Potential Role for CA-SP in Nucleating Retroviral Capsid Maturation

Matthew R. England,a John G. Purdy,aIra J. Ropson,b Paula M. Dalessio,b Rebecca C. Cravena

Department of Microbiology and Immunology,a and Department of Biochemistry and Molecular Biology,b The Pennsylvania State University College of Medicine, Hershey, Pennsylvania, USA

ABSTRACT

During virion maturation, the Rous sarcoma virus (RSV) capsid protein is cleaved from the Gag protein as the proteolytic intermediate CA-SP. Further trimming at two C-terminal sites removes the spacer peptide (SP), producing the mature capsid proteins CA and CA-S. Abundant genetic and structural evidence shows that the SP plays a critical role in stabilizing hexameric Gag interactions that form immature particles. Freeing of CA-SP from Gag breaks immature interfaces and initiates the formation of mature capsids. The transient persistence of CA-SP in maturing virions and the identification of second-site mutations in SP that restore infectivity to maturation-defective mutant viruses led us to hypothesize that SP may play an important role in promoting the assembly of mature capsids. This study presents a biophysical and biochemical characterization of CA-SP and its assembly behavior. Our results confirm cryo-electron microscopy (cryo-EM) structures reported previously by Keller et al. (J. Virol. 87:13655–13664, 2013, doi:10.1128/JVI.01408-13) showing that monomeric CA-SP is fully capable of assembling into capsid-like structures identical to those formed by CA. Furthermore, SP confers aggressive assembly kinetics, which is suggestive of higher-affinity CA-SP interactions than observed with either of the mature capsid proteins. This aggressive assembly is largely independent of the SP amino acid sequence, but the formation of well-ordered particles is sensitive to the presence of the N-terminal β-hairpin. Additionally, CA-SP can nucleate the assembly of CA and CA-S. These results suggest a model in which CA-SP, once separated from the Gag lattice, can actively promote the interactions that form mature capsids and provide a nucleation point for mature capsid assembly.

IMPORTANCE

The spacer peptide is a documented target for antiretroviral therapy. This study examines the biochemical and biophysical properties of CA-SP, an intermediate form of the retrovirus capsid protein. The results demonstrate a previously unrecognized activity of SP in promoting capsid assembly during maturation.

The CA protein is the major structural protein in retroviruses, forming the capsid structure in mature virions (1–3). CA is processed from the polyprotein Gag by the viral protease (PR), whose activity is initiated coincident with or immediately upon virus budding from the cell surface. The highly complex rearrangement process that ensues leads to the condensation of a virus budding from the cell surface. The highly complex rearrangement process that ensues leads to the condensation of a virus budding from the cell surface.

The release of the mature capsid subunit CA from Gag is a multistep process that in the alpharetroviruses and lentiretroviruses requires cleavage at a unique upstream site at the N terminus of CA and 2 to 3 cleavages between CA and the downstream NC domain (Fig. 1) (2, 10, 20–23). The initial cleavages at the N termini of CA and NC free NC to begin the formation of the RNP and also yield immature capsid proteins that bear a short spacer peptide (SP) at the C terminus. These intermediates, known as CA-SP in Rous sarcoma virus (RSV) (an alpharetrovirus) and CA-SP1 in human immunodeficiency virus type 1 (HIV-1) (a lentivirus), persist for several hours (10, 24, 25). In RSV, PR gradually removes either 9 or 12 residues from the C terminus of CA-SP, producing a mixture of two mature species: CA (237 amino acids) and CA-S (240 residues) (20). HIV-1 generally contains only a single species of CA (CA/p24) in wild-type (WT) virus, although a cryptic cleavage can yield an alternative CA form (9, 10). The fully matured RSV and HIV proteins are highly similar alpha-helical proteins, consisting of an N-terminal domain (NTD) and a C-terminal domain (CTD) separated by a short linker. A short unstructured region follows the last helix in the CTD (26–32).

Abundant evidence supports a critical role for the SP sequence both in the earliest events of Gag assembly and in forming stabilizing interactions in the immature Gag lattice. In immature Gag shells of HIV, RSV, and M-PMV (Mason-Pfizer monkey virus), cryo-electron tomographic studies identified intrahexameric SP interactions that stabilize rings of Gag molecules, while the CA CTD and NTD make interhexameric contacts to build the larger lattice (11, 12, 33–36). Biophysical studies of monomeric Gag fragments and synthetic SPs suggest that the region is more ex-
Genetic studies of RSV provide a strong argument that the transient presence of SP at the end of CA influences the outcome of maturation. A second-site mutation (S241L) in SP is capable of partially suppressing the capsid assembly defect caused by a primary mutation (F167Y) in the CTD that compromises the ability of CA to initiate capsid formation (45). Since little is known about the self-interaction properties of the CA-SP processing intermediate, we undertook a detailed analysis of purified RSV CA-SP to compare its biophysical and assembly properties to those of the well-characterized CA protein (46, 47). These findings document a clear difference in the assembly behaviors of CA and CA-SP, which suggests that the transient presence of CA-SP in maturing virions may actually promote the formation of capsids by nucleating mature CA interactions and provide a biological rationale for the conservation of staggered cleavages.

MATERIALS AND METHODS

Protein expression and purification. RSV Prague C CA-S (240 residues) and CA-SP (249 residues) were cloned into pET-24L (+) and expressed in *Escherichia coli* BL21(DE3), as described previously (46–49). Proteins were purified by ammonium sulfate precipitation, DEAE cation exchange, and size exclusion chromatography (46, 47). Purified monomeric proteins were stored in SEC buffer (20 mM Tris, 150 mM NaCl, 0.1 mM EDTA [pH 7.6]). The CA CTD and the CTD bearing SP were expressed as 6×His-tagged proteins and purified as described previously (50). The following extinction coefficient values were used: 24,980 M⁻¹ cm⁻¹ (RSV CA, CA-S, and CA-SP) and 6,990 M⁻¹ cm⁻¹ (RSV CTD and CTD-SP).

*In vitro* assembly and data analysis. CA assembly was initiated in 50-μl reaction mixtures (80 μM protein, 50 mM Tris, 150 mM NaCl, 0.1 mM EDTA) by the addition of sodium phosphate (final concentration, 500 mM) at pH 8.0 and 25°C. The development of turbidity was monitored at 450 nm in at least three independent experiments (46, 47). The kinetic data presented are averages of replicate curves (usually 3) generated on the same day. Reaction products were applied onto Formvar/carbon-coated grids, stained with 2% uranyl acetate, and visualized on a JEOL JEM1400 transmission electron microscope. Digital images were captured by using a Gatan charge-coupled-device (CCD) camera.

Biophysical characterization. Circular dichroism (CD) spectra were collected between 190 and 260 nm for monomeric CA and CA-SP (0.15 mg/ml protein in 20 mM Tris, 150 mM NaCl, 0.1 mM EDTA) using a Jasco J-710 spectropolarimeter. To assess protein stability, alpha kinetic values were used: 24,980 M⁻¹ cm⁻¹ (RSV CA, CA-S, and CA-SP) and 6,990 M⁻¹ cm⁻¹ (RSV CTD and CTD-SP). The dimerization affinity of CTD and CTD-SP proteins was measured at pH 3.7. Low pH triggers the dimerization of the isolated CTD by neutralizing aspartate residues at the dimer interface and also promotes the assembly of full-length CA into capsid-like structures identical to those formed in sodium phosphate at neutral pH (50, 51).

RESULTS

Monomeric CA-SP and CA-S. Recombinant CA-SP, 249-residue processing intermediate, and 240-residue mature CA-S proteins were expressed in *E. coli* and purified for comparison with the mature CA protein (237 residue) that has been extensively characterized *in vitro* (46–48, 52). Purified CA-S and CA-SP, like CA, were almost exclusively monomeric (Fig. 2A). The CA-SP protein eluted slightly faster from the size exclusion chromatography column than either CA or CA-S, as expected from its higher mass. In SDS-PAGE gels, purified CA-SP exhibited the same anomalously fast migration that was previously described for virus-infected cells (Fig. 2B), suggesting that CA-SP adopts a more compact structure in SDS than do the two shorter capsid proteins (20, 25).

The biophysical properties of monomeric CA-S and CA-SP

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**FIG 1** Proteolytic processing during maturation. A multistep sequence of cleavages of immature Gag molecules first produces the capsid intermediate CA-SP. Over 3 to 6 h, 9 or 12 spacer peptide residues are removed, leaving CA and CA-S to form the mature capsid. The gray arrow shows the initial cleavage generating CA-SP (249 amino acids), while black arrows represent the terminal SP cleavages resulting in the mature capsid proteins CA (237 amino acids) and CA-S (240 amino acids).

MINUTES

HOURS

CA-SP

CA-S

CA

PLTDQGIAAMSSAIQPLIMAVN

249

240

237

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**TABLE 1** Characteristics of mutant capsid lattices.*

<table>
<thead>
<tr>
<th>Mutant</th>
<th>Description</th>
<th>Assembly vs. CA</th>
<th>CTD-SP</th>
<th>CA-SP</th>
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<tr>
<td>CA-C62</td>
<td>C62 delete</td>
<td>+</td>
<td>+</td>
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<tr>
<td>CA-C62</td>
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<tr>
<td>CA-C62</td>
<td>C62 delete</td>
<td>+</td>
<td>+</td>
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</tr>
</tbody>
</table>

*Bold indicates significant differences from wild-type CA assembly. CA-C62, CA-C62, CA-C62, CA-C62, CA-C62.
were indistinguishable from those of CA. The circular dichroism spectra for CA, CA-S, and CA-SP were superimposable, suggesting that all three proteins are properly folded and have similar secondary structures (Fig. 2C). The midpoint of unfolding in the presence of the denaturant guanidine hydrochloride was the same \((/H110112.2 \text{ M})\) for CA and CA-SP, indicating that SP has no effect on the stability of the monomeric protein (Fig. 2D). In addition, the SP extension had no significant effect on the dimerization of the CTD, as measured by isothermal titration calorimetry (Fig. 3).

**SP confers distinctive protein assembly properties.** CA-S represents nearly half of the capsid protein in mature RSV virions (20). Considering that as much as 80% of the total available capsid protein is incorporated into the core during maturation, it is expected that both CA and CA-S contribute to the capsid shell (4). The self-interaction ability of CA-S was tested in vitro by using a sodium phosphate-triggered assembly reaction previously developed for RSV CA (47). Under standard conditions of 80 \(\mu\text{M}\) protein and 500 mM sodium phosphate at pH 8.0, the monomeric CA-S protein was indeed capable of assembly (Fig. 4A). The kinetics of assembly, monitored turbidimetrically, were very slightly faster than those observed with CA alone, although this effect was not uniformly reproducible. In a coassembly reaction mixture containing CA and CA-S, the kinetics of assembly and the final turbidity were indistinguishable from those observed for single-protein reactions (Fig. 5A). The turbidity profiles in all cases resembled those of typical nucleation-driven reactions, with a distinct lag followed by a rapid increase.

Both mature capsid proteins (CA and CA-S) form abundant spheres with a diameter of 17 nm in sodium phosphate (Fig. 4B and C). Spheres with a diameter of 32 nm made up 1.7% \(1.1\%\) of the assembly products (Fig. 4B and C). In vitro-assembled protein (80 \(\mu\text{M}\)) was collected after the curves reached a plateau, and the samples were spun at 128,000 \(\times\) g for 30 min. The concentration of unassembled protein was determined by using a Nanodrop-3000 instrument. Error bars represent 4 replicates. \(*, P < 0.01; **, P < 0.005.\)

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**FIG 2** Purification and characterization of capsid proteins. (A) CA, CA-S, and CA-SP expressed and purified from *E. coli* were analyzed by size exclusion chromatography using a Superdex S75 column. (B) Electrophoretic migration in 15% SDS-PAGE. CA-SP migrates anomalously fast, as described for CA-SP in vivo. (C) Secondary structures of full-length CA, CA-S (not shown), and CA-SP were compared by circular dichroism spectroscopy. (D) The stability of each protein in guanidine hydrochloride (Gdn-HCl) was determined by monitoring \(\alpha\)-helical content at 222 nm.

**FIG 3** Effects of SP on CTD-CTD dimerization. The dissociation constants (Kd) of the purified CTD and CTD-SP dimers were determined at low pH (3.7) by isothermal titration calorimetry. NDH, normalized \(\Delta\text{H}\) (cal/mol per injection).

**FIG 4** Kinetics of CA, CA-S, and CA-SP assembly. (A) In vitro assembly of CA, CA-S, and CA-SP at 40 \(\mu\text{M}\) and 80 \(\mu\text{M}\) was triggered with 500 mM sodium phosphate, and the turbidity at 450 nm was monitored. OD\(_{450}\), optical density at 450 nm. (B to D) Resulting assembly products of CA (B), CA-S (C), and CA-SP (D) were examined by negative-stain transmission electron microscopy. Bar = 100 nm. (E) In vitro-assembled protein (80 \(\mu\text{M}\)) was collected after the curves reached a plateau, and the samples were spun at 128,000 \(\times\) g for 30 min. The concentration of unassembled protein was determined by using a Nanodrop-3000 instrument. Error bars represent 4 replicates. \(*, P < 0.01; **, P < 0.005.\)

**FIG 5** Coassembly of CA, CA-S, and CA-SP in vitro. (A and B) Various ratios of CA, CA-S, and CA-SP (A) and CA and CA-SP (B) were mixed and assembled as described in the legend of Fig. 4. (C and D) Transmission electron microscopy images of CA, CA-S, and CA-SP (60:40:0; \(\times\) in panel A) (C) and CA and CA-SP (95:5; \(\triangle\) in panel B) (D) are shown. Bar = 100 nm.
in authentic virions were seen rarely (46). The coassembled CA (not shown) that resemble the capsid-related structures observed in authentic virions. Tubes and larger angular and multilamellar structures (Fig. 5C). Thus, the 3-amino-acid extension slightly stimulates assembly but does not alter the types of particles that form.

The behavior of the cleavage intermediate CA-SP was strikingly different from that of either the mature CA or CA-S protein. The presence of the extra 9 spacer peptide residues conferred very fast assembly behavior (Fig. 4A). Under standard conditions, CA-SP assembled rapidly, with no discernible lag phase. As with CA and CA-S, CA-SP self-assembly was protein concentration dependent, with the lag and growth phases becoming clear with reduction of the protein concentration (data not shown). The dramatic difference between CA-SP and the mature CA and CA-S proteins was most evident at a protein concentration of 40 μM, where the mature proteins failed to produce any detectable turbidity (Fig. 4A). Consistent with this, the concentration of protein remaining unassembled (resistant to pelleting at 128,000 × g) was ~6 μM for CA-SP, about 3-fold and 2-fold lower than those measured for CA and CA-S, respectively (Fig. 4E), suggesting a lower critical concentration for nucleation of assembly.

CA-SP nucleates mature CA and CA-S assembly. To test whether CA-SP could initiate the assembly of mature CA and CA-S in vitro, immature and mature proteins were mixed together in various ratios, keeping the total protein concentration constant at 80 μM, prior to the addition of sodium phosphate. In all reaction mixtures containing CA-SP, assembly was robust and proceeded with a shorter lag than either CA or CA-S (Fig. 5A). In reaction mixtures containing CA and CA-SP, a proportion of immature protein, as low as 5% of the total, was able to stimulate the assembly of CA (Fig. 5B). The coassembly reactions yielded small spheres that resembled those formed by CA alone (Fig. 5D). Thus, it appears that the presence of CA-SP can nucleate the assembly of the shorter mature proteins.

CA and CA-SP form structurally similar assembly products. Electron microscopy (EM) examination of the CA-SP assembly products showed structures resembling those of CA and CA-S and included abundant spherical particles 17 nm in diameter and multilayered particles 32 nm in diameter (Fig. 4D). In a typical experiment, the larger particles represented 7.8% ± 1.7% (n = 857) of the population, slightly higher than the frequency observed for CA or CA-S (see above). In addition, CA-SP formed occasional tubular and capsid-like structures (not shown). Three-dimensional single-particle reconstructions from 200 negatively stained 17-nm particles and from 60 of the 32-nm particles (not shown) revealed that they are consistent with T=1 and T=3 icosahedral symmetry, respectively, similar to those reported previously for CA (5, 53). These results were further extended in a collaborative study by high-resolution cryoelectron microscopy, T=1 particles at a 8.5-Å resolution showed that CA-CA interfaces in in vitro-assembled CA-SP are nearly indistinguishable from those in CA particles and that the spacer peptide itself did not contribute regular interactions within the particles (21). We note that similar assembly behaviors were previously described for N-terminally histidine-tagged CA, CA-S, and CA-SP proteins assembled on nickel-modified lipids (54). It is not clear whether there were any unique characteristics of the spherical particles that formed under those conditions.

The similarity of CA and CA-SP assembly was confirmed by testing the influence of mutations known to specifically cripple the ability of the CA protein to build capsid structures. The conservative F167Y substitution in the first α-helix of the CTD is a well-characterized mutation that cripples the nucleation of capsid formation in vitro and in maturing virions while allowing apparently normal Gag function (18, 45, 55). This mutation destroyed the ability of CA-SP to assemble in vitro (data not shown), as was shown previously for CA. Next, the importance of the N-terminal β-hairpin was tested. The formation of this structure, which forms upon the cleavage of CA from Gag and is a hallmark of mature capsid assembly, can be prevented by the substitution of an alanine at the asparagine at position 52 in helix 3, which serves as a docking site for the N-terminal proline in mature capsid proteins (19, 46, 56, 57). Monomeric D52A CA develops turbidity with rapid kinetics but is unable to form organized particles (46) (Fig. 6A and B). The same substitution in CA-SP likewise caused rapid precipitation without organized particle formation (Fig. 6A and C). The assembly-destroying activity of the D52A mutation was prevented, however, by an extension of CA-SP by 8 residues of the NC protein so that the entire length of the known Gag interaction domain, spanning the SP sequence and flanking regions, was present. Both the WT and D52A forms of CA-SP-8NC assembled rapidly under standard conditions (Fig. 6A) and produced spheres that appeared by negative-stain EM to resemble the 17- and 32-nm structures formed by CA-SP (Fig. 6D and E). In addition, both proteins formed a new subclass of larger, less regular particles that made up 4.6% ± 0.9% (CA-SP-8NC) (n = 2,083) and 3.8% ± 1.1% (D52 CA-SP-8NC) (n = 1,464) of the total population, suggesting some tendency of the longer proteins to form interactions not seen with the CA-SP protein (Fig. 6D and E, arrows). The slight increase in assembly kinetics of the D52A protein suggests that an unfolded N terminus promotes associations. Taken together, these findings indicate that CA-SP assemblies with properties characteristic of the mature capsid protein rather than its Gag precursor.

Probing of the amino acid sequence of SP. A series of mutations in the SP sequence were created to assess the importance of specific residues for the unique behavior of CA-SP (Fig. 7). None of the single- or triple-alanine substitutions tested disrupted CA-SP assembly. Each mutation displayed the rapid assembly kinetics characteristic of CA-SP and produced similar populations of small icosahedral particles. Compared to WT CA-SP, each of the triple-alanine substitution mutants assembled slightly more slowly, although the final products were similar (Fig. 7B to E). To create potentially more disruptive alterations, clusters of 3 residues were replaced with prolines. Each of the three triple-proline mutants developed turbidity with the rapid kinetics of wild-type CA-SP (Fig. 7A and C). In contrast to the alanine mutants, however, the P1 triple-proline mutant completely failed to produce well-ordered particles (Fig. 7F), while P2 and P3 produced some smaller spherical particles along with abundant amounts of precipitated protein (not shown). These observations suggest that the influence of SP on protein assembly is largely independent of the amino acid sequence but may be influenced by unknown structural properties of the spacer peptide.

DISCUSSION

Retroviral maturation in RSV begins in a protein environment that is in flux. Due to the staggered timing of cleavages that separate the CA and NC domains in Gag, the declining pools of the...
longer CA-SP intermediate coexist for some hours with the accumulating mature CA and CA-S proteins. This persistence of CA-SP in the maturing virion as well as the conservation of such staggered cleavages across retrovirus subfamilies are consistent with the idea that the SP plays a role (or even multiple roles) in controlling the outcome of the maturation process. The underlying basis of the slow trimming of spacer peptide from CA-SP is incompletely understood. In the case of HIV-1 PR, slow cleavage at the CA-SP1 site is due in part to its amino acid sequence but is also due to unknown “context” features of the protein (23). The biological consequences of staggered cleavages, however, have been examined in detail by electron microscopy with HIV-1 (9, 11, 21, 22). Cleavage at the SP1/NC junction is sufficient to allow condensation of the nucleocapsid to proceed. If cleavage of SP1 from CA is prevented, much of the CA-SP1 remains entangled in remnants of the Gag lattice, and the building of a proper capsid is aborted. Thus, it is clear that one consequence of the staggered cleavages in the spacer region is the temporal control of the formation of the different layers of the viral core.

In addition to controlling the timing of the maturation process, previous studies of RSV and HIV (45, 58) suggest that the transient presence of CA-SP in the maturing virion also exerts a direct influence on capsid protein structure that promotes the formation of mature CA-CA contacts. An F167Y substitution in the CTD hydrophobic core allows apparently normal Gag function but cripples capsid maturation by a potent effect on the formation of the mature CTD-CTD dimer interface. The subtle perturbation of CTD structure caused by the F167Y mutation can be accommodated by the cooperative action of any one of several interface-stabilizing mutations elsewhere in CA that can promote the nucleation of capsid assembly and restore infectivity (16, 46, 50, 55). Relevant to this study, F167Y lethality can also be partially suppressed by a compensatory substitution in the spacer peptide (S241L), suggesting that the unique protein interactions that build the mature capsid may be initiated prior to the removal of SP (45). Similarly, it was observed that a replication-crippling NTD mutation in HIV, H62F at a position contributing to the mature interdomain interface, is suppressed by a compensating change in SP1 (58).

The transient presence of SP at the end of the capsid protein

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**FIG 6** Assembly of CA-SP-8NC and proteins with the D52A substitution. (A) *In vitro* assembly of proteins was completed as described in the legend of Fig. 4. (B to E) EM images of D52A CA (B), D52A CA-SP (C), CA-SP-8NC (D), and D52A CA-SP-8NC (E) are shown, as described in the legend of Fig. 4. Black arrows show a new class of larger, angular particles seen in CA-SP-8NC constructs. Bar = 100 nm.

**FIG 7** Effects of mutations on CA-SP assembly. (A to C) The ability of CA-SP to assemble with single- and triple-alanine substitutions or triple-proline substitutions was analyzed as described in the legend of Fig. 4. (D and E) Representative EM images of assembly-competent mutant proteins are shown for the M240A (D) and CA-SP (A1) (E) proteins. (F) Only CA-SP (P1) failed to produce recognizable spheres. Bar = 100 nm.
could influence the outcome of capsid formation in at least two different ways. The SP sequence may exert a negative regulatory effect on CA-SP to prevent mature-type interfaces from forming until the final cleavage removes SP at the appropriate stage of maturation. Alternatively, SP may actively promote the formation of a CA-SP intermolecular interaction that is a critical intermediate step of capsid maturation. Genetic studies alone cannot distinguish between these possibilities. Therefore, in the current study, we have utilized an in vitro capsid assembly assay, developed and characterized by our laboratory, to test directly the influence of SP on the formation of mature CA–CA interfaces.

The results presented here demonstrate that E. coli-expressed CA-SP protein, identical in amino acid sequence to the maturation intermediate detected in maturing virions, is capable of robust assembly in vitro. The CA-SP protein forms structures that resemble closely the mature capsid-like structures formed by the RSV CA protein, including the unique NTD–CTD interface that distinguishes the mature capsid lattice from the CA interactions formed by Gag (21). The powerful inhibitory effects of the D52A and F167Y mutations, which are known to prevent normal CA capsid assembly, further support the interpretation that CA-SP possesses assembly properties that closely mirror those of CA.

Thus, we conclude that the extra C-terminal residues that comprise the spacer peptide do not confer an inherent inhibitory effect on CA-SP. Rather, the assembly competence of CA-SP in vitro predicts that CA-SP generated in maturing virions early upon the activation of proteolytic processing is also competent to begin capsid formation, at least once it is freed from entanglement in the remnants of the Gag lattice. Consistent with this is the observation of partially complete capsid-like structures in HIV virions in which the trimming of CA–SP1 to CA is interrupted (21, 22).

No convincing difference in the assembly abilities of CA and CA–S, the 3-residue-longer mature form of capsid protein, were uncovered in this work. In fact, there is no evidence to date supporting the possibility that the two species make unique contributions to capsid formation. Instead, the presence of the nine further residues of SP confers an aggressive assembly capability and CA-SP particles form under standard conditions with little lag. The presence of CA–SP is also able to nucleate the aggressive assembly of the mature capsid protein in trans, suggesting that CA–SP, after release from Gag, can quickly begin building mature protein contacts and may serve as the initiation point of capsid assembly. Such an activity is consistent with the identification of spacer peptide mutations that promote capsid maturation in HIV and RSV.

What properties of CA-SP confer its unique assembly behavior in vitro? We considered the possibility that either the SP sequence may form a direct intersubunit bridge to help promote protein assembly or, alternatively, the peptide may make an intrasubunit interaction with the CTD that influences allosterically the affinity of the CTD interactions. Either scenario would be consistent with observations of HIV suggesting that in the Gag lattice, the SP1 sequence lies in close proximity to the CTD, specifically the major homology region (MHR) (21, 60, 61). However, the relative insensitivity of CA-SP assembly to alanine or proline substitutions in the spacer peptide seems to argue against such scenarios that require a direct docking of SP against the CTD. This is in contrast to the in vitro assembly of Gag protein, which was largely intolerant of SP mutations (36).

It appears likely that the mere presence of extra amino acids appended to the end of the capsid protein promotes CA–CA interactions by influencing the structure of a critical upstream element in the CTD, possibly the last alpha-helix, which is known to contribute to both CTD–CTD interactions and the CTD–NTD interdomain interface. Although a previous nuclear magnetic resonance (NMR) analysis of the RSV CA, CA–S, and CA–SP proteins (53) did not detect any obvious effect of SP on the structure of the alpha-helical bundle, a detailed analysis of protein dynamics will be needed for the detection of any such structural effect of SP. Retroviral capsid proteins are characterized by an unusual degree of internal dynamics in regions that are important to their assembly/disassembly capabilities, e.g., the CTD and the flexible loop region on the outside surface of the NTD (62–64). In particular, a recent solid-state NMR comparison of assembled HIV–1 CA and CA–SP1 proteins provided detailed evidence that the presence of SP1 causes long-distance structural effects within these regions of the CTD and the NTD and provided strong support for this interpretation of the RSV CA–SP results presented here (65).

The maturation process that produces a functional capsid is a complex event that requires a finely tuned balance of many molecular events: the temporal pattern of PR cleavage kinetics at staggered sites, the severing of assembly domains that stabilize the Gag lattice of immature virions, and the establishment of novel contacts to build the mature capsid. The results presented here demonstrate that the intermediate CA–SP, once freed from the Gag lattice, may promote the nucleation of the mature capsid and suggest that the transient persistence of CA–SP in maturing virions may be evolutionarily conserved in part for its importance in the outcome of maturation.

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