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Association of Reovirus Outer Capsid Proteins σ3 and μ1 Causes a Conformational Change That Renders σ3 Protease Sensitive†

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Association of the reovirus proteins σ3 and μ1 influences viral entry, initiation of outer capsid assembly, and modulation of the effect of σ3 on cellular translation. In this study, we have addressed whether structural changes occur in σ3 as a result of its interaction with μ1. Using differences in protease sensitivity to detect conformationally distinct forms of σ3, we showed that association of σ3 with μ1 caused a conformational change in σ3 that converted it from a protease-resistant to a protease-sensitive structure and occurred posttranslationally. The effect of μ1 on the structure of σ3 was stoichiometric. Our results are consistent with a model in which σ3's association with μ1 shifts its function from translational control to assembly of an outer capsid in which σ3 is folded into the protease-sensitive conformation that is required for its cleavage during the next round of infection.

The reovirus outer capsid is composed of 600 copies each of the σ3 and μ1 proteins (8). In the capsid, μ1 exists primarily as a 72-kDa fragment, known as μ1C, that is generated by cleavage of μ1 near its amino terminus (15, 28, 40). The μ1 protein, which is myristoylated (28), is believed to mediate penetration of cellular membranes during entry (19, 26–28) and plays a role in transcriptase activation (5). The σ3 protein, a 41-kDa zinc metalloprotein (30), occupies the outermost position on the viral capsid (8) and is implicated in viral stability (6, 7). During viral entry, σ3 must be proteolytically removed from the virion (3, 35, 37). Within infected cells, σ3 has been implicated in viral effects on cellular translation (31), and expression of σ3 in transfected cells stimulates translation of a cotransfected reporter gene (11). The σ3 protein binds double-stranded RNA (dsRNA) (12), and it is believed that the ability to sequester dsRNA and inhibit the interferon-induced, dsRNA-activated inhibitory kinase (PKR) mediates σ3's stimulatory effect on translation (13).

The association of μ1 and σ3 plays an important role in the functions of these proteins. Studies in which the M2 and S4 genes, encoding μ1 and σ3, respectively (20, 25), were cotransfected into cells demonstrate that the presence of μ1 eliminates the ability of σ3 to stimulate translation, suggesting that the formation of σ3-μ1 protein complexes interferes with σ3's ability to bind dsRNA and inhibit the PKR pathway (38). Association of σ3 and μ1 also is required for outer capsid assembly. A temperature-sensitive mutant with a lesion that maps to the S4 gene (24) produces only core particles at the nonpermissive temperature (9, 23), indicating that the ability of μ1 to form the icosahedral lattice of the outer capsid (8) is dependent on the formation of σ3-μ1 complexes. Despite the evidence that the association of σ3 and μ1 has important functional consequences, it is not known if this association causes structural changes in either protein.

In this study, we examined the structural consequences of the association of μ1 with σ3. We expressed ^{35}S-labeled σ3 by in vitro transcription and translation from plasmid pCITE-2b-S4T3D, which was constructed by subcloning a cDNA copy of the T3D S4 gene (17) into pCITE-2b (Novagen, Madison, Wis.). In vitro transcription was carried out by the Ribomax (Promega) method. In vitro translation was carried out with the Flexi Rabbit Reticulocyte Lysate System (Promega).

Analysis of the translation product by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) (18) and fluorography revealed a single, ^{35}S-labeled protein band with the same mobility as virion σ3 (data not shown). To confirm that translated σ3 was not misfolded, we looked at the ability of seven anti-σ3 monoclonal antibodies to precipitate the protein. Experimental evidence indicates that only one of the seven antibodies recognizes a linear determinant maintained in denatured σ3, whereas the other six antibodies recognize epitopes that are dependent on protein conformation (39). Proteins were immunoprecipitated, using a previously described procedure (39), from a buffer consisting of 0.1 M NaCl, 1 mM EDTA, 10 mM Tris (pH 7.5), and 1% Nonidet P-40 (14). Translated σ3 was recognized by all seven anti-σ3 antibodies but not by a monoclonal antibody directed against μ1 (data not shown).

Our finding that conformation-dependent monoclonal antibodies directed against virion σ3 recognize translated σ3 indicates that elements of native tertiary structure are retained in translated σ3. The σ3 in virions is known to be protease sensitive (2, 16, 32). Early in infection, virions must be converted to intermediate subviral particles (ISVPs) by the proteolytic removal of σ3 and carboxy-terminal cleavage of μ1 and μ1C into two smaller fragments (3, 26, 35–37).

To determine whether σ3 is folded into a protease-sensitive conformation during or just after translation or whether this happens upon capsid assembly, we examined the protease sensitivity of translated and virion σ3. Folded domains of proteins are generally insensitive to protease digestion, whereas polypeptide segments that link folded domains are often susceptible. Changes in protease sensitivity reflect alterations in protein conformation (4). Purified ^{35}S-labeled virions, obtained as previously described (10), and translated σ3 were

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† Dedicated to the memory of Bernard N. Fields, who continues to serve as a spiritual mentor.

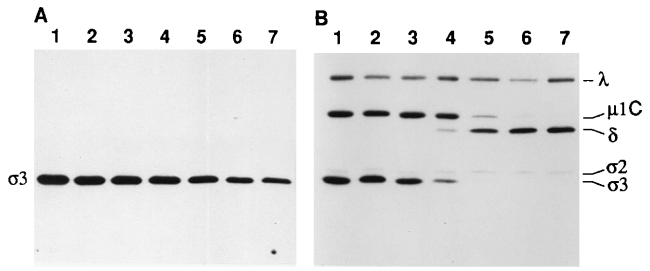

FIG. 1. Proteinase K digestion of translated σ3 and virions. Translated σ3 (98,000 trichloroacetic acid-precipitable ^{35}S cpm) and T3D virions (280,000 ^{35}S cpm) were digested with 20 μg of proteinase K per ml for 2 h at 30°C. Samples were analyzed by SDS-PAGE (10% resolving gel) and fluorography. Digestion samples of translated σ3 and virions are shown in panels A and B, respectively. Lane 1 contains the untreated control. Samples were removed at 0 (immediately after protease addition and incubation at 30°C; lane 2), 10 (lane 3), 30 (lane 4), 60 (lane 5), 90 (lane 6), and 120 (lane 7) min.

digested in virion storage buffer (10) with 20 μg of proteinase K (Sigma Chemical Co.) per ml for 2 h at 30°C. Reticulocyte lysate was added to all samples, and unlabeled virions were added to translated samples to balance the protein concentration. Samples were removed at intervals, and the digestion was stopped by the addition of 1 mM phenylmethylsulfonyl fluoride (Gibco BRL). Samples were analyzed by SDS-PAGE and fluorography (Fig. 1).

We found that translated σ3 (Fig. 1A) was considerably less protease sensitive than virion σ3 (Fig. 1B). A similar result was obtained if digestion was performed with trypsin (data not shown). Translated σ3 could be completely converted to a protease-sensitive form by the addition of 0.5% SDS, demonstrating that it was not misfolded to such a degree that it was permanently resistant to protease (data not shown). Incubation of translated σ3 and virions in the absence of protease did not affect the quantity of σ3 (data not shown), indicating that no contaminating proteases were present in the samples.

The finding that translated σ3 was less protease sensitive than virion σ3 suggested that σ3 undergoes a conformational change subsequent to its translation. To investigate directly whether translated σ3 would assume a protease-sensitive conformation upon binding to the viral particle, we developed an assay based on the finding that σ3 from infected cells can recoat subviral particles (1). ^{35}S-labeled infected cell extracts were made by using a previously described procedure (12), with the following modifications. Monolayers of L cells were infected with T3D at a multiplicity of infection of 50, and at 12 h postinfection, 100 μCi of Tran^{35}S-label per ml was added for 2 h at 37°C. ISVPs were obtained as described by Nibert and Fields (26). To recoat ISVPs, 2.5 ml of extract was incubated with 2×10^{12} ISVPs for 1 h at room temperature and sedimented in a preformed CsCl gradient (1.25 to 1.45 g/cm^3) in a Beckman 50.1 rotor at 35,000 rpm for 3 h at 4°C. The gradient was fractionated, and fractions containing the radioactive peak corresponding to recoated ISVPs were pooled and concentrated. The recoated ISVPs were analyzed by SDS-PAGE. Proteins of the ISVP were visualized by staining the gel (data not shown), and labeled protein bound to the ISVP was visualized by fluorography (Fig. 2A). This analysis confirmed the finding of Astell and colleagues (1) that σ3 from reovirus-infected cell extracts (Fig. 2A, lane 1) can recoat ISVPs (Fig. 2A, lane 3).

Next, we examined the ability of translated σ3 to recoat ISVPs. Radiolabeled, translated σ3 was mixed with 5×10^{10} ISVPs in virion storage buffer, and the recoated particles were purified and analyzed as described above. Like σ3 from infected cells, translated σ3 recoated ISVPs (Fig. 2B, lane 2). This finding provided further evidence that translated σ3 shares functional properties with native σ3 from infected cells. Recoating was not stoichiometric with either translated σ3 or σ3 from infected cell extract.

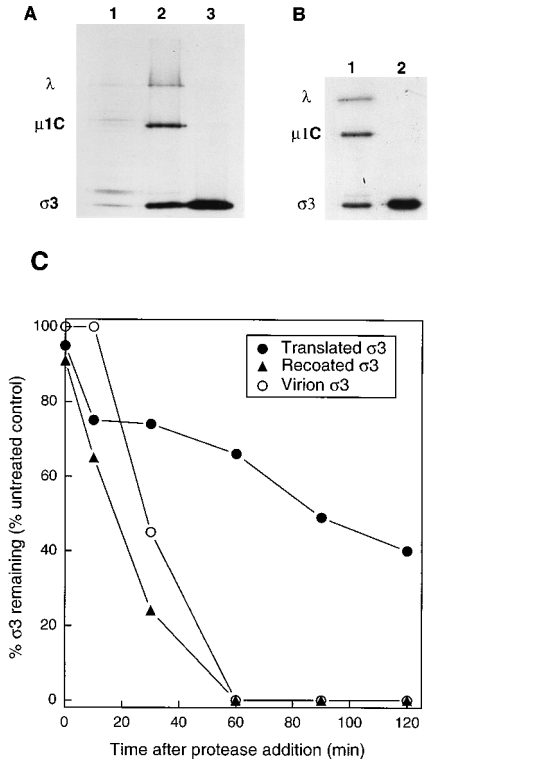

FIG. 2. Proteinase K digestion of translated σ3, recoated ISVPs, and virions. (A) ^{35}S-labeled, reovirus-infected cell extract (lane 1) was incubated with ISVPs. The resulting particles were purified and analyzed by SDS-PAGE (10% resolving gel) and fluorography (lane 3). An untreated sample of ^{35}S-labeled virions was also analyzed (lane 2). The positions of the viral proteins are indicated on the left. (B) Untreated ^{35}S-labeled virions (lane 1) and ISVPs recoated with ^{35}S-labeled, translated σ3 (lane 2) were analyzed by SDS-PAGE (10% resolving gel) and fluorography. Positions of viral proteins are indicated on the left. (C) ISVPs recoated with translated σ3 (280,000 ^{35}S cpm), translated σ3 (98,000 trichloroacetic acid-precipitable ^{35}S cpm from the sample used for ISVP recoating), and virions (280,000 ^{35}S cpm) were digested with 20 μg of proteinase K per ml for 2 h at 30°C. Samples were removed at intervals and analyzed by SDS-PAGE (10% resolving gel) and fluorography. Bands representing σ3 were quantitated by densitometry.

Using ISVPs recoated with translated σ3, we assessed whether association of σ3 with the viral particle altered its conformation. We used the proteinase K digestion assay to compare the protease sensitivities of translated σ3, σ3 in virions, and translated σ3 bound to ISVPs. Bands representing σ3 were quantitated by densitometry. Densitometric analysis was performed on a Macintosh IIsi computer using the public domain NIH Image program (written by Wayne Rasband at the National Institutes of Health and available from the Internet by anonymous ftp from zippy.nimh.nih.gov or on floppy disk from NTIS, 5285 Port Royal Rd., Springfield, VA 22161, part no. PB93-504868). The percentage of σ3 that remained over time was calculated and is represented graphically in Fig. 2C. Association with the ISVP rendered translated σ3 protease sensitive to the same degree as virion σ3. These results indicated that σ3 undergoes a conformational change upon association with the ISVP and provided evidence that the protease-resistant conformation of newly translated σ3 is not irreversible.

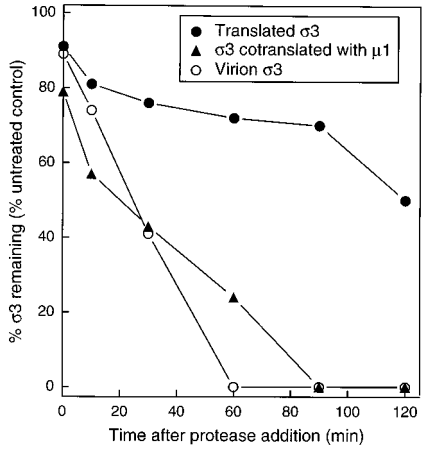

FIG. 3. Proteinase K digestion of translated σ3, cotranslated σ3 and μ1, and virions. Translated σ3 (98,000 trichloroacetic acid-precipitable ^{35}S cpm), cotranslated σ3 and μ1 (280,000 trichloroacetic acid-precipitable ^{35}S cpm), and virions (280,000 ^{35}S cpm) were digested with 20 μg of proteinase K per ml for 2 h at 30°C. Samples were analyzed by SDS-PAGE (10% resolving gel) and fluorography. Bands representing σ3 were quantitated by densitometry, and the percentage of σ3 remaining, relative to the level in the untreated control, was calculated and represented graphically. The time zero value for σ3 cotranslated with μ1 was lower than the time zero values for virion and translated σ3 as a result of the presence of truncated products of μ1 that interfered with quantitation of intact σ3. These truncated μ1 products were completely digested upon addition of protease (data not shown).

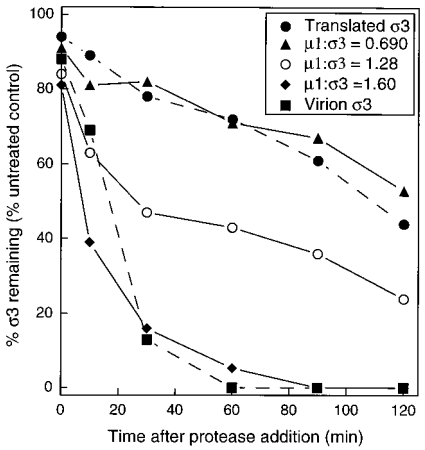

FIG. 4. Proteinase K digestion of translated σ3, cotranslated σ3 and μ1 at various ratios, and virions. For this experiment, all translated samples were labeled with [^{35}S]methionine. Translated σ3 (98,000 trichloroacetic acid-precipitable ^{35}S cpm), cotranslated σ3 and μ1 (280,000 trichloroacetic acid-precipitable ^{35}S cpm), and virions (280,000 ^{35}S cpm) were digested with 20 μg of proteinase K per ml for 2 h at 30°C. Samples were analyzed by SDS-PAGE (10% resolving gel) and fluorography. Bands representing σ3 were quantitated by densitometry, and the percentage of σ3 remaining, relative to the level in the untreated control, was calculated and represented graphically. For the cotranslation samples, the σ3 and μ1 bands in the untreated control were quantitated by densitometry, corrected for numbers of methionines in each protein, and divided to obtain a ratio. The positive and negative controls, virions and translated σ3, respectively, are indicated by dashed lines.

In the ISVP, the most exterior major protein is μ1 (8), which has been cleaved into three fragments (26, 28, 36, 40). Thus, the conformational change that occurs when σ3 binds to the ISVP is likely to have resulted from interaction with the cleaved form of μ1, which has been shown by cryoelectron microscopy to largely retain the conformation of μ1 and μ1C found in virions (8). To determine whether the conformational change in σ3 could be mediated by soluble, intact μ1, we compared the protease sensitivity of σ3 cotranslated with μ1 with that of σ3 translated alone and σ3 in virions. To generate μ1 by in vitro transcription and translation, we introduced a cDNA copy of the T3D M2 gene (15) into pCITE-2b. Cotranslation of σ3 with μ1 resulted in σ3 that was as protease sensitive as virion-bound σ3 (Fig. 3), suggesting that association of σ3 with μ1 is sufficient to confer a conformational change on σ3. The conformational change in σ3 can be detected in cotranslation samples within 15 min of the initiation of translation, the earliest time point assayed (data not shown). Cotranslation of σ3 with chloramphenicol acetyltransferase did not produce σ3 that was protease sensitive, demonstrating that this finding was specific for μ1 (data not shown). Protease digestion and subsequent immunoprecipitation of σ3 and μ1 from infected cell extracts revealed that at late times during infection, σ3 is similarly protease sensitive (data not shown).

Our results suggested that association of σ3 and μ1 as early as 15 min following the initiation of translation mediates a conformational change in σ3. To determine whether μ1 could mediate a change in preexisting σ3, the protease digestion was repeated with σ3 and μ1 that were translated separately and then mixed for 1 h at 30°C. Mixing of σ3 and μ1 rendered σ3 protease sensitive (data not shown), indicating that a conformational change in σ3 could be mediated by μ1 posttranslationally. To determine whether the μ1-mediated change in σ3 involved formation of a μ1-σ3 complex, we used monoclonal antibodies to immunoprecipitate σ3 and μ1 that had been cotranslated or individually translated and mixed. Cotranslated and mixed samples were immunoprecipitated in a buffer containing 0.05% Nonidet P-40 to maintain protein complexes. Our analysis indicated that both the μ1-specific monoclonal antibody 10H2 and the σ3-specific monoclonal antibody 4F2 coprecipitated σ3 and μ1 (data not shown). Thus, for both cotranslated and mixed σ3 and μ1, the conformational change in σ3 was associated with σ3-μ1 complex formation.

In the outer capsid, σ3 and μ1 are present in a 1:1 stoichiometry (15), and it has been suggested that formation of a complex containing one molecule each of σ3 and μ1 blocks the ability of σ3 to bind dsRNA (38). To determine whether a 1:1 complex of σ3 and μ1 is required to confer a conformational change on σ3, we cotranslated σ3 and μ1 at different relative ratios and examined the protease sensitivity of the resulting σ3. Densitometric analysis of the σ3 and μ1 bands was performed. Values representing band intensity were corrected for the relative number of methionine residues in each protein to determine the ratios of μ1 to σ3. We found that as the number of μ1 molecules increased relative to the number of σ3 molecules, σ3 became more protease sensitive (Fig. 4). Thus, the ability of μ1 to confer protease sensitivity on σ3 is dependent on their relative stoichiometries and appears to require at least equal numbers of σ3 and μ1 molecules. Limitations in quantitation by densitometry do not allow us to precisely determine the relative amounts of σ3 and μ1 in our cotranslated samples. However, our finding suggests that higher-order structures are being formed when a ratio of 1:1 or higher is achieved. Such structures may be required for μ1 to efficiently confer a conformational change on σ3 and could serve to initiate the assembly of the outer capsid.

To determine whether the μ1-mediated conformational change folds σ3 into a conformation similar to that of σ3 in virions, we examined the intermediate peptides formed upon proteinase K digestion of T1L σ3 in virions, on recoated

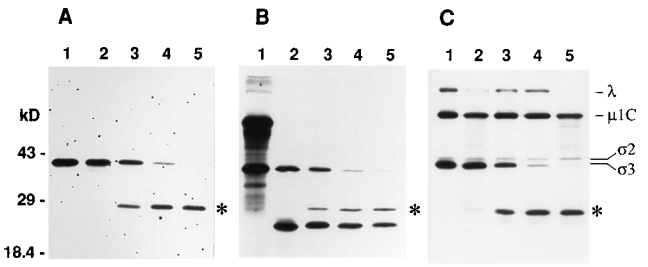

FIG. 5. Proteinase K digestion of T1L σ3-recoated ISVPs (A), T1L σ3 cotranslated with μ1 (B), and T1L virions (C). Samples (280,000 ^{35}S cpm) were digested with 100 μg of proteinase K per ml for 1 h at 5°C, and samples were analyzed by SDS-PAGE (10 and 12% resolving gels) and fluorography. In each panel, lane 1 contains the untreated control and lanes 2 through 5 contain samples removed at 0, 10, 30, and 60 min, respectively. The positions of protein size standards are indicated on the left, and the positions of the viral proteins are indicated on the right. Each asterisk marks the position of the 26- to 28-kDa peptide fragment produced by protease digestion. In the cotranslation sample (B), a predominant μ1 band, as well as two minor truncated forms of μ1, are produced by in vitro translation of the M2 gene. The additional band seen upon digestion of cotranslated σ3 and μ1 was generated from digestion of μ1 (data not shown).

ISVPs, and cotranslated with μ1. Limited proteolysis of gel-purified σ3 or bacterially expressed σ3 with V8 protease produces an amino-terminal 24-kDa fragment and a carboxy-terminal 16-kDa fragment (22, 29, 30). Digestion of virions with other proteases generates similarly sized fragments of σ3 (29). Intermediate digestion products of σ3 from T1L virions are relatively stable, whereas those produced from T3D are transient (29). T1L σ3 was prepared by in vitro transcription and translation from plasmid pCITE-2b-S4T1L. The results are shown in Fig. 5. The protease-sensitive form of σ3 on ISVPs and in cotranslated samples was degraded by proteinase K to yield the same approximately 26- to 28-kDa peptide seen when virion σ3 was digested (asterisks in Fig. 5). These results suggest that association of μ1 with soluble σ3 folds σ3 into the same protease-sensitive conformation in which it is found on virions.

In this report, we have demonstrated that binding of μ1 to σ3 results in a conformational change in σ3 that converts it from a protease-resistant form to a protease-sensitive form. Monoclonal antibodies directed against two nonlinear epitopes bind the in vitro-translated, protease-resistant form of σ3, as well as the protease-sensitive conformation of the protein found on virions (39) and in complexes containing translated σ3 and μ1 (34). These results suggest that the conformational change is a subtle one, rather than a gross rearrangement of σ3's tertiary structure. Information currently available regarding σ3's structure suggests a molecular mechanism for this conformational change. A number of studies have identified a region near the center of the σ3 protein that is hypersensitive to proteolytic cleavage (22, 29, 30). These data, along with cryoelectron microscopy analyses (8), suggest that the tertiary structure of σ3 consists of two domains joined by a polypeptide hinge. Our results suggest that the hinge region in newly translated σ3 is inaccessible to proteases and that the μ1-mediated conformational change in σ3 affects the hinge region, causing it to become accessible to proteases.

Proteolytic removal of σ3 is an early, essential step in the reovirus life cycle (37). We have demonstrated that association with soluble μ1 converts σ3 to a protease-sensitive structure. We have also used limited proteolysis to provide evidence that soluble σ3 complexed with μ1 is folded into the same conformation as σ3 in virions. Our results suggest that the σ3-μ1 interaction is important for folding σ3 into a conformation that allows its proteolytic cleavage during entry. Similarly, studies showing that extragenic revertants of mutations in the M2 gene map to the S4 gene suggest that the σ3-μ1 association is important for μ1's ability to function during entry (9, 21, 24). Thus, association of σ3 and μ1 in a specific conformation appears to be important for the formation of an outer capsid that is competent for entry.

Our studies are consistent with a model in which formation of a 1:1 complex of σ3 and μ1 and the subsequent conformational change in σ3 serve as a functional switch that converts σ3's function from translational control to assembly of the outer capsid onto nascent core particles. Association of σ3 and μ1 folds σ3 in progeny virions into a conformation that renders it susceptible to proteolysis during the next round of infection. Our findings raise the interesting possibility that the negative effect of the M2 gene on the translational effects of the S4 gene (38) is the result of the conformational change in σ3 rather than competition by μ1 for the dsRNA-binding site on σ3. Recent studies from our laboratory suggest that μ1 and dsRNA do not share identical binding sites on σ3 (33). Ongoing studies on the effect of μ1 on dsRNA binding by σ3 will further our understanding of the significance of σ3-μ1 interactions during reovirus infection.

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