyte lysate, and the products were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under conditions which allowed for the identification of oligomeric forms of  $\sigma$ 1. A total of four oligomeric protein bands (corresponding to A<sub>3</sub>, A<sub>2</sub>B<sub>1</sub>, A<sub>1</sub>B<sub>2</sub>, and B<sub>3</sub>, respectively) was consistently observed, which suggests that the protein is made up of three monomeric subunits. Biophysical characterization of purified  $\sigma$ 1 using column filtration and sucrose gradient sedimentation analysis confirmed the highly asymmetric shape of  $\sigma$ 1 and allowed us to determine the molecular weight of the native protein to be ~132K (a trimer). Similar biophysical analysis on the two tryptic fragments of the  $\sigma$ 1 [N-terminal fibrous tail (26K monomeric molecular weight) and the C-terminal globular head (23K monomeric molecular weight)] yielded molecular weights of 77K and 64K, respectively, both again corresponding to trimers. We therefore conclude that protein  $\sigma$ 1 is a homotrimer and provide, with supportive experimental evidence, a rationale for the anomalous behavior of the oligomeric protein in SDS-polyacrylamide gels, which, coupled with chemical cross-linking studies, has in part led to the previous suggestion that  $\sigma$ 1 might be a higher order oligomer. © 1991 Academic Press, Inc.

### INTRODUCTION

Of all the proteins encoded by the double-stranded RNA genomic segments of reovirus, the  $\sigma$ 1 protein has been most thoroughly studied. Protein  $\sigma$ 1 is a minor outer capsid protein situated at each vertex of the outer capsid of the viral icosahedron (Lee *et al.*, 1981; Furlong *et al.*, 1988). This protein functions as the reovirus cell attachment protein (Lee *et al.*, 1981; Armstrong *et al.*, 1984) and the viral hemagglutinin (Weiner *et al.*, 1978; Yeung *et al.*, 1987).

In view of the important roles played by  $\sigma$ 1 in reovirus infection, considerable effort has been made to probe the structure-function relationships of this protein. The S1 gene segment, which encodes the  $\sigma$ 1 protein, has been sequenced for all three reovirus serotypes (Nagata *et al.*, 1984; Cashdollar *et al.*, 1985; Bassel-Duby *et al.*, 1985; Duncan *et al.*, 1990; Nibert *et al.*, 1990), and the deduced amino acid sequences have been analyzed to predict various structural motifs. Under electron microscopy the purified protein appears as a lollipop-shaped structure with a long fibrous tail topped with a globular head (Banerjee *et al.*, 1988; Furlong *et al.*, 1988; Fraser *et al.*, 1990). Similar structures are also seen extending from the surface of the

virion with the head region most distal from the virion (Furlong *et al.*, 1988). The head corresponds to the C-terminal portion of  $\sigma$ 1 which is predicted, from sequence analysis, to form a complex globular structure (Bassel-Duby *et al.*, 1985; Nibert *et al.*, 1990) and which contains the cell receptor recognition domain (Nagata *et al.*, 1987; Yeung *et al.*, 1989; Duncan *et al.*, 1991). Interestingly, treatment of  $\sigma$ 1 with trypsin cuts the protein approximately in half, essentially separating the head from the stalk of the lollipop, with the C-terminal half still fully capable of binding to cell receptors (Yeung *et al.*, 1989). The N-terminal half of the protein represents the fibrous stalk of the lollipop and contains a heptapeptide repeat of hydrophobic residues, suggestive of a coiled-coil structure (Bassel-Duby *et al.*, 1985; Nibert *et al.*, 1990). This heptad repeat region was subsequently shown to be responsible for  $\sigma$ 1 oligomer stability and to possess intrinsic oligomerization function (Banerjee and Joklik, 1990; Leone *et al.*, 1991a). That the N-terminus indeed represents the proximal end is also evident from the demonstration that the N-terminal one-quarter of  $\sigma$ 1 possesses an intrinsic virion-anchoring function (Mah *et al.*, 1990). The actual virion-anchoring domain is presumed to reside in the N-terminal hydrophobic tail (<20 residues) of the protein (Leone *et al.*, 1991b).

The oligomerization state of  $\sigma$ 1 has been a matter of

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controversy. Since the  $\sigma 1$  cell-binding function is manifested only by the oligomeric, but not the monomeric, form of  $\sigma 1$  (Leone et al., 1991c), it is important that the exact stoichiometry of the  $\sigma 1$  protein be known. Based on the icosahedral distribution of  $\sigma 1$  on the virion (Lee et al., 1981) and on earlier stoichiometric data (Smith et al., 1969), it was initially suggested that two molecules of  $\sigma 1$  were present on each vertex of the virion (Lee et al., 1981). Subsequent sequence analysis was also compatible with a dimeric model of  $\sigma 1$  (Bassel-Duby et al., 1985). More recently, however, a number of studies including SDS-PAGE analysis of nondissociated protein, chemical crosslinking, sequence analysis, and electron microscopy have supported a tetrameric model for the  $\sigma 1$  protein (Bassel-Duby et al., 1987; Nibert et al., 1990; Fraser et al., 1990). On the other hand, analysis of the N-terminal oligomerization domain by SDS-PAGE under nondissociating conditions suggests that it forms a three-stranded coiled coil (Leone et al., 1991a).

In the present study, we present evidence that the  $\sigma 1$  protein is trimeric. Specifically, mRNAs of full-length and various deletion mutants of the S1 gene were co-translated *in vitro* to produce homo-oligomeric and hetero-oligomeric  $\sigma 1$  complexes. After subjecting the resultant proteins to SDS-PAGE under nondissociating conditions, a total of four bands was generated, indicative of a protein composed of three monomeric units. Biophysical analyses of the oligomeric  $\sigma 1$  protein and of the oligomeric N- and C-terminal tryptic fragments have provided additional evidence that these proteins are trimeric. We further demonstrate a possible reason for the large molecular weight ( $\sim 200K$ ) previously ascribed to  $\sigma 1$  based on SDS-PAGE analysis under nondissociating or chemically cross-linked conditions.

## MATERIALS AND METHODS

### Plasmid construction

The plasmid pG4T3 which contains the full-length T3 S1 gene (455 codons) cloned into pGEM-4Z (Promega) has been previously described (Leone et al., 1991a; Duncan et al., 1991). Construction of a deletion mutant (d44) which lacked 44 codons at the 3'-terminus of the full-length S1 gene has also been described (Duncan et al., 1991). The mutant d111 with 142 amino acids (residues 224–365) deleted from the C-terminal portion of  $\sigma 1$  was generated by partial digestion of pG4T3 with *Bcl*I (nucleotide 678), followed by complete digestion with *Bgl*II (nucleotide 1104) and religation.

### *In vitro* transcription-translation

The plasmids were linearized with *Hind*III and used in *in vitro* transcription reactions to generate full-length or truncated S1 mRNAs. These mRNAs were translated either individually or in combination (ideally in equimolar amounts) *in vitro* in rabbit reticulocyte lysates according to the manufacturer's specifications (Promega). Typically, the translation mixture contained 50–100 ng of RNA and 20  $\mu$ Ci of [<sup>35</sup>S]methionine in a total volume of 25  $\mu$ l. Reactions were stopped after incubation for 45 min at 37° by the addition of 200  $\mu$ l of phosphate-buffered saline (PBS), pH 7.2. Protein sample buffer (5X) was then added (final 1X concentration was 50 mM Tris, pH 6.8, 1% SDS, 2%  $\beta$ -mercaptoethanol, 10% glycerol, and 0.01% bromophenol blue) and the samples were either boiled for 5 min (dissociating condition) or incubated for 30 min at 37° (nondissociating condition) prior to SDS-PAGE.

### SDS-PAGE

Discontinuous SDS-PAGE was performed using the protocol of Laemmli (1970). For the hetero-oligomer experiments, 7.5% polyacrylamide gels were used for running the nondissociated proteins and 10% gels were used for running the dissociated proteins. Gels containing [<sup>35</sup>S]methionine-labeled proteins were fixed and treated with DMSO-PPO, dried under vacuum, and exposed to XAR-5 film at  $-70^\circ$ .

The protein bands from hetero-oligomer experiments were located by exposing the dried, unfixed gel to X-ray film. The bands were then excised and rehydrated with Laemmli running buffer, and the proteins electroeluted using Laemmli running buffer and ISCO electroelution cups. Protein sample buffer (5X) was added to electroeluted proteins, and the samples were boiled for 5 min prior to SDS-PAGE.

### Temperature titration gels

Samples of purified intact and trypsinized  $\sigma 1$  (see below) were incubated in the presence of protein sample buffer (1X concentration as above) at 4°, 10°, 15°, 22°, and 37° for 30 min or boiled for 5 min prior to SDS-10% PAGE. These gels were electrophoresed at 4° in prechilled (4°) Laemmli running buffer, followed by fixing and staining by the Coomassie method of Zehr et al. (1989).

### Laser scanning densitometry

Densitometric analyses of autoradiograms and Coomassie-stained gels were carried out using a Soft laser scanning densitometer (Biomed Instruments, Inc.) and

Biomed laser software. Values for quantitation of autoradiographic bands from hetero-oligomer experiments were normalized to accommodate for the numbers of methionines present in the intact and truncated  $\sigma 1$  proteins. Specifically, the dIII deletion mutant contains only five of the eight methionines present in the intact  $\sigma 1$  protein and, as such, was normalized in the appropriate manner.

#### Sepharose CL-6B chromatography of intact $\sigma 1$

A Sepharose CL-6B column (2.5 × 95 cm) was prepared and equilibrated with buffer A [10 mM sodium phosphate (pH 8.0) and 0.1 M NaCl]. Molecular weight standards of known Stokes radii [thyroglobulin (85.0 Å), ferritin (61.0 Å), catalase (52.2 Å), calmodulin-dependent phosphodiesterase (44.4 Å), and bovine serum albumin (35.5 Å)] were prepared by dissolving 2 mg of each protein in 1 ml of buffer A and applied to the CL-6B column. Elution was carried out at 4° at a flow rate of 20 ml/hr. Eluted samples were collected in 1-ml fractions and analyzed by Bio-Rad protein assay.

Approximately 2 mg of purified  $\sigma 1$ , derived from a baculovirus expression vector system (R. Duncan, J. E. Strong, P. Gale, and P. W. K. Lee, unpublished data), was dissolved in 1 ml of buffer A and applied to the CL-6B column. Eluted samples were analyzed by Bio-Rad protein assay and by SDS-PAGE.

#### Sephacryl S-300 SF chromatography of $\sigma 1$ tryptic fragments

A Sephacryl S-300 SF column (2.5 × 95 cm) was prepared and equilibrated with buffer B [10 mM Tris buffer (pH 7.5) and 0.1 M NaCl]. Molecular weight standards of known Stokes radii [catalase (52.2 Å), aldolase (48.1 Å), bovine serum albumin (35.5 Å), ovalbumin (30.5 Å), chymotrypsinogen A (20.9 Å), and ribonuclease A (16.4 Å)] were prepared by dissolving 2 mg of each protein in 1 ml of buffer B and applied to the S-300 column. Elution was carried out at 4° at a flow rate of 20 ml/hr. Eluted samples were collected and analyzed as above.

Trypsin-cleaved  $\sigma 1$  was prepared by digesting 3 mg of purified  $\sigma 1$  for 30 min at 37° with washed insoluble trypsin (Sigma) (3.8 U) in 1.2 ml PBS. After pelleting the trypsin beads, TLCK was added to the supernatant to a final concentration of 0.2 mg/ml, and the supernatant was applied to the column.

#### Sucrose gradient sedimentation analysis

Linear 12-ml 5–20% sucrose gradients in buffer A were used for sedimentation analysis. Approximately 1 mg of intact  $\sigma 1$  or 2 mg of trypsin-cut  $\sigma 1$  in 300  $\mu$ l of

buffer A was applied to the gradients. Molecular weight standards of known sedimentation coefficients [catalase (11.30 S), aldolase (7.35 S), bovine serum albumin (4.30 S), ovalbumin (3.66 S), and chymotrypsinogen A (2.58 S)] were prepared by dissolving 1 mg of each protein in 300  $\mu$ l of buffer A and applied to parallel gradients. For intact  $\sigma 1$ , the samples were centrifuged at 38,000 rpm (Beckman SW41 rotor) for 24 hr at 4°. Trypsin-cut  $\sigma 1$  and standards were centrifuged at 38,000 rpm for 25 hr. All samples were fractionated and analyzed by Bio-Rad protein assay. Fractions containing intact and trypsin-cut  $\sigma 1$  proteins were subjected to SDS-PAGE to assess purity and, in the case of trypsin-cut samples, the gels were scanned with the Soft laser scanning densitometer (Biomed Instruments, Inc.) to determine the peak fraction.

#### Molecular weight determinations

Column filtration data are expressed in terms of  $K_{av}$ , a parameter which is defined as

$$K_{av} = (V_e - V_0)/(V_t - V_0), \quad (1)$$

where  $V_e$  is the elution volume of solute;  $V_0$  is the void volume of column; and  $V_t$  is the total bed volume of column.

Siegel and Monty (1966) have demonstrated a linear relationship between elution volume (expressed in terms of  $K_{av}$ ) and a theoretical value termed the Stokes radius for a variety of proteins. The relationship of the  $K_{av}$  to the Stokes radius is given by

$$(-\log K_{av})^{1/2} = \alpha(\beta + a), \quad (2)$$

where  $a$  is the Stokes radius, and  $\alpha$  and  $\beta$  are constants related to the intrinsic properties of the gel matrix.

The Stokes radius of a protein can be measured by passing the protein through a column which has been previously calibrated with markers of known Stokes radii. After determining the sedimentation coefficient of the protein by sucrose gradient centrifugation with standards of known sedimentation values, accurate estimates of the molecular weight of the protein can be calculated using

$$M = \frac{6\pi\eta N s}{(1 - \bar{v}\rho)} \quad (3)$$

where  $M$  is the molecular weight,  $a$  is the Stokes radius,  $s$  is the sedimentation coefficient,  $\bar{v}$  is the partial specific volume (actual value used = 0.732 cc/g),  $\eta$  is the viscosity of medium (value used = 1),  $\rho$  is the density of medium (value used = 1), and  $N$  is Avogadro's number.

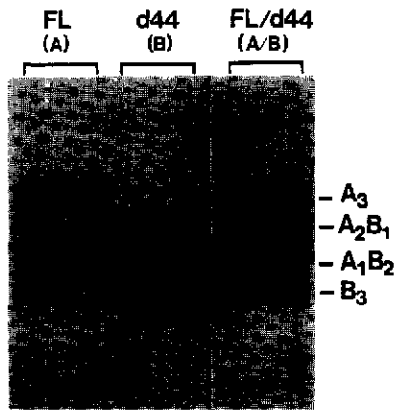


Fig. 1. Analysis of *in vitro* cotranslation products of full-length (FL) and 3'-truncated (d44) S1 mRNAs. Proteins were synthesized in the presence of [<sup>35</sup>S]methionine either individually or in combination as indicated (see Materials and Methods). Synthesized proteins were mixed with protein sample buffer, incubated at 37° for 30 min, and analyzed on SDS-PAGE. The predicted compositions of the protein bands are indicated on the right where A represents the full-length product and B represents the product of the d44 transcript.

The frictional ratio ( $f/f_0$ ) of the protein can then be estimated using

$$f/f_0 = a \left/ \left( \frac{3\bar{v}M}{4\pi N} \right)^{1/3} \right. \quad (4)$$

## RESULTS

### Hetero-oligomerization of full-length and truncated $\sigma 1$

To determine the oligomerization state of  $\sigma 1$ , we took advantage of the observation that both full-length and C-terminal truncated  $\sigma 1$  proteins are capable of forming oligomers in an *in vitro* translation system and that these oligomeric forms can be resolved from each other by SDS-PAGE under nondissociating conditions (Duncan *et al.*, 1991). It was therefore reasoned that cotranslation of the full-length mRNA and a C-terminal truncated S1 mRNA should lead to the formation of a set of homo-oligomeric and hetero-oligomeric complexes as a result of random association of monomeric proteins. The oligomerization state can then be determined from the number of hetero-oligomeric species present on SDS-PAGE.

The results from one such experiment using the full-length (FL)  $\sigma 1$  and a truncated  $\sigma 1$  with 44 amino acids deleted from the C-terminus (d44) are shown in Fig. 1. Both FL and d44 were detected as single protein bands (homo-oligomers) when synthesized individually, with the d44 oligomer migrating faster than the FL oligomer. When synthesized together, four protein

bands were seen. Two of the bands migrated at the same positions as the individually synthesized FL and d44, whereas the middle two bands migrated with intermediate mobilities. The formation of four oligomeric protein species with different migration rates is consistent with the notion that  $\sigma 1$  is composed of three subunits. Whereas the slowest and fastest migrating species represented homo-oligomers that contained only full-length ( $A_3$  in Fig. 1) or truncated proteins ( $B_3$  in Fig. 1), the intermediate complexes contained either two full-length and one truncated ( $A_2B_1$  in Fig. 1) or two truncated and one full-length protein ( $A_1B_2$  in Fig. 1) (see below).

The above experiment was repeated using FL and another deletion mutant (dIII) lacking 142 amino acids (residues 224–365) from the C-terminal portion of  $\sigma 1$  (Fig. 2A). Here again, cosynthesis of the full-length (A) and truncated proteins (C) produced two hetero-oligomeric complexes ( $A_2C_1$ , and  $A_1C_2$ ). The full-length complex was found to exist as two differently migrating species (labeled FL and FL\*). The band labeled FL represented the most dominant conformation, while FL\* represented an altered, misfolded, and more compact form of the full-length oligomer, also previously noted by Banerjee and Joklik (1990). [A comparable but somewhat diffused FL\* band was also detected in the gel used in Fig. 1, but is not shown since it migrated considerably faster than d44 ( $B_3$ )].

A number of other combinations of deletion and full-length transcripts were also attempted. In every case, only two hetero-oligomeric complexes, in addition to the two homo-oligomeric species, were formed (data not shown). This data strongly suggest that the oligomeric proteins are composed of three monomeric units.

To confirm this reasoning, and to dispel any suggestion that any single band might be made up of more than one homo-oligomeric or hetero-oligomeric species, each of the four bands from Fig. 2A was excised from the gel, electroeluted, boiled in SDS-containing buffer, and subjected to SDS-PAGE (Fig. 2B). The gels were then scanned and the molar ratio of the two proteins in each complex was determined, taking into consideration the difference in methionine content between FL and dIII. As expected, bands 1 and 4 contained almost exclusively FL and dIII, respectively (the minor contaminations observed were quite typical for analysis of this nature). The ratios of FL to dIII in bands 2 and 3 were found to be 2.0:1 and 1:2.2, respectively, in good agreement to what would be expected for a trimeric protein. Analysis of the excised FL\* band revealed that it contained only the full-length  $\sigma 1$  protein (data not shown).

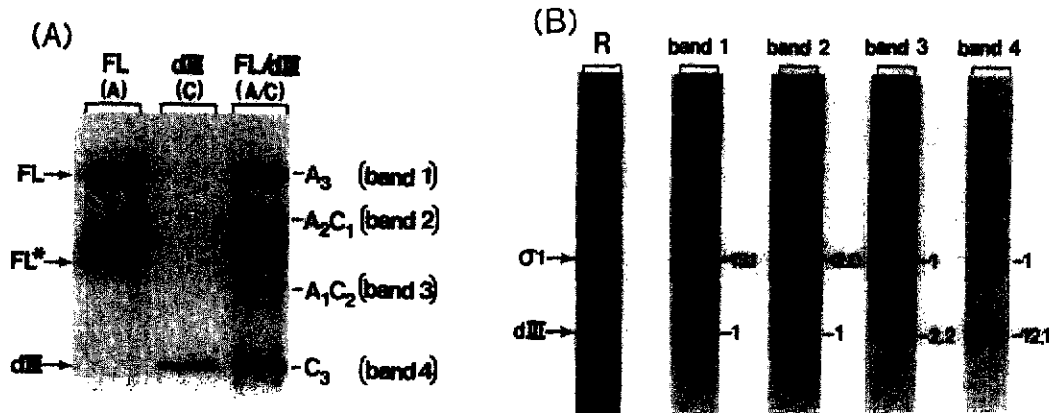


FIG. 2. (A) Analysis of *in vitro* cotranslation products of full-length (FL) and truncated (dIII) S1 mRNAs. These proteins were synthesized and analyzed as described in the legend to Fig. 1. The predicted compositions of the protein bands are indicated on the right where A and C represent the full-length and the dIII products, respectively. The FL\* band represents a misfolded form of the full-length oligomer. (B) Individual bands from (A) (labeled band 1–4) were excised, rehydrated in Laemmli running buffer, and electroeluted. After boiling the eluted proteins in protein sample buffer for 5 min, the samples were subjected to SDS-PAGE and autoradiography. The composition of each band (i.e., the ratio of FL:dIII polypeptides) was analyzed by laser scanning densitometry and the results are shown to the right of each lane. R represents purified [<sup>35</sup>S]methionine-labeled reovirus type 3.

### Determination of the molecular weights of intact $\sigma_1$ and N- and C-terminal tryptic fragments of $\sigma_1$

In order to confirm the trimeric model and analyze  $\sigma_1$  in its native state, the physical characteristics of the intact  $\sigma_1$  protein were determined using column filtration and sucrose gradient sedimentation analysis. Upon exclusion chromatography through a Sepharose CL-6B column that was calibrated with marker proteins of known Stokes radii, the  $\sigma_1$  oligomer eluted as a homogenous species with an estimated Stokes radius of 63.3 Å (Fig. 3A). Sedimentation in sucrose gradients (5–20%) together with standard proteins indicated that

protein  $\sigma_1$  had a sedimentation coefficient of  $4.94 \times 10^{-13}$  sec (Fig. 3B). The molecular weight of the oligomeric protein was then calculated to be 132K (Table 1) according to the method of Siegel and Monty (1966) [see Eq. (3)], making  $\sigma_1$  (49K monomeric molecular weight) a trimer. The highly asymmetric nature of this protein is indicated by its high frictional ratio ( $f/f_0$ ) of 1.88 (an  $f/f_0$  ratio of 1.5 is consistent with a prolate ellipsoid of axial ratio of about 8:1) and supported by the observation that its apparent molecular weight by column filtration ( $\sim 440$ K) greatly exceeds the calculated molecular weight.

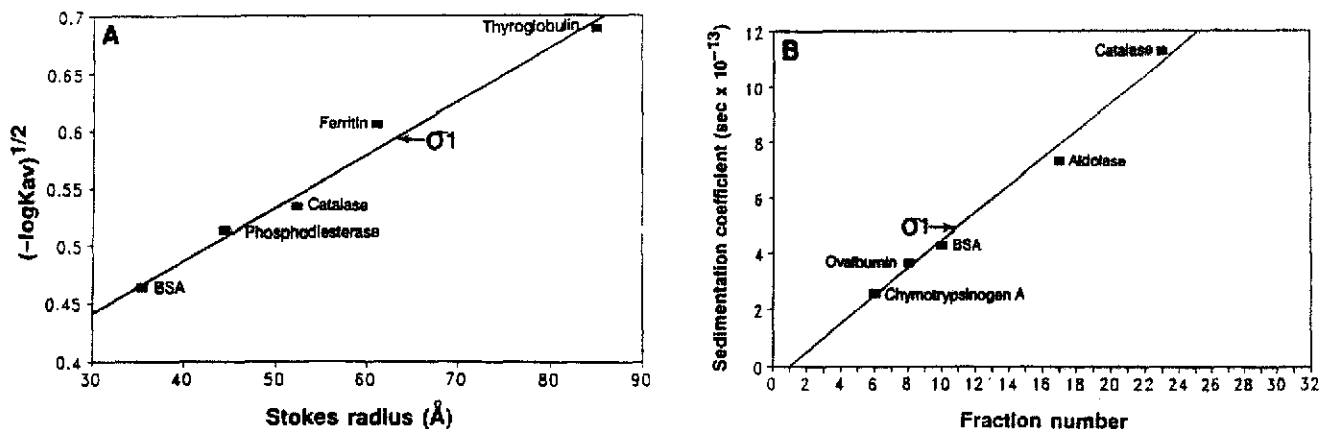


FIG. 3. Biophysical analysis of intact  $\sigma_1$ . (A) Sepharose CL-6B column exclusion chromatography of intact  $\sigma_1$ . Approximately 2 mg each of  $\sigma_1$  and marker proteins of known Stokes radii were applied to the CL-6B column, eluted with equilibration buffer into 1-ml fractions, and analyzed by Bio-Rad protein assay (see Materials and Methods). A calibration curve of the column was obtained by plotting  $(-\log K_{av})^{1/2}$  vs Stokes radii of the markers and used to determine the Stokes radius of  $\sigma_1$ . (B) Sucrose gradient sedimentation analysis of intact  $\sigma_1$ . Approximately 1 mg of  $\sigma_1$  or marker proteins was applied to 5–20% linear sucrose gradients and centrifuged at 38,000 rpm (Beckman SW41 rotor) for 24 hr at 4°. Gradient fractions were analyzed by Bio-Rad protein assay and SDS-PAGE. The sedimentation coefficient of  $\sigma_1$  was determined from the calibration curve obtained by plotting the sedimentation coefficients of marker proteins vs peak fraction number.

TABLE 1  
SUMMARY OF BIOPHYSICAL DATA ON INTACT  
AND TRYPTIC FRAGMENTS OF  $\sigma 1$

	Stokes radius <sup>a</sup> (Å)	S value <sup>b</sup> (sec $\times 10^{-13}$ )	$f/f_0$ <sup>d</sup>	Molecular weight <sup>c</sup> (kDa)	Oligomeric status <sup>e</sup>
$\sigma 1$	63.3	4.94	1.88	132	2.7
N-fragment	54.1	3.37	1.92	77	3.0
C-fragment	36.4	4.16	1.37	64	2.8

<sup>a</sup> Determined from Figs. 3A and 4A.

<sup>b</sup> Determined from Figs. 3B and 4B.

<sup>c</sup> Calculated by means of Eq. (3).

<sup>d</sup> Calculated by means of Eq. (4).

<sup>e</sup> Calculated using molecular weights of 49K, 26K, and 23K for monomeric  $\sigma 1$  and N- and C-terminal tryptic fragments, respectively.

It was previously shown that trypsin cuts  $\sigma 1$  approximately in the middle (after Arg<sub>245</sub>), generating a 26K N-terminal fragment and a 23K C-terminal fragment under dissociating conditions (Yeung *et al.*, 1989; Duncan *et al.*, 1991). The N-terminal fragment migrates as a trimer ( $\sim 80$ K) on SDS-PAGE under non-dissociating conditions (Leone *et al.*, 1991a), and the C-terminal fragment also exists as an oligomeric structure, albeit less stable than the N-terminal oligomeric fragment (Duncan *et al.*, 1991). It was therefore of interest to use the biophysical analysis described above to determine whether these two tryptic fragments, like intact  $\sigma 1$ , are also trimeric.

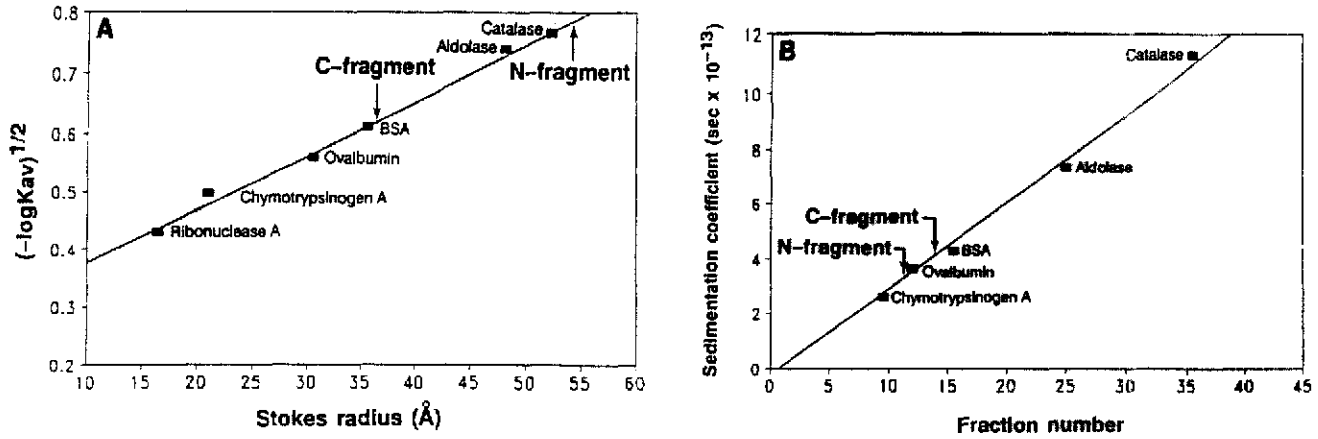
It was predicted that since the N-terminal tryptic fragment possesses rod-like properties, it should behave anomalously on column filtration and sedimentation analysis, whereas the C-terminal tryptic fragment, being mostly globular in nature, should behave almost ideally on both parameters. Such predictions were found to be correct. The N-terminal fragment eluted off the S-300 column with a Stokes radius of 54.1 Å (Fig. 4A), equivalent to a protein of greater than 230K molecular weight, whereas by sedimentation analysis the sedimentation coefficient of  $3.37 \times 10^{-13}$  sec (Fig. 4B) predicted a protein of less than 40K. When these values were combined in Eq. (3) (see Materials and Methods), a calculated molecular weight of 77K was obtained, which corresponds to three times the molecular weight (26K) of the monomeric subunit (Table 1). As expected, the N-terminal tryptic fragment is highly asymmetric, with an  $f/f_0$  value of 1.92. The C-terminal tryptic fragment behaved almost ideally since its Stokes radius, determined by S-300 column filtration to be 36.4 Å (Fig. 4A), and its sedimentation coefficient, determined by sucrose gradient centrifugation to be  $4.16 \times 10^{-13}$  sec (Fig. 4B), both predicted a protein

with a molecular weight similar to that of BSA (67K). Not surprisingly, the molecular weight of the C-terminal tryptic fragment was calculated to be 64K using Eq. (3), again indicative of a trimer (Table 1). The globular nature of this fragment is reflected by its calculated frictional ratio of 1.37, a value that is comparable to that for BSA ( $f/f_0 = 1.35$ ).

#### Effects of preelectrophoresis incubation temperature on the migration rates of intact $\sigma 1$ and $\sigma 1$ tryptic fragments

The previous suggestion that native  $\sigma 1$  is a tetramer was in part based on the observation that under non-dissociating conditions,  $\sigma 1$  migrates at a position corresponding to  $\sim 200$ K or higher (Bassel-Duby *et al.*, 1987; Banerjea and Joklik, 1990). We subsequently found that when trypsin-cut  $\sigma 1$  is subjected to the same treatment, the N-terminal fragment migrates as an oligomer (trimer), whereas the C-terminal fragment migrates as a monomer (Leone *et al.*, 1991a; also see Fig. 5). The differential effects of such treatment on the two  $\sigma 1$  tryptic fragments, when extrapolated to the intact protein, would be the generation of a hydra-like structure, with the stalk representing the trimeric, rod-shaped N-terminal half, and the tentacles representing the three dissociated and unfolded C-terminal strands (see Fig. 6). It is not difficult to envision how such a structure could manifest a retarded mobility in polyacrylamide gels.

Our present finding that the C-terminal tryptic fragment in its native state is indeed oligomeric (trimeric) has led us to reason that perhaps, under conditions which allow this fragment to remain nondissociated, intact  $\sigma 1$  would maintain its lollipop morphology and migrate at a rate more representative of its true molecular weight. This was indeed found to be the case (Fig. 5). By simply reducing the preelectrophoresis incubation temperature from 37° to 22° or lower, we detected a dramatic shift in the migration rate of the C-terminal tryptic fragment from the 23K (monomer) to the 55–60K (trimeric) position. The trimeric status of the N-terminal fragment remained unchanged, although a partial and minor shift in migration rate was observed. The same down-shift of temperature was found to cause intact  $\sigma 1$  to shift from the position of  $>200$ K (A form) to one slightly lower than where the reovirus  $\lambda$  proteins ( $\sim 150$ K) migrated (B form), the expected position for a trimeric  $\sigma 1$  protein. The absolute correlation between stabilization of the trimeric C-terminal tryptic fragment and enhanced mobility of the intact  $\sigma 1$  protein has led us to safely conclude that the  $\sigma 1$  A form ("hydra") migrates anomalously, resulting in the previous overestimation of its molecular weight. The somewhat hetero-



**FIG. 4.** Biophysical analysis of the tryptic fragments of  $\sigma 1$ . (A) Sephacryl S-300 column exclusion chromatography of  $\sigma 1$  tryptic fragments. Approximately 3 mg of  $\sigma 1$  was digested with 3.8 U of insoluble trypsin for 30 min at 37°. Trypsin-cut  $\sigma 1$  was then applied to the S-300 column previously calibrated with marker proteins. The proteins were eluted with equilibration buffer into 1-ml fractions and analyzed by Bio-Rad protein assay and SDS-PAGE (see Materials and Methods). A calibration curve of the column was obtained by plotting  $(-\log K_{av})^{1/2}$  vs Stokes radii of the markers and used to determine the Stokes radii of the N- and C-terminal  $\sigma 1$  tryptic fragments. (B) Sucrose gradient sedimentation analysis of the tryptic fragments of  $\sigma 1$ . Approximately 1 mg of trypsin-cut  $\sigma 1$  or marker proteins was applied to 5–20% linear sucrose gradients and centrifuged at 38,000 rpm (Beckman SW41 rotor) for 25 hr at 4°. Gradient fractions were analyzed by SDS-PAGE and laser scanning densitometry. The sedimentation coefficients of the N- and C-terminal  $\sigma 1$  tryptic fragments were determined from the calibration curve obtained by plotting the sedimentation coefficients of marker proteins vs peak fraction number.

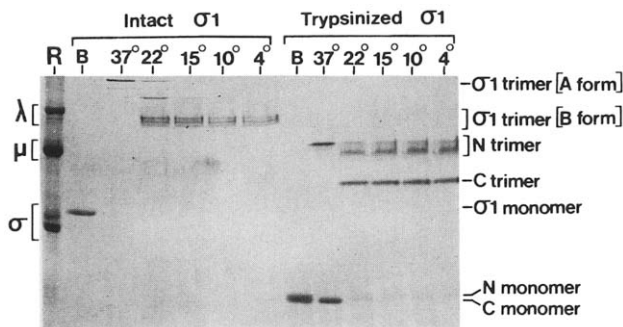
geneous nature of the  $\sigma 1$  B form is believed to reflect the partial and minor mobility shift of the N-terminal trimeric fragment upon temperature down-shift.

**DISCUSSION**

The oligomerization status of the reovirus cell attachment protein  $\sigma 1$  has been controversial. It was initially thought that protein  $\sigma 1$ , which is located at the 12 vertices of the viral icosahedron, exists as a dimer (Lee *et al.*, 1981; Bassel-Duby *et al.*, 1985; Yeung *et al.*, 1987). Subsequent studies based on migration rate in

gels, chemical cross-linking, electron microscopy, and sequence analysis have led to the suggestion that  $\sigma 1$  is a tetramer (Bassel-Duby *et al.*, 1987; Banerjea *et al.*, 1988; Banerjea and Joklik, 1990; Nibert *et al.*, 1990; Fraser *et al.*, 1990). Recently, we demonstrated that the N-terminal tryptic fragment of  $\sigma 1$  migrates as a trimer on SDS-PAGE under nondissociating conditions and that the N-terminal heptad repeat region possesses an intrinsic trimerization function (Leone *et al.*, 1991a). In the present study, we have used two independent approaches, one biochemical and the other biophysical, to demonstrate that  $\sigma 1$  is trimeric and provide a rationale for the anomalous behavior of the intact protein on SDS-PAGE (under certain nondissociating conditions and not under others) which has in part led to the previous suggestion that it is a higher order oligomer.

The cosynthesis of full-length and C-terminal truncated  $\sigma 1$  proteins has allowed us to identify hetero-oligomers, the number of which reflects the oligomerization status of  $\sigma 1$ . If  $\sigma 1$  were a dimer, for example, only one hetero-oligomer would be formed and, together with the two homo-oligomers, a total of three bands would be observed on SDS-PAGE. Similarly, if  $\sigma 1$  were a tetramer, five complexes would be produced. The fact that four oligomeric species are consistently found for any of the combinations of full-length and truncated proteins tested is a strong indication that  $\sigma 1$  is composed of three monomeric subunits. It deserves mention in this regard that if the assortment is indeed random, the distribution of the four oligomeric species ( $A_3$ ,



**FIG. 5.** Effects of preelectrophoresis incubation temperature on intact  $\sigma 1$  and  $\sigma 1$  tryptic fragments. Purified  $\sigma 1$  and trypsin-cut  $\sigma 1$  were either boiled for 5 min (B) or incubated at 37°, 22°, 15°, 10°, and 4° for 30 min in SDS-containing protein sample buffer. SDS-PAGE was performed at 4°, followed by fixing and Coomassie staining. R represents purified reovirus type 3 under dissociating conditions.



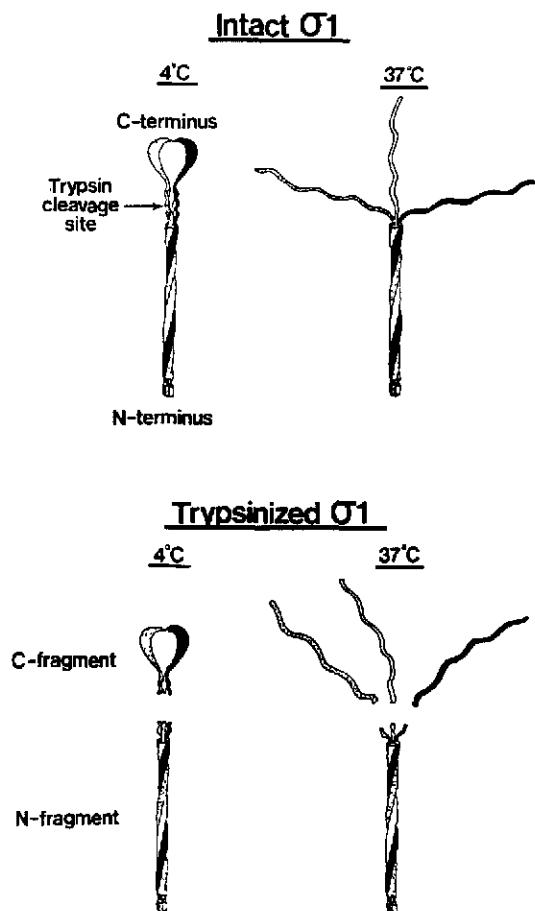


FIG. 6. Schematic diagram showing the configuration and oligomerization state of intact  $\sigma 1$  and  $\sigma 1$  tryptic fragments at different temperatures (in SDS-containing protein sample buffer). The transition from the "lollipop" to "hydra" configuration for intact  $\sigma 1$  is correlated with the dissociation of the trimeric C-terminal tryptic fragment (the globular head) into its monomeric subunits.

$A_2B_1$ ,  $A_1B_2$ , and  $B_3$  in Fig. 1) would be in the ratio of 1:3:3:1 (assuming equimolar amounts of A and B were synthesized). This has not been found to be the case. Rather, we consistently observed a preference for homotrimer over heterotrimer formation. Although we have not completely ruled out the possibility that, for whatever reason, homotrimers are assembled more efficiently or are more stable than heterotrimers, recent studies (unpublished) suggest that trimerization may not occur in solution and most likely involves a scaffold in close proximity to the site of protein synthesis. Newly synthesized monomeric  $\sigma 1$  would be "recruited" onto this scaffold where they are assembled into trimers and subsequently released.

Results from the biophysical analysis of both intact and trypsin-cut  $\sigma 1$  confirm the trimer model as well as the asymmetric nature of this protein. Based on sequence analysis, Bassel-Duby *et al.* (1985) first predicted that the  $\sigma 1$  protein consists of a long fibrous tail

topped with a globular head. Such predictions have subsequently been verified by electron microscopy (Banerjee *et al.*, 1988; Furlong *et al.*, 1988; Fraser *et al.*, 1990). The asymmetry of  $\sigma 1$  is further indicated in the present study from its high frictional ratio and manifested by its abnormal migration on density gradient sedimentation and column filtration. We have also confirmed that the asymmetric shape is confined almost entirely to the N-terminal half of the protein since the C-terminal tryptic fragment behaved almost ideally by both parameters.

The need for the two halves of  $\sigma 1$  to assume unique structural characteristics can readily be seen when one considers their distinct functional roles. The N-terminal one-third of  $\sigma 1$ , which is highly  $\alpha$ -helical and contains a long heptad repeat of hydrophobic residues, possesses intrinsic oligomerization function (Banerjee and Joklik, 1990; Leone *et al.*, 1991a) and, as such, confers oligomer stability to the intact  $\sigma 1$  protein (Leone *et al.*, 1991a). These observations suggest that the heptad repeat region is likely where trimerization of the intact  $\sigma 1$  protein initiates and that trimerization of this region results in the formation of a stable extension which presumably facilitates access of the C-terminal region to the cellular receptor. More importantly, trimerization of the heptad repeat appears to be crucial for the generation of a competent virion-anchorage domain which likely resides in the N-terminal hydrophobic tail (Leone *et al.*, 1991b), as well as the subsequent trimerization and proper folding of the C-terminal globular head (G. Leone and P. W. K. Lee, unpublished data). The significance of trimerization in  $\sigma 1$  function is also indicated by our observation that monomeric  $\sigma 1$  synthesized *in vitro* is nonfunctional (Leone *et al.*, 1991c) and that when expressed as a truncated protein (i.e., lacking the N-terminal oligomerization domain), the C-terminal half of  $\sigma 1$  manifests a drastically reduced cell-binding activity (Nagata *et al.*, 1987). However, once formed, the C-terminal trimeric head is a compact, protease-resistant structure which is stabilized by intersubunit interactions (Duncan *et al.*, 1991) and retains full cell-binding function even when separated from the N-terminal half of the protein (Yeung *et al.*, 1989). The trimeric nature of the C-terminal globular head also raises the question as to the stoichiometry of the  $\sigma 1$ -receptor interaction. At present, it is not known whether each subunit possesses its own receptor-binding site or whether the three subunits contribute to a single receptor-recognition domain.

The difference in oligomer stability between the N- and the C-terminal halves of  $\sigma 1$  is accountable for the anomalous behavior of the intact  $\sigma 1$  protein in polyacrylamide gels. We have found that under conditions previously used to detect  $\sigma 1$  oligomers, the C-terminal

globular head dissociates into separate subunits, whereas the N-terminal fibrous portion maintains its trimeric state. The resulting structure would therefore resemble a hydra (see Fig. 6) whose mobility in polyacrylamide gels should be drastically retarded. However, under less stringent conditions where oligomer integrity of both the N- and the C-terminal portions is maintained,  $\sigma 1$  would assume a more native structure, possibly resembling a lollipop, and should migrate at a position more representative of its true molecular weight. This has indeed been found to be the case: the "hydra" structure migrates at  $>200K$ , whereas the "lollipop" structure migrates at  $\sim 150K$ , the theoretical position for a trimeric  $\sigma 1$ . The dependence of mobility (in polyacrylamide gels) on  $\sigma 1$  structure can also be used to explain the chemical cross-linking data previously presented as partial evidence for a tetrameric model of  $\sigma 1$  (Bassel-Duby *et al.*, 1987). One can envision how intersubunit cross-linking of the  $\sigma 1$  protein along the N-terminal fiber, but not between the C-terminal subunits, could produce a protein which would very much resemble a hydra under dissociating conditions. Such a protein would have a retarded mobility and would therefore resemble a very large protein. However, if the C-terminal subunits were additionally interlinked, the same protein would be more compact and therefore migrate faster. Thus, the number of bands and the apparent migration rates of these cross-linked proteins would not necessarily be a function of their actual oligomeric status, but rather a function of the conditions of cross-linking.

In consideration of our conclusion that the reovirus  $\sigma 1$  protein is a trimer, it is noteworthy that a number of 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 appear to be trimeric. It remains to be seen whether the trimeric state reflects any unifying concepts in the virus attachment process.

Finally, it is interesting to note that Smith *et al.* (1969) originally estimated that  $\sigma 1$  accounts for  $\sim 1\%$  of virion proteins and that there are approximately 31 molecules of  $\sigma 1$  per virion. Our results suggest that there are in fact 36 molecules of  $\sigma 1$  per virion (a trimer at each of the 12 vertices). Thus, in a way, the story of  $\sigma 1$  stoichiometry has come full circle.

#### ACKNOWLEDGMENT

This work was supported by the Medical Research Council of Canada. G.L. and R.D. are recipients of the Alberta Heritage Founda-

tion for Medical Research (AHFMR) Studentship and Fellowship, respectively. P.W.K.L. is an AHFMR Scholar

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