Signals in APOBEC3F N-terminal and C-terminal Deaminase Domains Each Contribute to Encapsidation in HIV-1 Virions and Are Both Required for HIV-1 Restriction*

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Background: The antiretroviral activity of cellular proteins APOBEC3F and APOBEC3G requires their inclusion within HIV-1 virions.

Results: Unlike APOBEC3G, signals in N- and C-terminal deaminase domains of APOBEC3F each contribute to virion encapsidation.

Conclusion: A3F employs an additional mechanism for virion encapsidation that is absent in A3G.

Significance: Elucidation of the mechanisms of APOBEC3F and APOBEC3G encapsidation may suggest new strategies to decrease HIV-1 replication.

Human cytidine deaminases APOBEC3F (A3F) and APOBEC3G (A3G) inhibit human immunodeficiency virus type-1 (HIV-1) replication. In the absence of HIV-1 Vif, A3F and/or A3G are incorporated into assembling virions and exert antiviral functions in subsequently infected target cells. Encapsidation of A3F or A3G within the protease-matured virion core following their incorporation into virions is hypothesized to be important for the antiviral function of these proteins. In this report, we demonstrated that A3F was quantitatively encapsidated in the mature virion core. In distinct contrast, A3G was distributed both within and outside of the virion core. Analysis of a series of A3F-A3G chimeras comprised of exchanged N- and C-terminal deaminase domains identified a 14 amino acid segment in the A3F C-terminal deaminase domain that contributed to preferential encapsidation and anti-HIV activity. Amino acid residue L306 in this C-terminal segment was determined to be necessary, but not sufficient, for these effects. Amino acid residue W126 in the N-terminal deaminase domain was determined also to contribute to preferential encapsidation and antiviral activity of A3F. Analysis of the A3F (W126A L306A) double mutant revealed that both residues are required for full antiviral function. The results reported here advance our understanding of the mechanisms of A3F virion encapsidation and antiviral function and may lead to innovative strategies to inhibit HIV-1 replication.

Human cytidine deaminases APOBEC3F (A3F) and APOBEC3G (A3G) are expressed in CD4+ T cells, the natural targets of HIV-1 infection. Each protein contains two cytidine deaminase motifs, located in homologous N-terminal and C-terminal domains. However, only the C-terminal deaminase domain is catalytically active, deaminating cytidine residues in single-stranded DNA substrates (1–3). In the absence of the HIV-1 virion infectivity factor (Vif),3 A3F and/or A3G are incorporated into assembling virions and exert potent antiviral function in subsequently infected target cells by both cytidine deaminase-dependent and independent mechanisms. Some published studies indicate that cytidine deaminase activity is required for the antiviral effect of A3G (4–7). Others have reported that mutants with non-functional C-terminal cytidine deaminase active sites in A3F, and in A3G to a lesser extent, are still capable of inhibiting HIV-1 replication (5, 8–11).

Incorporation of A3F or A3G into HIV-1 virions is known to involve interactions with the nucleocapsid (NC) domain of Gag and RNA (12–19). A role for both HIV-1 genomic RNA and cellular 7SL RNA in this process has been demonstrated and debated (15–20). Substitutions at amino acids Y124 and W127 in the N-terminal deaminase domain of A3G substantially reduce virion incorporation (19, 21, 22). An analogous substitution at amino acid W126 of A3F is reported to result in a similar reduction in virion incorporation (16). The reduction in incorporation of mutant A3F and A3G was correlated with the loss of interaction with cellular 7SL RNA (15, 16). Both A3F and A3G also interact with HIV-1 integrase (23), a known component of virion cores and the replication machinery (24, 25). Furthermore, virion A3G has been demonstrated to associate with viral nucleoprotein complexes in virion cores (26, 27) and both A3G and A3F fractionated with cores in another report (28). However, the specific protein-protein and/or protein-RNA interactions required for A3F and A3G incorporation into virions and subsequent localization within the protease-matured virion core (encapsidation) remain incompletely understood.

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The anti-HIV activity of A3F and A3G is hypothesized to depend on the association of these proteins with the replicative machinery encapsidated in the virion core. Because the antiviral potency of A3G is reported to be at least 10-fold greater than A3F (9, 29), we sought to determine whether greater virion encapsidation of A3G, relative to A3F, might explain the disparity in antiviral function of these proteins. In this communication, we report that the majority of A3F incorporated into virions fractionated with the protease-matured core of viral particles, in distinct contrast to a smaller proportion of virion A3G being found in the core. This result excluded quantitatively better virion encapsidation as a mechanistic explanation for the greater antiviral activity of A3G, relative to A3F (9, 29).

We demonstrated that residues W126 and L306 each contributed to A3F encapsidation and both were required for full anti-HIV activity. We also discuss that the stronger virion core association of A3F is related to the presence of unique C-terminal deaminase domain sequences in A3F that enhance encapsidation, in addition to N-terminal deaminase domain signals found in both A3F and A3G (21, 30).

EXPERIMENTAL PROCEDURES

Cell Lines and Culture Conditions—HEK293T cells were obtained from Chris Aiken and the TZM-bl cells were obtained through the NIH AIDS Research and Reference Reagent Program from John C. Kappes, Xiaoyun Wu, and Tranzyme, Inc. The TZM-bl indicator cell line, used for infectivity assays, is a genetically engineered HeLa cell clone expressing CD4, CXCR4, CCR5, and Tat-responsive firefly luciferase and Escherichia coli β-galactosidase under the control of an HIV-1 long terminal repeat. HEK293T and TZM-bl cells were cultivated in DMEM (containing 4.5 g/liter glucose, L-glutamine, and sodium pyruvate) medium plus 10% fetal calf serum, 50 IU/ml penicillin, and 50 μg/ml streptomycin at 37 °C and 5% CO2.

Plasmids—A full-length infectious HIV-1 clone, pNL4–3 (31) was obtained from Chris Aiken. An isogenic pNL4–3 Vif-deletion mutant in which tandem nonsense mutations were introduced in codons 26 and 27 of the Vif ORF was constructed by PCR using appropriate oligonucleotide primers and insertion in the NotI and EcoRI sites of pCEM-15 (32) using appropriate oligonucleotide primers constructed by PCR from pCEM-15 (32) using appropriate oligonucleotide primers encoded to A3F encapsidation and both were required for full anti-HIV activity. We also discuss that the stronger virion core association of A3F is related to the presence of unique C-terminal deaminase domain sequences in A3F that enhance encapsidation, in addition to N-terminal deaminase domain signals found in both A3F and A3G (21, 30).

plasmid DNA, as indicated in individual figure legends, using linear polyethyleneimine (PEI; 25 kDa; Polysciences, Inc.) as described (35). Culture supernatants were collected 48 h after transfection and cellular debris was removed either by filtration through a 0.45 μm filter or centrifugation at 2500 × g for 30 min. HIV-1 particles were then concentrated by ultracentrifugation (100,000 × g for 3 h at 4 °C) through a 20% sucrose cushion (w/v) in STE buffer (10 mM Tris-HCl, pH 7.4, 100 mM NaCl, 1 mM EDTA). Pelleted virions were resuspended in 300 μl of STE buffer, and subjected to ultracentrifugation (130,000 × g for 16 h at 4 °C) through a layer of 1% Triton X-100 into a linear 30–70% (w/v) sucrose density gradient as described (37). After centrifugation, 1-ml fractions were collected from the top of the gradient and stored at −20 °C. Specific proteins in individual fractions were analyzed by SDS-polyacrylamide gel electrophoresis and Western blotting. One-tenth milliliter (0.1 ml) of each fraction was diluted with 0.1 ml STE buffer and the protein precipitated with an equal volume of 20% trichloroacetic acid on ice for 30 min. The protein precipitate was washed twice with 0.3 ml acetone, air dried, and dissolved in 25 μl of 2× SDS-protein sample buffer (100 mM Tris-HCl, pH 6.8, 4 mM Na2EDTA, 4% SDS, 4% 2-mercaptoethanol, 20% glycerol, 0.1% bromphenol blue). Samples were heated at 100 °C for 5 min and 5 μl of each was fractionated by electrophoresis through a 12.5% SDS-polyacrylamide gel. After electrophoresis, separated proteins were transferred to an Immobilon-P membrane (Millipore) and processed for Western blot digestion with XbaI and EcoRI; A3F fragments were then ligated into pcDNA3.1(−) digested with Xba1 and HindIII. Digested A3G and A3F fragments were digested with EcoRI and HindIII. FLAG-tagged chimeric A3F-A3G expression plasmids were generously provided by Vinay Pathak (33). A3F mutants were constructed by using appropriate mutagenic primers. Mutations were confirmed by DNA sequencing. The DNA sequences encoding A3F amino acid residues 162–373 and HIV-1 nucleocapsid were amplified by PCR using appropriate oligonucleotide primers followed by insertion in the mammalian expression plasmid pTT5 (35) in-frame with the coding sequence for Shistosoma japonicum glutathione S-transferase (GST). The sequences of primers used for the construction of the GST-A3F and HIV-1 nucleocapsid chimeras, A3G-A3F chimeras and A3F mutants are available on request.

Antibodies—The following antibodies were obtained through the NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH: HIV-1 IN monoclonal antibody (8G4) from Dag E. Helland (36), anti-ApoC17 polyclonal antibody which recognizes the 17 C-terminal residues of human APOBEC3G from Klaus Strebel (26), and anti-APOBEC3F(C18) from Michael Malim (9). Anti-β-actin monoclonal antibody (clone AC-74) was from Sigma. Anti-p24 monoclonal antibody 183-H12–5C was from Vanderbilt-Meharry Center for AIDS Research Virology Core.
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RESULTS

A3F Quantitatively Associates with the Virion Core during the Process of HIV-1 Particle Maturation—Because the antiviral functions of A3F and A3G are hypothesized to depend on the association of these proteins with the replicative machinery located within the mature virion core, we sought to determine whether greater encapsidation might contribute to the ~10-fold greater antiviral activity of A3G, relative to A3F (9, 29). Virions containing each protein were fractionated by ultracentrifugation through a layer of Triton X-100 atop a 30–70% linear sucrose density gradient (Fig. 1). Virion fractionation by this method results in recovery of mature cores at a density of 1.24–1.26 g/ml in gradient fractions 9 and 10 (37). Core localization at this density was validated by demonstrating p24Gag, integrase, and genomic RNA in these fractions (Fig. 1, A and B). We demonstrated that A3F quantitatively fractionated with the virion core, whereas A3G was more broadly distributed across gradient fractions (Fig. 1, A and B), a result that was contrary to our hypothesis.

We next analyzed the distribution of A3F and A3G in virions produced when the intracellular concentration of each protein was substantially reduced to evaluate whether overexpression of A3 proteins might result in aberrant intravirion localization (27) (Fig. 2). Cellular expression levels were adjusted by altering the A3 plasmid/provirus molar ratio in co-transfections from 0.5 to 0.1, as had been done in a previous report (27). Western blotting of cellular and viral lysates demonstrated the expected reduction in A3F and A3G protein levels under these experimental conditions (Fig. 2A). Sucrose density gradient analysis of virions produced under the conditions of reduced A3G expression (0.1 molar ratio) demonstrated peak concentrations of A3G in fraction 2 and virion core fractions 9 and 10. However, a substantial proportion of A3G was not associated with cores (in fractions 3–8, Fig. 2C), as was observed before under conditions in which the protein was overexpressed (Fig. 1B). In contrast, the nearly quantitative co-fractionation of A3F with the virion core (Fig. 1A) was not altered with reduction in the intracellular levels of the protein (Fig. 2B). In sum, these results demonstrated that virion core localization of A3F and A3G were substantially different and indicated that the 10-fold dif-

Viral Infectivity Assay—TZM-bl indicator cells were plated at a density of 10,000 cells/well in a 96 well culture plate 24 h prior to infection and incubated at 37 °C (5% CO2). The day of infection, the culture medium was removed, and the cells inoculated in triplicate with 100 μl of 2-fold serial dilutions of viral supernatants in culture medium containing 20 µg/ml DEAE-dextran and incubated at 37 °C (5% CO2). After 24 h of incubation, culture medium was then removed and replaced with 100 μl of Britelite Plus luciferase assay substrate (PerkinElmer). Following 5 min of incubation at room temperature, 70 μl of each cell lysate was transferred to a 96-well OptiPlate 96 (PerkinElmer) and luminescence was measured in a VICTOR X2 Multilabel Reader (PerkinElmer). The effect on inhibition of virion infectivity of the combined A3F mutations W126A and L306A was evaluated using Compusyn software following the methods described by Chou (38).

GST Pull-downs—HEK293T cells were plated at a density of 6 × 10⁵ cells/well in a 6-well culture plate 24 h prior to transfection with either 0.2 μg of pTT5-GST or 1 μg of pTT5-GST-NC and 1 μg of pcDNA-A3F, pcDNA-A3F(W126A), or pcDNA-A3F(L306A) plasmid DNA using linear polyethyleneimine (PEI; 25 kDa; Polysciences, Inc.) as described (35). Forty-eight hours after transfection, cells were lysed in 250 μl of cell lysis buffer ((1× Dulbecco’s Phosphate-buffered Saline (Mediatech, Inc.), 1 mM Na₂EDTA, 0.5% Triton X-100 (v/v), and complete mini protease inhibitor mixture without Na₂EDTA (Roche)) and centrifuged at 10,000 × g for 5 min at 4 °C. Cell lysates were added to glutathione-agarose beads (25 μl packed bead volume) and incubated for 3 h at 4 °C with constant rotation. Beads were washed three times for 10 min with 500 μl of cell lysis buffer. Washed beads were combined with an 25 μl of 2× SDS-protein sample buffer (100 mM Tris-HCl, pH 6.8, 4 mM Na₂EDTA, 4% SDS, 4% 2-mercaptoethanol, 20% glycerol, 0.1% bromphenol blue), heated at 100 °C for 5 min and analyzed by electrophoresis through a 12.5% SDS-polyacrylamide gel. After electrophoresis, separated proteins were transferred to an Immobilon-P membrane (Millipore) and processed for Western blot analysis using protein-specific antibodies with chemiluminescent detection.
ference in the antiviral potency of A3F versus A3G is not strictly dependent on a difference in virion encapsidation of these proteins (29).

The C-terminal Deaminase Domain of A3F Is Necessary for Encapsidation of the Protein in the Virion Core—Our results demonstrating that A3F was quantitatively associated with virion core fractions, in distinct contrast to A3G, suggested that the determinants involved in encapsidation of each of these proteins might differ. Therefore, study of A3F might add to our understanding of the mechanisms involved in virion encapsidation of A3G (16, 21). A3F chimeras were detected with A3F(C18) polyclonal antibody, A3G was detected using A3G polyclonal antibody Apo-C17. Viral capsid and integrase were detected with p24Gag and integrase monoclonal antibodies.

In contrast, the majority of the A3G1–155-F151–373 chimeric protein fractionated with virion core fractions (Fig. 3A, lower panel). The demonstration that both chimeric proteins were efficiently incorporated into virions supports the previously reported role for the N-terminal deaminase domains of both A3G and A3F in this process (16, 21). However, the profile of intravirion distribution of the A3G1–155-F151–373 chimeric protein, which was identical to that of full-length A3F (Figs. 1A and 2B), suggested that the A3F C-terminal domain contained unique sequences that enhanced A3F encapsidation, in addition to N-terminal domain signals found in both A3G and A3F. To map the location of A3F C-terminal residues that enhanced virion core encapsidation, we constructed a series of A3G-F chimeras in which A3F C-terminal sequences were systematically reduced with a concomitant increase in A3G N-terminal sequences (Fig. 3B).

FIGURE 2. Reduction in cellular expression levels does not alter the distinctive patterns of A3F and A3G intravirion localization. HEK293T cells were co-transfected with 15 μg of proviral plasmid pNL4–3(Vif−) and either A3F or A3G expression plasmids. A, analysis of A3F and A3G levels in cellular and viral lysates by Western blotting. B and C, intravirion localization of A3F and A3G was analyzed by sucrose density gradient centrifugation as described in “Experimental Procedures.” A3 plasmid/provirus molar ratios were 0.5 and 0.1 as indicated. B and C, intravirion localization of A3F and A3G was analyzed by sucrose density gradient centrifugation as described in “Experimental Procedures.” A3 plasmid/provirus molar ratio was 0.1. Shown are Western blots of A3F or A3G, viral capsid (p24Gag), and integrase (p32IN) in individual gradient fractions 1–12. A3F was detected with A3F(C18) polyclonal antibody, A3G was detected using A3G polyclonal antibody Apo-C17. Viral capsid and integrase were detected with p24Gag and integrase monoclonal antibodies.

FIGURE 3. The A3F C-terminal deaminase domain contains signals that direct A3F association with the virion core. HEK293T cells were co-transfected with 15 μg of proviral plasmid pNL4–3(Vif−) and 3 μg of either A3F-G or A3G-F expression plasmids (A3 plasmid/provirus molar ratio was 0.5). Virions were concentrated from culture supernatants 48 h after transfection and fractionated by sucrose density gradient centrifugation as described in “Experimental Procedures.” A–C, Western blots of chimeric proteins and viral capsid (p24Gag) in individual gradient fractions 1–12. A3G-F chimeras were detected with A3G polyclonal antibody Apo-C17. A3F-G chimeras were detected with A3F(C18) polyclonal antibody. Viral capsid and integrase were detected with p24Gag and integrase monoclonal antibodies. The identity of the each chimeric protein with included A3F and A3G amino acid residues is shown to the left of their respective Western blot.
A3F, whereas A3G1–342–F336–373 and A3G1–356–F350–373 progressively lost preferential core localization. This indicated that the A3F C-terminal segment comprised of amino acid residues E289 to F336 contained signals directing encapsidation of A3F in the virion core.

To confirm the involvement of this region in core association, we analyzed a previously described series of A3F-G chimeras generously provided by Vinay Pathak (33). These expression plasmids contain varying lengths of A3F N-terminal sequences fused to A3G C-terminal sequences (Fig. 3C). Analysis of the intravirion localization of each chimera demonstrated that the A3F-G chimera containing A3F amino acid residues 1–282 (A3F1–282-G291–384) and 1–300 (A3F1–300–G309–384) resembled wild type A3G in intravirion distribution, whereas the chimera that included A3F amino acid residues 1–314 (A3F1–314-G322–384) was concentrated in virion core fractions similar to wild type A3F. These results allowed us to narrow the location of A3F C-terminal sequences that enhanced A3F encapsidation to the 14 amino acid residues between T300 and Y314.

Assessment of the Antiviral Function of A3 Chimeric Proteins—The relationship between antiviral function and intravirion localization of the various A3 chimeric proteins was assessed. HEK293T cells were co-transfected with NL4–3(Vif–) provirus and expression plasmids encoding A3 chimeric proteins. Forty-eight hours after transfection, the level of A3 chimeric protein incorporation into virions was analyzed and the infectivity of virions in culture supernatants was determined (Fig. 4).

Western blotting of viral lysates demonstrated that each A3G-F chimeric protein was more poorly incorporated into virions than A3F, with the most dramatic effect observed for A3G1–162–F168–384 (Fig. 4A). The antiviral activity of A3G1–296–F289–373 was substantially lower than A3F and the other A3G-F chimeric proteins (Fig. 4B). However, because of its very low level in virions, the relationship between antiviral function and intravirion localization of this chimera was difficult to evaluate. The antiviral activity of A3G1–155–F151–373 was similar to A3F (after correction for its slightly lower level in virions), which was consistent with the quantitative encapsidation of this protein. The antiviral activities of A3G1–342–F336–373 and A3G1–356–F350–373 were greater than A3F and nearly equivalent to A3G. These chimeras each contain a high overall percentage of A3G residues, including the enzymatically active A3G C-terminal cytidine deaminase motif. Consequently, A3G1–342–F336–373 and A3G1–356–F350–373 acted like full-length A3G in both antiviral function and pattern of intravirion distribution (Fig. 3B).

The A3F1–162–G168–384, A3F1–282–G291–384, and A3F1–300–G309–384 chimeric proteins demonstrated relatively low antiviral activity. In contrast, the antiviral activity of the A3F1–314–G322–384 chimeric protein was nearly identical to A3G and 2-fold greater than A3F (Fig. 4B). Western blotting of viral lysates demonstrated that virion incorporation of these chimeric proteins was similar to A3G indicating that the observed differences in their antiviral activity was not due to variability in intravirion protein levels (Fig. 4A). The substantial increase in antiviral activity of A3F1–314–G322–384 relative to A3F1–300–G309–384 demonstrated the importance of A3F residues 300–314, previously implicated in the stronger encapsidation of A3F than A3G, for A3F antiviral function (Fig. 3C).

A3F Amino Acid Residue L306 Is Necessary for A3F Encapsulation—To determine which amino acid residues within the identified C-terminal segment comprised of residues T300 to Y314 promoted A3F encapsidation and better antiviral activity, a series of single residue alanine substitutions were introduced into this region (Fig. 5A). Analysis of the intravirion localization of each mutant protein demonstrated that virion core association was identical to wild type A3F, with the exception of the L306A mutant in which a substantial portion of virion incorporated protein was found in gradient fractions outside of the virion core (Fig. 5B and data not shown).

Encapsulation of A3F Requires Amino Acid Residue W126 in the N-terminal Deaminase Domain in Addition to Amino Acid Residue L306—To determine whether A3F L306 was both necessary and sufficient for A3F encapsidation, we analyzed virion localization of a glutathione S-transferase (GST)-A3F C-terminal deaminase domain chimera in which the A3F N-terminal deaminase domain was replaced by GST (Fig. 6A). This analysis demonstrated that the majority of incorporated GST-A3F162–373 was found in fractions 4–6, outside of the virion core. Thus, the A3F C-terminal deaminase domain with the L306 residue alone is not sufficient to direct preferential encapsidation of the protein in the virion core. Importantly, GST-A3F162–373 was also shown to completely lack antiviral activity (Fig. 4B), demonstrating the requirement for both N-terminal and C-terminal deaminase domains for A3F antiviral function.
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Substitutions at amino acids Y124 and W127 in the N-terminal domain of A3G substantially reduce virion incorporation (21). An analogous substitution at amino acid W126 of A3F was reported to result in a similar reduction in virion incorporation (16). Alignment of the A3G and A3F N-terminal deaminase domains in this region is shown in Fig. 6B. To assess the potential involvement of A3F amino acid residues Y123 and W126 in virion encapsidation, we analyzed the intravirion distribution of virion localization of A3F mutants T300A, F302A, W310A, and J314A were identical to wild type A3F and are not shown.

Amino acid residue L306 in the C-terminal deaminase domain is necessary for A3F encapsidation. A, amino acid sequence of wild type A3F residues 300–314 with alanine substitution mutations indicated. B, HEK293T cells were co-transfected with 15 μg of proviral plasmid pNL4–3(Vif–) and 3 μg of an expression plasmid encoding GST-A3F162–373 chimeric protein. Virions were concentrated from culture supernatants 48 h after transfection and fractionated by sucrose density gradient centrifugation as described in “Experimental Procedures.” Shown are Western blots of GST-A3F162–373 and viral capsid (p24Gag) in individual gradient fractions 1–12. The results of this analysis demonstrated that virion localization of A3F mutants T300A, F302A, W310A, and Y314A were identical to wild type A3F and are not shown.

N-terminal and C-terminal Deaminase Domain Mutations Affect A3F Antiviral Potency—Because A3F amino acid residues W126 and L306 were each demonstrated individually to contribute to A3F encapsidation, we sought to determine the effect of these mutations on A3F antiviral function. HEK293T cells were co-transfected with NL4–3(Vif–) provirus and expression plasmids encoding A3F wild type and mutant proteins Y123A, W126A, R305A, L306A, and Y307A. Forty-eight hours after transfection, the infectivity of virions in culture supernatants was determined (Fig. 7A). This analysis showed that the antiviral activity of each A3F mutant, with the exception of A3F(Y123A), was reduced relative to wild type A3F. Western blotting of viral lysates demonstrated that the level of incorporation of each mutant protein into virions was similar to wild type A3F, with the exception of A3F(W126A), which was lower than wild type (Fig. 7B). Therefore, reduced virion incorporation of A3F(W126A) accounted, at least in part, for the lower antiviral activity of this protein. Although mutation of A3F residues W126, R305, L306, and Y307 reduced A3F antiviral potency, a substantial level of antiviral function remained that was apparently independent of the specific localization of these proteins within the virion (Fig. 7A). As shown in Figs. 5B and 6B, A3F(L306A) and A3F(W126A) were not as strongly associated with the virion core as wild type A3F, while encapsidation of A3F(R305A) and A3F(Y307A) was similar to wild type A3F. In sum, these results demonstrated the importance of residues W126, R305, L306, and Y307 for maximum A3F antiviral activity, but also suggested that virion core localization was not the sole determinant of A3F antiviral function.

Mutation of both W126 and L306 Residues in A3F Results in Complete Loss of Antiviral Function without further Affecting Virion Incorporation and Encapsulation—Because mutation of either W126 or L306 did not eliminate A3F antiviral function, we determined the consequence of mutating both W126 and L306 on A3F antiviral activity and virion localization. HEK293T
cells were co-transfected with NL4–3(Vif–) provirus and expression plasmids encoding A3F wild type or mutant proteins W126A, L306A, and the double mutant W126A L306A. Forty-eight hours after transfection, the infectivity of virions in culture supernatants was determined (Fig. 8A). The results of this analysis demonstrated that the antiviral activity of A3F(W126A L306A) was severely compromised even though the protein was well incorporated into virions (Fig. 8B). While the level of antiviral activity of each single mutant was reduced relative to wild type A3F, the magnitude of the decrease in antiviral activity of the A3F(W126A L306A) double mutant was consistent with synergistic impairment of A3F antiviral function (Combination Index of 0.05 indicating strong synergy as calculated by Compusyn software) (38). Fractionation of virions containing A3F(W126A L306A) in sucrose density gradients demonstrated a pattern of intravirion localization very similar to the W126A single mutant protein (Fig. 8C); the double mutant was not markedly less well localized into cores than either single mutant (Figs. 5B and 6B).

**Mutation of A3F Amino Acid Residue L306 Does Not Affect A3F Interaction with HIV-1 Nucleocapsid**—Mutation of A3F amino acid residue W126 results in poor virion incorporation of the protein as a consequence of reduced RNA-dependent interaction with HIV-1 nucleocapsid (16). Loss of A3F(W126A) interaction with nucleocapsid, which is found in the mature virion core, also provides a hypothetical mechanistic explanation for our data demonstrating that virion incorporated A3F(W126A) was not encapsidated (Fig. 6B). Because mutation of amino acid residue L306 also results in loss of A3F encapsidation (Fig. 5B), we sought to assess the potential involvement of this residue in nucleocapsid interaction using a GST pull-down assay (Fig. 9). Cell lysates containing a GST-nucleocapsid chimera and either wild type A3F, A3F(W126A) or A3F(L306A) were incubated with glutathione-agarose beads and the level of interaction determined by Western blotting. This analysis confirmed the previously reported role of A3F amino acid residue W126 in nucleocapsid binding and demonstrated that the A3F-nucleocapsid interaction was not compromised by the L306A mutation.

**DISCUSSION**

In the absence of the HIV-1 virion infectivity factor (Vif), A3F, and/or A3G are incorporated into virion cores (26–28). Incorporation of A3F and A3G into virions is known to be dependent on their interaction with RNA and the viral nucleocapsid protein (12–19). Although both proteins inhibit HIV-1 replication in subsequently infected target cells by cytidine deaminase-dependent and independent mechanisms, the substantial disparity in their antiviral activities led us to determine whether A3G was better encapsidated within the protease-matured virion core following incorporation into virions than A3F.

In the present study, sucrose density gradient analysis of virions produced from HEK293T cells expressing either A3F or A3G demonstrated a striking difference in the pattern of intravirion localization of these proteins. A3F was found quantitatively associated with the virion core. In contrast to A3F, a substantial portion of A3G was found outside of the virion core and only a fraction of intravirion A3G was encapsidated (Fig. 1B). We considered that this finding might be the result of A3G overexpression. Therefore, we analyzed the distribution of A3G in virions produced under the conditions described by Soros et al. (27), in which the intracellular A3G concentration more closely matched physiological levels observed in lymphocytes. The results of this analysis demonstrated that, even at lower A3G expression levels, a relatively large proportion of A3G in virions still was found in fractions outside the core, in distinct contrast to A3F’s preferential encapsidation at these lower expression levels (Fig. 2, B and C). This result demonstrated that the striking disparity in the encapsidation of A3F versus A3G was not simply an artifact of protein overexpression and suggested there might be a fundamental difference in the determinants of virion encapsidation of each of these proteins. The greater virion core localization of A3F than A3G disproved our starting hypothesis that A3G’s greater activity as an antiviral might be due to relatively greater core localization, but allowed characterization of signals determining the greater core localization of A3F.

We discovered that dual signals, one each in N-terminal and C-terminal deaminase domains, contribute to encapsidation in HIV-1 virions. The presence of two encapsidation signals in A3F provides a mechanistic explanation for the relatively stronger association of A3F with the virion core than A3G, as A3G has been reported to contain a single signal for virion incorporation and encapsidation in its N-terminal deaminase domain (3, 13, 21, 30, 39). It is apparent from earlier studies extensively investigating A3G virion incorporation that substitutions introduced into the A3G N-terminal deaminase domain reduce
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FIGURE 8. Mutation of both W126 and L306 residues in A3F results in complete loss of antiviral function without further affecting virion incorporation and encapsidation. HEK293T cells were co-transfected with 2.5 μg of proviral plasmid pNL4–3(Vif−) and either 250 or 500 ng of A3F wild type or mutant expression plasmids. A, relative infectivity of virions in collected culture medium was determined using the TZM-bl indicator cell line as described in “Experimental Procedures.” Shown is the mean percentage viral infectivity of triplicate determinations (relative to the infectivity of viruses produced in the absence of A3F), plus or minus the standard deviation, normalized to viral p24 levels measured in culture supernatants by capsid-specific ELISA (41). B, analysis of A3F wild type and mutant protein levels in viral and cellular lysates by Western blotting. C, sucrose density gradient analysis of the intravirion localization of A3F(W126A L306A). HEK293T cells were co-transfected with 15 μg of proviral plasmid pNL4–3(Vif−) and 5 μg of A3F(W126A L306A) expression plasmid. Virions, collected from culture supernatants 48 h after transfection, were fractionated by sucrose density gradient centrifugation as described in “Experimental Procedures.” Shown are Western blots of A3F(W126A L306A) and viral capsid (p24Gag) in individual gradient fractions 1–12 detected with A3F(C18) polyclonal antibody and p24Gag monoclonal antibody, respectively.

FIGURE 9. Mutation of amino acid residue L306 does not affect A3F interaction with HIV-1 nucleocapsid. Cellular lysates of HEK293T cells expressing GST-nucleocapsid (GST-NC) or GST and either A3F, A3F(W126A) or A3F(L306A) were incubated with glutathione-agarose beads as described in “Experimental Procedures.” The cellular expression level and interaction of wild type and mutant A3F proteins with HIV-1 nucleocapsid was analyzed by SDS-PAGE and Western blotting. GST-NC and GST were detected with a GST polyclonal antibody. Wild type and mutant A3F proteins with HIV-1 nucleocapsid was analyzed by SDS-PAGE and Western blotting. GST-NC and GST were detected with a GST polyclonal antibody. Wild type and mutant A3F was detected with the A3F(C18) polyclonal antibody.

virion incorporation by ablating the ability of the protein to bind RNA which provides a bridging interaction with HIV-1 nucleocapsid (3, 15, 21, 30). The sequence requirements for incorporation of A3F into virions have not been studied as comprehensively as A3G. However, there is one report showing that mutation of A3F N-terminal deaminase domain residue W126, which is homologous to A3G W127, results in reduced protein incorporation into virions due to loss of RNA-dependent interaction with nucleocapsid (16). We confirmed the involvement of A3F W126 in incorporation of this protein into virions (16) and have extended this analysis to demonstrate that this residue was also necessary for encapsidation of the protein that was incorporated into the virion. In this report, we demonstrate that the homologous A3G N-terminal deaminase domain can replace the A3F N-terminal deaminase domain without altering the quantitative incorporation and encapsidation of the A3G-F chimeric protein (Fig. 3). This result suggests that the homologous A3F and A3 G-terminal deaminase domains perform similar functions in mediating sequential steps in the virion incorporation and encapsidation of these proteins. In contrast, the GST-A3F162–373 chimeric protein was incorporated into virions, but neither encapsidated (Fig. 6A) nor active as an antiviral (Fig. 4), underscoring the importance of the N-terminal deaminase domain of both proteins for virion incorporation, encapsidation, and antiviral function.

In addition to A3F residue W126 in the N-terminal deaminase domain, we demonstrated that A3F C-terminal deaminase domain residue L306 is also necessary for virion encapsidation of the protein and that the A3G C-terminal deaminase domain could not substitute for this function (Fig. 3A). The A3F C-terminal deaminase domain is evolutionarily distinct from the A3G C-terminal deaminase domain (40), and we suggest that A3G lost the C-terminal deaminase domain encapsidation signal during A3 evolution, while retaining the N-terminal deaminase domain signal shared with A3F. In contrast to the A3F(W126A) mutant, mutation of L306A neither ablated the ability of this protein to bind HIV-1 nucleocapsid (Fig. 9), nor affected its incorporation into virions (Fig. 7). This result suggests that this residue might be involved in an interaction of A3F with another viral factor involved in virion particle maturation other than the HIV-1 nucleocapsid. A3F has been reported to interact with HIV-1 integrase that is a component of virion cores (23). However, we have determined that wild type A3F was incorporated normally and encapsidated in HIV-1 virions generated from proviral vectors with deletions in HIV-1 integrase, or in both HIV-1 integrase and reverse transcriptase (data not shown). The matrix domain of HIV-1 Gag has been shown to mediate RNA-dependent packaging of APOBEC3C (A3C) into virions (42) and in another report, A3C was shown to be associated with virion cores (43). Since the single deaminase domain of A3C shares 77% amino acid identity with the A3F C-terminal deaminase domain, the interaction of A3F with the matrix domain of HIV-1 Gag warrants investigation. However, determination of which viral, or even cellular, proteins play a role in A3 encapsidation is needed, as is
more detailed characterization of the physical association of A3F with the virion core.

The effect on A3F encapsidation of the W126A and L306A mutations were similar when they were each present individually, as well as when they were present together in the W126A L306A double mutant. The double mutant did not have a synergistically detrimental effect on encapsidation. In contrast, while the single W126A and L306A mutants of A3F each had reduced antiviral activity, the A3F(W126A L306A) double mutant lost all antiviral activity (Fig. 8A). A mathematical analysis indicated synergistic impairment of antiviral function when the critical residues in each domain were both mutated. Synergy of two factors is generally evident when each attacks different steps in the same mechanistic pathway, rather than different pathways. Therefore, these results suggest the hypothesis that the signals in A3F N-terminal and C-terminal deaminase domains each perform different mechanistic roles in virion incorporation and encapsidation, but that both of these contribute to the same pathway mediating A3F’s antiviral mechanism. The current results provide an important step toward better characterization of the mechanisms of A3 antiviral function and encapsidation, as well as the differences in these biological mechanisms between A3F and A3G. This will inform new interventions against HIV-1.

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