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NOTES

Necessity of the Spacer Peptide between CA and NC in the Rous Sarcoma Virus Gag Protein

REBECCA C. CRAVEN,[1]* ALPHONSE E. LEURE-DUPREE,[2] CHRISTINE R. ERDIE,[3] CAROL B. WILSON,[1] AND JOHN W. WILLS[1]

Department of Microbiology and Immunology[1] and Department of Neuroscience and Anatomy,[2] Hershey Medical Center, The Pennsylvania State University College of Medicine, P.O. Box 850, Hershey, Pennsylvania 17033, and Louisiana State University School of Medicine, Shreveport, Louisiana 71130[3]

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A mutant of Rous sarcoma virus was constructed in which the nine amino acids that separate the CA and NC sequences in the Gag protein were deleted. The spacer peptide deletion mutant produced particles containing the normal complement of viral RNA and all of the viral proteins, including reverse transcriptase. Though electron microscopy revealed particles of normal morphology, the particles were noninfectious. The normally slow maturation of the CA protein, which involves cleavage of the spacer peptide from the carboxy terminus, was bypassed in this mutant, and the association between CA and the internal components of the core appears to have been disrupted. The results suggest that the spacer peptide has an essential role in directing folding and/or oligomerization of the CA subunits within the capsid structure.

The retroviral CA protein was given its descriptive name because it forms a capsid-like structure inside the envelope of the mature virion. Contained within this shell is the ribonucleoprotein (RNP) complex consisting of viral RNA, tRNA primer, the nucleocapsid (NC) protein, and the two enzymes of replication: reverse transcriptase (RT) and integrase (3, 16, 26). This large assemblage represents the virion core that is seen in electron micrographs of retroviruses (13) and which enters the cytoplasm of the host cell during the initial phase of infection (5).

The CA protein is synthesized as part of a polyprotein precursor known as the Gag protein whose function is to direct virion assembly. Once assembled into particles, the precursor is cleaved by the viral protease (PR) to form the mature structural proteins (9, 16, 28). In the case of Rous sarcoma virus (RSV; a member of the avian sarcoma-leukosis group), the major cleavage products are MA (matrix), p10, CA, NC, and PR (Fig. 1A) as well as several small peptides. These proteins have all been purified, and the sequences of their termini have been determined (2, 9, 24, 28). A comparison of the protein and DNA sequence data indicates that the carboxy-terminal residue of the mature CA is separated from the amino terminus of NC by nine amino acids (2, 18, 24). It appears, therefore, that a peptide consisting of nine residues (SSAIQPLIM; the spacer peptide [SP]) is released by the action of PR on the Gag protein. Since the presence of SP in virions has never been demonstrated, it is possible that multiple cleavage events occur in this region.

It has been proposed that SP is cleaved relatively slowly from the CA protein so that an immature form of CA, with the nine-amino-acid SP attached, is present transiently within maturing virions (1). In support of this hypothesis, multiple forms of CA have been detected by labeling *gag*-expressing cells with [^{35}S]methionine (Fig. 1B and C; see also reference 1, Fig. 3, and Fig. 4). The fastest-migrating species, CA1, is visible in the medium after a 15-min pulse; two slower-moving species appear during the next hour (1). After 5 h or more, CA3 (the slowest-migrating form) becomes predominant and only a small amount of the intermediate product, CA2, remains (data not shown). Thus, CA maturation is a multistep process with a rapid phase in which the CA1 is cleaved from the Gag protein, followed by a slower, postbudding phase during which CA1 is converted to CA2 and CA3. Though the molecular differences between the three CA species have not been determined, it seems likely that they are generated by proteolysis since the appearance of CA2 and CA3 is delayed for mutants with reduced PR activity (1). This model of proteolytic maturation of CA does not explain why three, rather than two, CA species are seen; however, it is possible that CA2 is a product of cleavage within the SP sequence. It seems rather unlikely that posttranslational modifications other than proteolysis could account for CA2 and CA3 because these products appear after budding—the responsible enzymatic activity and the moiety to be added would have to be packaged into the virion. The model also does not explain why the shift in mobility is opposite that expected for loss of molecular mass, but this might reflect subtle differences in secondary structure or differences in sodium dodecyl sulfate (SDS) binding during electrophoresis.

Effects of a spacer deletion on particle assembly and maturation. If the model described above is correct, then deletion of SP by mutagenesis of the *gag* gene should result in the appearance of only CA3, if assembly and particle release were to occur at all. To test this hypothesis, a precise deletion of the nine codons (Fig. 1A) was constructed in an expression plasmid, pSV.Myr1, which encodes a hybrid Gag protein whose first 10 amino acids are those of the Src

* Corresponding author.

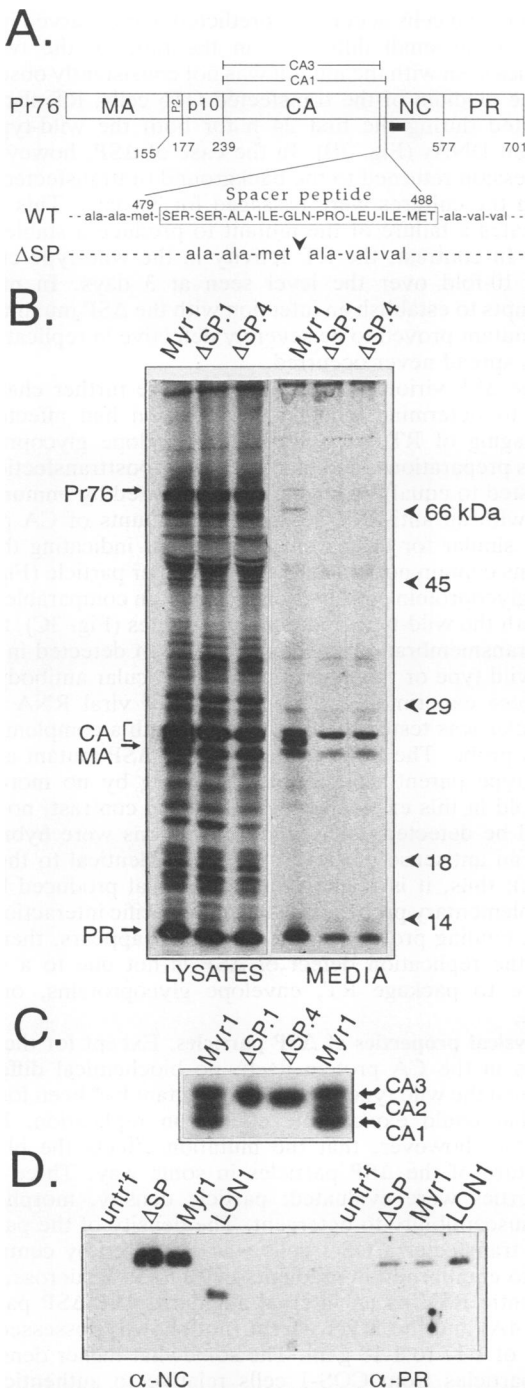

FIG. 1. The ΔSP mutant of RSV. (A) The Gag protein (Pr76) is illustrated. Numbers below the precursor indicate the carboxy-terminal residue of each cleavage product. In addition, the amino acid sequence of SP (boxed) and the flanking regions of CA and NC are shown. The arrowhead marks the predicted cleavage site formed by joining CA directly to NC in the ΔSP mutant. The black bar beneath the NC region of Pr76 marks the position of the 24-amino-acid deletion (residues 494 to 517) in the LON1 mutant. WT, wild type. (B) The ΔSP mutation was constructed by oligonucleotide-directed mutagenesis of the wild-type *gag* gene, and the *Bgl*II-*Bss*HII fragment (nucleotides 1630 to 2724) was transferred into the pSV.Myr1 expression vector, replacing the wild-type fragment (29). COS-1 cells were transfected with pSV.Myr1 and with two clones of pSV.Myr1.ΔSP. Forty-eight hours later, the cultures were incubated for 2.5 h with [^{35}S]methionine. The labeled Gag proteins were immunoprecipitated from cell lysates and medium samples with anti-RSV serum and analyzed by electrophoresis in a 13-cm-long SDS–12% polyacrylamide gel (acrylamide/bisacrylamide ratio, 29.2:0.8) followed by fluorography. Positions of the molecular size markers and the major viral proteins are indicated. (C) The Myr1 parent and ΔSP mutant were analyzed as described for panel B except that proteins from the medium samples were separated on a 25-cm-long 12% polyacrylamide gel and the 27-kDa region of the fluorogram was enlarged to show the three capsid species. (D) The NC and PR proteins of the ΔSP mutant were analyzed by Western blotting (immunoblotting). Forty-eight hours after transfection with plasmids containing the parental, ΔSP, or LON1 allele, unlabeled particles were harvested from the medium by ultracentrifugation. Duplicate samples were separated on a 15% gel, transferred to nitrocellulose, and then probed with either an anti-NC (α-NC) or anti-PR (α-PR) serum. Bound antibodies were detected by chemiluminescence (ECL System; Amersham) following incubation in a secondary antibody conjugated to horseradish peroxidase. The region of the gel containing NC and PR is shown. The NC and PR proteins of the wild type and the ΔSP mutant comigrate under these electrophoretic conditions. The different mobilities of NC and PR in the LON1 mutant (which bears a 25-amino-acid deletion in NC) demonstrate the specificity of the two sera. untr'f, untransfected.

oncoprotein, pp60^{src} (29). To examine the effects of the mutation on particle assembly and budding, COS-1 cells were transfected with plasmids carrying either the parental or the ΔSP allele and labeled for 2.5 h with [^{35}S]methionine, and the Gag proteins were immunoprecipitated with an anti-RSV serum (29). A difference in CA maturation was immediately obvious. Though the three CA species were visible in the case of the Myr1 parent (Fig. 1B and C), the CA protein of the ΔSP mutant appeared as only a single band, migrating very close to that of the wild-type CA3. Even with a very short (30-min) labeling period, only the single CA band could be seen in the medium or as a cleavage product in the cell lysates (not shown), implying that the novel CA-NC junction was cleaved with rapid kinetics. Furthermore, the NC protein of the mutant appeared to be indistinguishable from that of the parent in electrophoretic behavior (Fig. 1D), suggesting that cleavage occurs precisely at this junction, though this has not yet been confirmed by protein sequencing. Clearly, the ΔSP mutation did not block the assembly of the mutant Gag protein or its release from the transfected cells, and the kinetics of cleavage at other sites appears to be unaltered by the mutation, as judged by the presence of MA (as p23) and PR in the medium and the presence of the uncleaved Gag protein only in the lysates. The behavior of the ΔSP mutant proves that the appearance of CA1 and CA2 during the maturation of the wild-type particles is dependent upon the presence of SP and provides strong support for the hypothesis that the removal of SP converts CA1 into CA3.

Effects of the ΔSP mutation on virus infectivity. Since the deletion of SP had no obvious effects on particle assembly and maturation other than the subtle effect on CA, it was of interest to examine whether the mutation would have any effect on viral infectivity. To address this, the ΔSP mutation was transferred into the RSV genome carried in pBH-RCAN.HiSV (10) and transfected into quail cells of the QT6 line (19). Briefly, 6×10^5 cells were placed in 60-mm-diameter dishes containing F-10 medium (GIBCO-BRL) supplemented with 10% tryptose phosphate broth, 5% fetal calf serum, and 1% chicken serum. Twenty-four hours later, the medium was changed to Dulbecco's modified Eagle

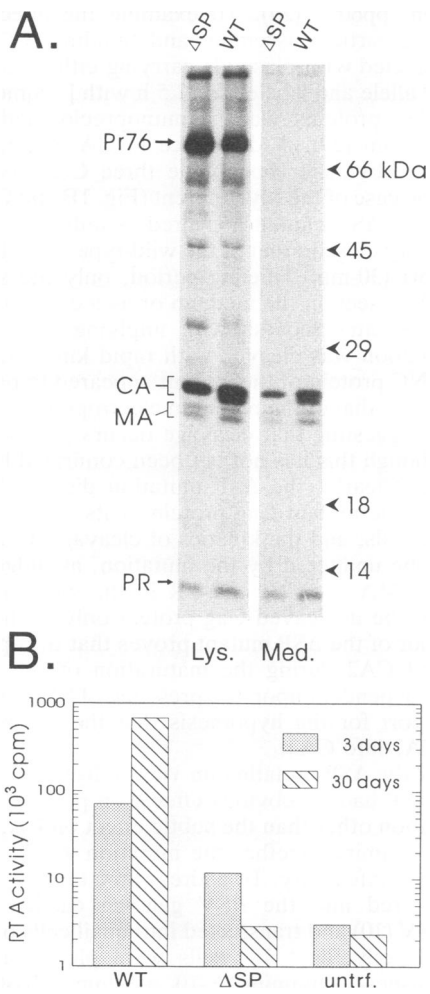

FIG. 2. Behavior of the ΔSP mutant in QT6 quail cells. (A) Quail cells were transfected as described in the text with RSV genomes derived from pBH.RCAN.HiSV (10). RC.V8 (wild type [WT]) is an infectious viral vector in which the *gag* gene (the *Sst*I-to-*Hpa*I fragment; nucleotides 255 to 2731) was replaced with that of the Prague C strain of virus. RC.ΔSP (ΔSP) is identical except for deletion of the codons for the SP. At 24 h posttransfection, the cells were labeled with [^{35}S]methionine and the Gag proteins were collected and analyzed as for Fig. 1B. (B) Three days after transfection with wild-type and ΔSP DNAs, virus particles were collected from the culture medium by ultracentrifugation and assayed for RT activity (7). The transfected cells were cultured for 4 weeks and passaged approximately every 5 days, after which the RT assay was repeated 3 days after the last passage.

medium (GIBCO-BRL) containing 10% fetal calf serum, and then 10 to 20 μg of DNA in the form of a calcium phosphate precipitate was added according to standard protocols (22). After 4 to 12 h of incubation at 37°C in 5% CO_2, the cells were returned to their usual supplemented F-10 medium; no treatment with dimethyl sulfloxide, glycerol, or chloroquine was required. The expression of viral proteins was evaluated at 24 h posttransfection.

The Gag protein profiles of the wild-type parent and the mutant (Fig. 2A) were identical to those observed in the COS-1 cell experiments described above (Fig. 1B) in that the CA protein of the ΔSP mutant appeared to migrate with CA3 of the wild type. Thus, the behavior of the Gag proteins in mammalian cells accurately predicted that observed in avian cells. (The small difference in the ratio of the two MA species seen with the mutant was not consistently observed.) In the medium of the transfected QT6 cells, RT also accumulated during the first 24 h for both the wild-type and mutant DNAs (Fig. 2B). In the case of ΔSP, however, RT expression returned to the background (untransfected) level when the cultures were passaged for 30 days. This finding indicates a failure of the mutant to produce a stable infection. In contrast, the RT activity in the wild-type cultures rose 10-fold over the level seen at 3 days. In multiple attempts to establish an infection with the ΔSP mutant virus, the mutant proved to be severely defective in replication, as virus spread never occurred.

The ΔSP virions from QT6 cells were further characterized to determine whether the deletion had affected the packaging of RT, viral RNA, or envelope glycoproteins. Virus preparations, harvested at 3 days posttransfection and adjusted to equal RT levels, were analyzed by immunoblotting with an anti-RSV serum. The amounts of CA protein were similar for the parent and mutant, indicating that the virions contain normal amounts of RT per particle (Fig. 3B). The glycoprotein gp85 was also present in comparable levels in both the wild-type and mutant particles (Fig. 3C), though the transmembrane protein gp37 was not detected in either the wild type or the mutant by the particular antibody used in these experiments. The presence of viral RNA in the particles was tested by hybridization with a complementary RNA probe. The RNA contents of the ΔSP mutant and the wild-type parent were similar, differing by no more than twofold in this experiment (Fig. 3A). In contrast, no signal could be detected when wild-type virions were hybridized with an antisense probe (of sequence identical to the viral RNA); thus, it is unlikely that the signal produced by the complementary probe is due to a nonspecific interaction with RNA-binding proteins in the virions. It appears, therefore, that the replication defect of ΔSP is not due to a simple failure to package RT, envelope glycoproteins, or viral RNA.

Physical properties of ΔSP particles. Except for the alterations in the CA protein itself, no biochemical difference between the wild type and the ΔSP mutant had been found so far that could explain the effects on replication. It was possible, however, that the mutation affects the physical structure of the ΔSP particles in some way. Three virion properties were evaluated: particle density, morphology, and susceptibility to detergent. The density of the particles from transfected COS-1 cells was measured by centrifugation to equilibrium in gradients of 10 to 50% sucrose, using authentic RSV as an internal standard. The ΔSP particles (Fig. 4A) and the Myr1 parent (not shown) possessed densities of 1.17 to 1.18 g/ml. The somewhat higher density of the particles from COS-1 cells relative to authentic RSV (1.15 to 1.16 g/ml) has been reported previously for particles containing the Myr0 form of the Gag protein, which contains a fully wild-type amino acid sequence (30). The difference presumably is due to the fact that the particles made in COS-1 cells do not contain the products of the *pol* and *env* genes. In any case, it is clear that the ΔSP mutation did not alter the density.

Next, the sensitivity of wild-type and mutant particles to detergent was examined. Immature RSV cores (containing uncleaved Gag proteins) are known to be resistant to treatments with Triton X-100 or deoxycholate (25). In contrast, detergent treatment of mature virions has been shown to produce either (with mild treatment) a core containing the

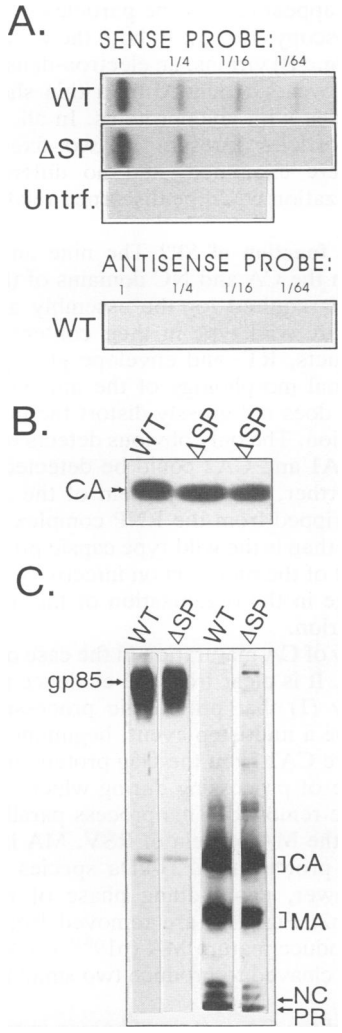

FIG. 3. Biochemical composition of ΔSP virions. (A) Viral RNA was detected by a Northern (RNA) slot blot technique (22). QT6 cells were transfected with wild-type (WT) and ΔSP plasmid DNA; 3 days later, virions were harvested and concentrated as described for Fig. 2. The virus preparations were diluted with phosphate-buffered saline to normalize the RT activities of the different samples. Fourfold dilutions of the standardized preparations were applied to nitrocellulose under vacuum in a slot blot apparatus. An RNA probe complementary to viral RNA (the sense probe) was produced by in vitro transcription of a pGEM plasmid (Promega) bearing *gag* sequences. An antisense probe (of the same sense as the viral RNA) was used as a control for nonspecific binding of RNA to the filter. Hybridization was performed according to Promega protocols. Untrf., untransfected. (B) Standardized preparations of virions were analyzed for CA protein by Western blotting (immunoblotting) as described for Fig. 1D except that the primary antibody was an anti-RSV serum. (C) The envelope glycoprotein (gp85) present in standardized preparations of virions was detected by immunoblotting with an anti-Env serum (left two lanes), and Gag proteins were detected with anti-RSV serum (right two lanes).

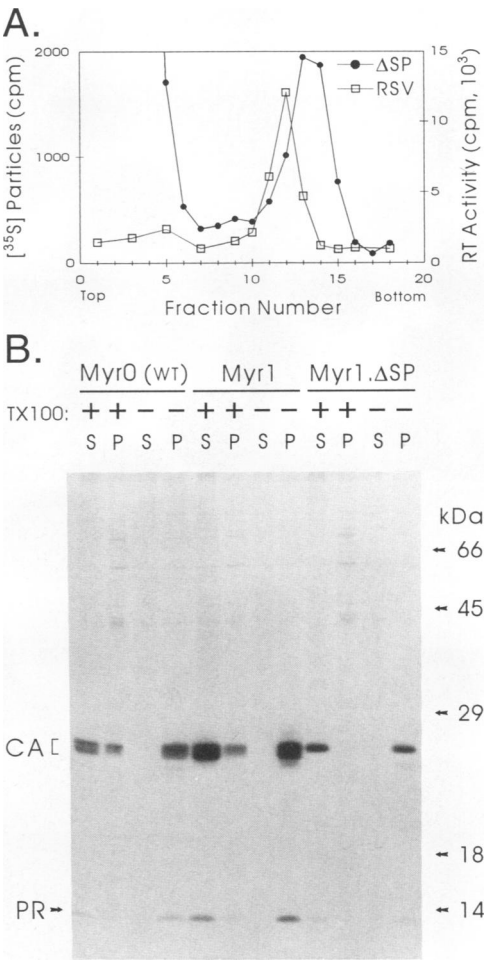

FIG. 4. Physical characteristics of the ΔSP mutant particles produced in COS-1 cells. (A) Particle density was analyzed by sucrose gradient centrifugation. Particles were labeled with [^{35}S]methionine as described for Fig. 1B. A sample of unlabeled, authentic RSV was added, and the mixture was subjected to sedimentation in a gradient of 10 to 50% sucrose as described previously (30). Radioactivity near the top of the tube represents unincorporated [^{35}S]methionine. The position of the authentic RSV in the gradient was determined by RT assay, and that of ΔSP was determined by scintillation counting. (B) COS-1 cells were transfected with pSV.Myr0, with pSV.Myr1, and with pSV.Myr1.ΔSP. After radiolabeling, each sample of medium was divided, and 1% Triton X-100 (TX100) was added to one half. After a 10-min incubation at 37°C, each sample was layered over 10% sucrose and centrifuged at 150,000 × *g* for 40 min at 10°C. Labeled Gag proteins were collected from the pellets (P) and supernatant fractions (S) by immunoprecipitation with anti-RSV serum and analyzed as in Fig. 1B.

CA, NC, and PR proteins as well as the RNA or (with harsher treatment) an RNP complex containing primarily RNA and NC (3, 26). To examine the effect of the ΔSP mutation on particle stability, [^{35}S]methionine-labeled particles produced by transfected COS-1 cells were treated with 1% Triton X-100 for 10 min at 37°C, and the material that remained particulate was pelleted through a layer of 10% sucrose. The Gag proteins present in the supernatant and pellet fractions were analyzed by immunoprecipitation with anti-RSV serum (Fig. 4B). Under these conditions, the Gag proteins of untreated Myr1 particles were present only in the pelleted material, while the detergent-treated particles were largely solubilized. Virtually all of the MA protein, as well as the majority of the PR and CA proteins, was found in the soluble fraction; however, a small fraction of the CA and PR (10 to 25%) was recovered from the pellet after detergent treatment. The pattern was reproducibly observed whether

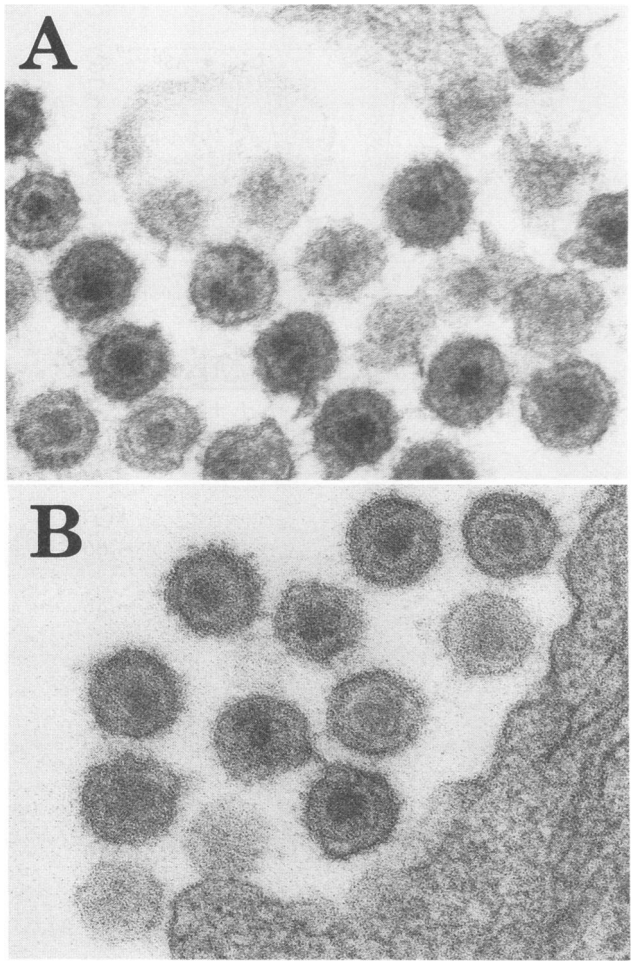

FIG. 5. Virion morphology. Cell lines stably expressing the wild-type (A) or ΔSP (B) genome were obtained after hygromycin selection of transfected QT6 cells. Cells were grown on nitrocellulose filters (Millicell-HA inserts; Millipore) and fixed for 1 h with 3% glutaraldehyde in a buffer of 0.1 M cacodylate, 1.5 mM $CaCl_2$, and 1% sucrose. The fixed cells were postfixed for 0.5 h with 1% osmium tetroxide in 0.1 M cacodylate buffer and then dehyrated and embedded in Epon 812. Thin sections were stained with alcoholic uranyl acetate and lead citrate and examined in a Philips 400 electron microscope. Magnification, ×121,000.

the Myr1 or the Myr0 form of Gag was tested (nine or five repetitions, respectively). Interestingly, the detergent-resistant fraction was highly enriched for the mature form of CA (CA3); the CA1 or immature species was selectively lost during detergent treatment. In contrast to the Myr0 and Myr1 particles, the ΔSP mutant was much more sensitive to detergent. In five repetitions of the experiment, no CA protein was found in the pellet after detergent treatment, indicating that the capsid structure is less stable than in the wild type. Wild-type and mutant virions produced in QT6 cells exhibited the same patterns of detergent sensitivity as did the particles formed in COS-1 cells (data not shown). Furthermore, the material that pelleted after detergent treatment contained the majority of the NC protein, suggesting that the RNP structure was still intact in the ΔSP mutant. Presumably, RT activity and viral RNA were also present in the pellets, though this was not tested.

Finally, the appearance of the particles was examined by electron microscopy (Fig. 5). In both the wild-type (Fig. 5A) and mutant (Fig. 5B) virions, an electron-dense nucleoid was seen, and this was surrounded by a thin shell and a thick envelope studded with glycoproteins. In all, approximately 100 mutant particles (present in two fixed and stained specimens) were examined, and no differences in their internal organization could be discerned relative to the wild type.

What is the function of SP? The nine amino acids that reside between the CA and NC domains of the Gag protein clearly are not required for the assembly and budding of particles that are wild type in their content of RNA, Gag cleavage products, RT, and envelope glycoprotein. Moreover, the normal morphology of the mutant indicates that removal of SP does not grossly distort the internal arrangement of the virion. The only obvious defects relate to the CA protein. No CA1 and CA2 could be detected during virion maturation; further, the CA protein of the ΔSP mutant is more easily stripped from the RNP complex by mild detergent treatment than is the wild-type capsid protein. Thus, the powerful effect of the mutation on infectivity must be due to a subtle change in the organization of the internal components of the virion.

The pathway of CA maturation in the case of RSV appears to be complex. It is clear from the evidence presented here and previously (1) that proteolytic processing of the CA protein must be a multistep event, beginning with cleavage of the immature CA1 from the Gag protein and followed by a slower phase of processing during which the nine amino acids of SP are removed. This process parallels that of the maturation of the MA protein of RSV. MA is first released from the Gag protein as a 23-kDa species ($p23^{MA}$); then during the slower, postbudding phase of maturation, 22 amino acids (p2; Fig. 1A) are removed from the carboxy terminus to produce mature MA ($p19^{MA}$). Interestingly, the p2 sequence is cleaved to produce two small peptides found in virions (20).

Maturation of the CA protein of human immunodeficiency virus (HIV) appears to follow a pathway similar to that for RSV CA. It is initially released as a 25-kDa species (p25) that is slowly converted into p24 (17, 27). This maturation has been interpreted to be due to the removal of 14 amino acids from the carboxy terminus of the protein after particle formation. Indeed, alteration of the cleavage site at the carboxy terminus of p24 resulted in the appearance of the larger CA protein (14). For neither RSV nor HIV, however, has the primary structure of the immature form of CA (CA1 in RSV or p25 in HIV-1) been confirmed by protein sequencing.

If removal of the nine amino acids of the SP from an immature capsid protein accounts for the conversion of CA1 to CA3, then what is the nature of CA2? The kinetics of appearance of this protein suggest that it is an intermediate in a processing cascade: CA1 → CA2 → CA3. Perhaps some of the CA1 molecules are cleaved within SP to yield CA2, which is further trimmed to form the mature protein. In this regard, it is interesting that SP contains a sequence that in theory could be cleaved by PR: Ser-Ala-Ile-Gln*Pro-Leu-Ile, where the asterisk represents the proposed scissile bond (6). Except for the glutamine at P1, each of these residues exists in the corresponding position in one or more naturally occurring RSV cleavage sites; furthermore, the glutamine might be consistent with slow processing, because peptide substrates containing large, polar residues (arginine or glu-

tamic acid) at this position are cleaved with low efficiency (6).

As mentioned earlier, it seems unlikely that the postbudding maturation events associated with the RSV CA species would be due to protein modifications other than proteolysis. Although the CA protein of HIV-1 has been reported to exist in multiple phosphorylated forms (15, 27), this probably has little to do with the difference between the p24 and p25 species. In the case of RSV, the mature CA protein could not be labeled with $^{32}P_i$ (data not shown); nevertheless, a transient modification of CA1 or CA2 remains a formal possibility.

Though it appears that the CA-NC junction of the ΔSP mutant is cleaved accurately, the virus is replication defective. The data presented here, taken together, imply that the presence of SP on the immature CA protein and its removal late in the maturation pathway are essential for the proper folding and/or oligomerization of the CA proteins within the capsid shell or that SP is required for other modifications of CA that are in turn involved in proper capsid assembly. This conclusion is supported by two observations. First, the more mature forms of CA (CA2 and CA3), rather than the immature CA1, remain associated with the RNP complex of the wild-type virion core after mild detergent treatment; second, the ΔSP virions, which have not proceeded through the normal CA maturation cascade, exhibit decreased core stability compared with the wild type.

There are interesting similarities between this model for capsid maturation and the so-called maturation cleavages that have been shown to occur in picornaviruses (11) and, more recently, in nodaviruses (12, 23). In both cases, cleavage of a capsid protein occurs as the final step in virus assembly and converts provirions into mature, infectious viral particles. Accompanying the maturation cleavage is a dramatic increase in the stability of the particles. It would be interesting to examine the stability of RSV particles in which the removal of SP is prevented by mutation of the CA-SP cleavage site. If unstable, then particles of RSV that contain the immature CA1 protein should probably be designated provirions, too.

Interactions between the CA protein and the RNP complex in the wild-type virion may have an important function in the initial steps of the infection cycle. It has been demonstrated that a portion of the CA protein of murine leukemia virus remains associated with the RNP as it goes through the process of synthesizing an integration-competent DNA product (5). The phenomenon of restriction of murine leukemia virus replication by the *Fv-1* allele of mice (4, 8, 21) also suggests that the CA protein plays an important role during the steps immediately preceding integration. Therefore, the loss of infectivity of ΔSP may be due to the instability of its core and loss of interactions between the CA protein and the RNP. The block to replication in this mutant is under investigation.

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