

Resistance of human T cell leukemia virus type 1 to APOBEC3G restriction is mediated by elements in nucleocapsid

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Human T cell leukemia virus type 1 (HTLV-1) has evolved a remarkable strategy to thwart the antiviral effects of the cellular cytidine deaminase APOBEC3G (hA3G). HTLV-1 infects T lymphocytes *in vivo*, where, like HIV-1, it is likely to encounter hA3G. HIV-1 counteracts the innate antiviral activity of hA3G by producing an accessory protein, Vif, which hastens the degradation of hA3G. In contrast, HTLV-1 does not encode a Vif homologue; instead, HTLV-1 has evolved a cis-acting mechanism to prevent hA3G restriction. We demonstrate here that a peptide motif in the C terminus of the HTLV-1 nucleocapsid (NC) domain inhibits hA3G packaging into nascent virions. Mutation of amino acids within this region resulted in increased levels of hA3G incorporation into virions and increased susceptibility to hA3G restriction. Elements within the C-terminal extension of the NC domain are highly conserved among the primate T cell leukemia viruses, but this extension is absent in all other retroviral NC proteins.

retrovirus

Cells have evolved numerous strategies to restrict infection by pathogens such as viruses. In turn, viruses that are disseminated in a particular host have evolved mechanisms to resist cellular restriction factors. A good example of antiviral restriction emerged from studies aimed at understanding the function of HIV-1 Vif. It was known that Vif-deficient HIV-1 could replicate in some cell lines but not others, and that this phenotype depended on the virus-producer cell rather than the target cell. The cellular protein responsible for this effect was cloned and identified as CEM15, also known as APOBEC3G (1). Human APOBEC3G (hA3G) belongs to a family of cytidine deaminases and is a broadly acting antiviral restriction factor (1, 2). In addition to inhibiting the replication of Vif-deficient HIV-1, hA3G has been shown to inhibit the replication of other exogenous retroviruses, endogenous retroviruses, retrotransposons, and hepatitis B virus (3–12).

hA3G can be packaged into retrovirus particles and then inhibit virus replication in the target cell by catalyzing the conversion of cytosine to uracil in minus-strand DNA during reverse transcription (resulting in G-to-A hypermutation of the provirus) (3, 6, 13–15). Although cytosine deaminase activity appears to correlate with antiviral activity of hA3G, there is evidence that other factors may contribute to antiviral activity, and it is still unclear which stage of the virus infectious cycle is primarily affected (16–18). The exact mechanism by which hA3G is packaged into virions is also unresolved but it does appear to require both RNA and viral nucleocapsid (NC) protein (19–22). HIV-1 counteracts the effects of hA3G with the accessory protein Vif, whose primary function is to target hA3G for proteasomal degradation, thereby preventing encapsidation of hA3G into virus particles (23–28). Primate foamy viruses resist the inhibitory effects of hA3G with the accessory protein Bet, which interacts with hA3G and prevents its packaging into virus particles, but by a different mechanism than HIV-1 Vif (8). It is not clear whether or how retroviruses, which do not encode Vif homologues, resist APOBEC restriction. Human T cell

leukemia virus type 1 (HTLV-1) is particularly interesting in this regard because, like HIV-1, it infects human T lymphocytes, which express hA3G.

HTLV-1 appears to resist the antiviral effects of hA3G. Mahieux *et al.* (10) recently reported that G-to-A mutations were not detected in the proviruses from HTLV-1-infected patients, and <0.1% of proviruses generated after *in vitro* infections contained G-to-A hypermutations. In addition, two other studies (11, 12) showed that HTLV-1 was relatively resistant to hA3G despite the fact that hA3G could be found in HTLV-1 particles; however, it should be noted that hA3G packaging was not quantified in those studies. We report here that HTLV-1 is less susceptible to hA3G inhibition compared with HIV-1 because HTLV-1 packages less hA3G into virus particles than HIV-1. We ruled out the possibility that HTLV-1 expresses an accessory protein that interferes with hA3G packaging or activity, and we show that the determinant for resistance to hA3G resides in the HTLV-1 structural protein Gag. Specifically, a motif in the C-terminal region of the NC domain of Gag acts in cis to exclude hA3G from HTLV-1 particles.

Results

Previous studies indicated that HTLV-1 somehow resists antiviral effects of hA3G (10–12). It seemed unlikely that HTLV-1 does so by expressing a transacting accessory protein like Vif, because chronically HTLV-1-infected T cells accumulate hA3G and restrict HIV Δ vif replication (29). We formally excluded a transacting mechanism by performing the following experiments. First, cotransfection of either full-length HTLV-1 or HTLV-1 Tax/Rex expression plasmids with HIV Δ vif vectors and hA3G expression plasmid failed to rescue HIV Δ vif replication in single-cycle infectivity assays (data not shown). Second, HTLV-1 mutants with a deletion of the pX ORFs, which encode putative accessory proteins, packaged the same amount of hA3G as wild-type virus. Also, when used as packaging plasmids for single-cycle replication assays, the pX mutants showed the same susceptibility to hA3G inhibition as wild-type vectors (data not shown). Finally, as shown below, Gag alone determines the amount of hA3G that is packaged into HTLV-1 versus HIV-1 particles.

To delineate the mechanism by which HTLV-1 evades hA3G restriction, we used viral vectors in single-cycle replication assays that make it possible to quantify viral infection and replication (30). HTLV-1 or HIV Δ vif virus-like particles (VLPs), pseudotyped with vesicular stomatitis virus G protein, were produced in 293T cells cotransfected with varied amounts of a

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Abbreviations: HTLV-1, human T cell leukemia virus type 1; hA3G, human APOBEC3G; VLP, virus-like particle; MA, matrix; CA, capsid; NC, nucleocapsid.

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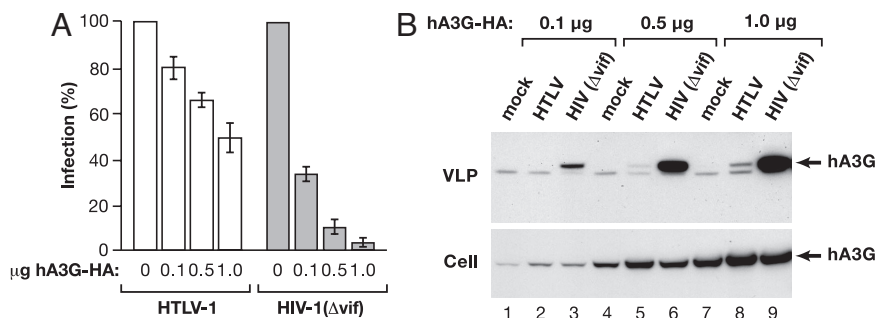


Fig. 1. Differences in the inhibition of HTLV-1 versus HIV-1 Δ vif by hA3G correlate with the amount of hA3G incorporated into VLPs. (A) Single-cycle replication assays were performed with VLPs produced in cells cotransfected with varied amounts of hA3G-HA expression plasmid. Infection is expressed as the percent transduction of luciferase relative to no-hA3G control. Because HTLV-1 VLPs have a low specific infectivity, HIV Δ vif VLPs were diluted 1:1,000 before infection to give comparable luciferase activities for the two viruses. (B) Cells were cotransfected with the indicated amount of hA3G-HA plasmid in combination with either empty vector (mock), HTLV-1 vectors, or HIV Δ vif vectors. VLPs were concentrated from supernatants by centrifugation. Cell and VLP extracts were immunoblotted and probed with anti-HA antibody. Concentrations of HTLV-1 and HIV-1 Gag proteins in VLP extracts were determined by ELISA, and equal amounts of Gag were used for immunoblotting. Arrows indicate the position of the hA3G-HA, which is the upper band in VLP lysates.

hemagglutinin (HA)-tagged hA3G expression plasmid. Both HTLV-1 and HIV-1 transfer vectors encode a GFP-luciferase fusion protein so that infection of HeLa cells can be monitored by assaying for luciferase activity. As shown in Fig. 1A, and in agreement with previous studies, expression of hA3G significantly diminished infectivity of HIV Δ vif, but had only a modest effect on HTLV-1 infection. When cotransfected with 1 μ g of hA3G plasmid, HIV Δ vif infection was reduced \approx 20-fold, but HTLV-1 infection was reduced by only 2-fold. We next compared the amount of HA-tagged hA3G protein that was packaged into HTLV-1 and HIV Δ vif VLPs by immunoblotting with an anti-HA antibody (Fig. 1B). Gag protein concentrations in VLP lysates were quantified by either HTLV-1 p19 (matrix, MA) or HIV-1 p24 (capsid, CA) ELISA, and equal amounts of Gag protein were loaded in each lane of SDS-protein gels. Phosphor-image analysis of immunoblots indicated that HTLV-1 particles packaged \approx 20 times less hA3G than HIV Δ vif VLPs. As in chronically infected cell lines, HTLV-1 expression did not di-

minish hA3G accumulation in transfected cells. These experiments indicated that HTLV-1 particles incorporated less hA3G compared with HIV Δ vif particles and therefore HTLV-1 resists the inhibitory effects of hA3G because HTLV-1 does not efficiently package hA3G.

The HTLV-1 Gag protein, composed of MA, CA, and NC domains (Fig. 2), is sufficient for the assembly and release of VLPs. We compared the amount of hA3G that was packaged into VLPs produced from Gag expression plasmids that encode either wild-type, mutant, or chimeric versions of the HTLV-1 Gag protein (Fig. 2). Immunoblots of VLP lysates revealed that hA3G was not efficiently packaged into VLPs containing wild-type HTLV-1 Gag (Fig. 2, lane 1, WT). In contrast, hA3G was detected in large amounts in VLPs containing HIV-1 Gag (data not shown) and in VLPs containing a chimeric Gag composed of HTLV-1 MA/CA and HIV-1 NC domains (Fig. 2, lane 8, NC4.0). Thus, the amounts of hA3G packaged into VLPs produced from the Gag expression plasmids were similar to the amounts packaged into VLPs produced

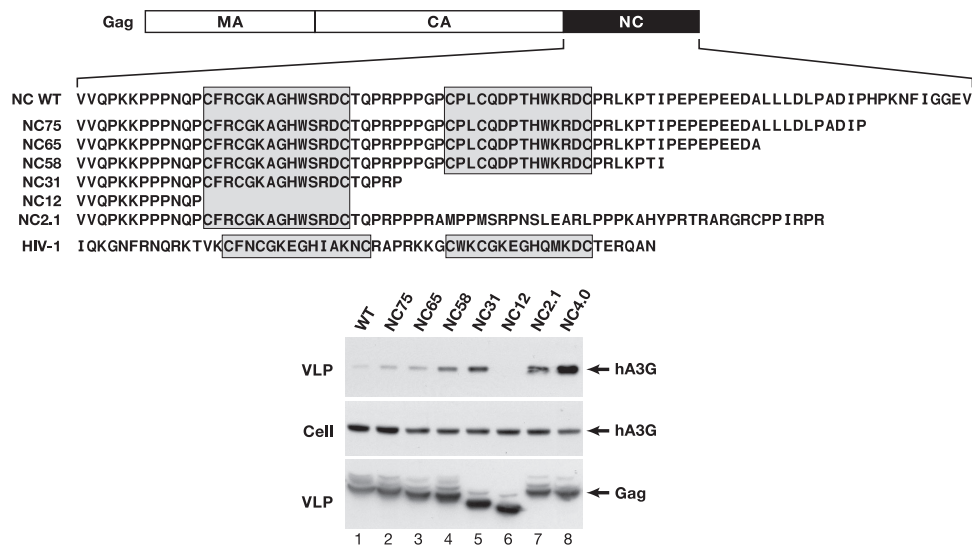


Fig. 2. Packaging of hA3G into HTLV-1 particles is determined by the NC domain of Gag. The organization of HTLV-1 Gag is shown above the amino acid sequences of wild-type and mutated NC domains. The Zn-finger motifs in NC are shaded. The wild-type or modified HTLV-1 gag genes were subcloned into a mammalian expression plasmid. A series of C-terminal truncations of the NC domain are shown below the wild-type NC sequence. The plasmid designated as NC2.1 has a frameshift mutation between the Zn-finger motifs that replaces the C-terminal half of NC with the indicated sequence. The Gag protein designated as NC4.0 has the HTLV-1 MA and CA domains fused to the HIV-1 NC domain, whose sequence is shown. 293T cells were transfected with 1 μ g of hA3G-HA expression plasmid and the indicated Gag expression plasmids. Immunoblots of cell and VLP lysates were probed with anti-hA3G antibody or anti-HTLV-1 p19(MA) antibody. Loading of equal amounts of Gag protein in VLP lysates was based on HTLV-1 p19(MA) ELISA determination.

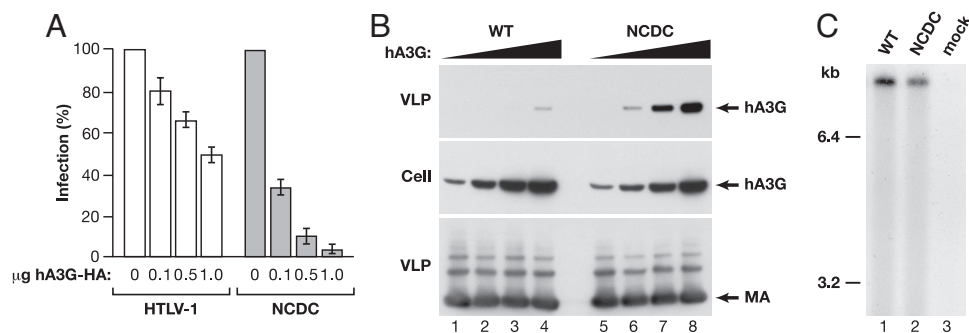


Fig. 3. A peptide region near the C terminus of HTLV-1 NC acts to exclude hA3G from VLPs. (A) A 20-aa region (PTIPEPEPEEDALLDLPAD) near the C terminus of NC was deleted in pCMVHT- Δ Env to yield the NCDC mutant. Single-cycle replication assays were performed with VLPs produced from cells transfected with HTLV-1 or NCDC vectors and the indicated amounts of hA3G-HA plasmid. Infection is expressed as percent transduction relative to no-hA3G control. (B) Immunoblots of cell and VLP lysates from 293T cells transfected with HTLV-1 or NCDC vectors in combination with 0 μ g (lanes 1 and 5), 0.1 μ g (lanes 2 and 6), 0.5 μ g (lanes 3 and 7), or 1.0 μ g (lanes 4 and 8) of hA3G expression plasmid. Immunoblots were probed with anti-hA3G antibody or anti-HTLV-1 p19(MA) monoclonal antibody. (C) Viral poly(A)⁺ mRNA was prepared from virions produced from cells transfected with wild-type or NCDC versions of the infectious clone of HTLV-1. Northern blots were probed with a ³²P-labeled *gag* gene fragment. The positions of RNA size markers, which were run on the same gel, are indicated.

from whole-virus expression plasmids (Fig. 1B). Moreover, the difference in hA3G packaging between HIV-1 and HTLV-1 VLPs is determined by the NC domain of Gag.

As shown in the sequence alignments at the top of Fig. 2, HTLV-1 and HIV-1 NC proteins differ dramatically in the amino acid sequence after the two zinc finger motifs. HTLV-1 NC has a long C-terminal tail rich in proline, glutamic acid, and leucine residues. Deletions of 10–20 aa from the C terminus of the 85-residue HTLV-1 NC protein were accompanied by a slight increase in hA3G packaging into VLPs (Fig. 2, lane 2, NC75 and lane 3, NC65). The amount of hA3G incorporated into VLPs increased by \approx 8-fold when 29 aa were removed from the C terminus of NC in the NC58 mutant (Fig. 2, lane 4). The amount of hA3G detected in VLPs was also high in mutant NC31, which contains only one zinc finger motif (Fig. 2, lane 5). Deletion of both zinc fingers in NC12 abolished hA3G packaging (Fig. 2, lane 6). Furthermore, hA3G was efficiently packaged by a Gag mutant (NC2.1) in which the C-terminal half of NC (including the second zinc finger) was replaced by a 38-aa-long peptide rich in proline and basic amino acid residues (Fig. 2, lane 7). These data indicate that the first zinc finger of HTLV-1 NC is sufficient for hA3G packaging and that a motif in the C terminus of NC acts to prevent hA3G packaging into the virus particle.

To determine how the C terminus of NC affects HTLV-1 replication and hA3G inhibition, we constructed an HTLV-1 expression plasmid with a 20-aa deletion in this region (PTIPEPEPEEDALLDLPAD), which is designated as NCDC. In the absence of hA3G, NCDC infectivity was \approx 40% of wild-type infectivity in single-cycle replication assays. VLPs expressed in cells cotransfected with increasing amounts of hA3G plasmid revealed that the NCDC mutant was \approx 20 times more sensitive to hA3G than wild-type virus (Fig. 3A). Immunoblots revealed that NCDC VLPs packaged significantly higher levels of hA3G than wild-type HTLV-1 particles (Fig. 3B), consistent with the increased susceptibility of NCDC to hA3G inhibition. Because retroviral NC proteins bind to RNA and are responsible for viral RNA encapsidation, we determined the amount of viral mRNA in wild-type and NCDC VLPs by Northern blot and phosphorimaging analyses (Fig. 3C). These experiments revealed that the ratio of viral mRNA-to-Gag protein [determined by HTLV-1 p19(MA) ELISA] for NCDC and WT VLPs differed by $<$ 5%, indicating that the deletion mutation did not affect viral RNA packaging. Together, these data indicate that elements in the C-terminal region of HTLV-1 NC counteract hA3G inhibition of

virus replication by preventing hA3G packaging into virus particles.

To define amino acids in the C terminus of NC that mediate susceptibility to hA3G, we constructed site-directed mutants. An alignment of HTLV-1, HTLV-2, and HTLV-3 (31) NC proteins revealed several highly conserved motifs within otherwise divergent C termini (Fig. 4A). These consisted of a cluster of acidic amino acids and an adjacent LLLDL sequence, which were contained within the region deleted in the

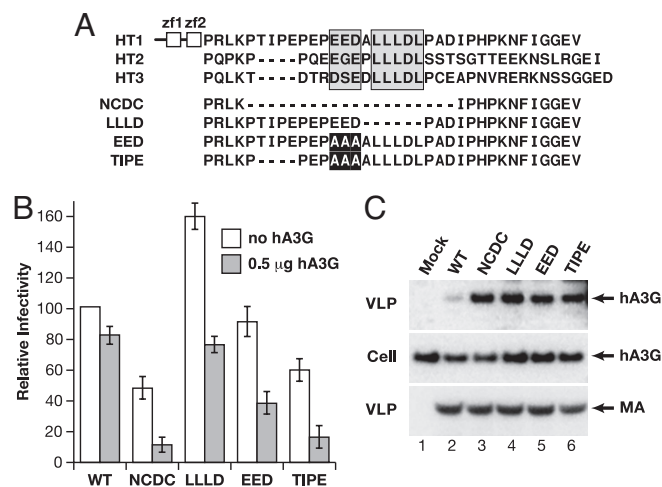


Fig. 4. Conserved motifs in the C-terminal region of the NC domain mediate HTLV-1 resistance to hA3G. (A) Amino acid sequence alignment of the C-terminal portions of HTLV-1, HTLV-2, and HTLV-3 (31) NC domains is shown; the highly conserved N terminus, containing two zinc finger motifs (zf1 and zf2) is depicted for HTLV-1 at the top. A cluster of acidic amino acids and a conserved LLLDL motif are shaded. Shown below the sequence alignment are the mutated HTLV-1 NC domains that were cloned into the pCMVHT- Δ Env packaging vector. Deleted amino acids are indicated by dashed lines, and alanine substitutions are highlighted. (B) Wild-type and mutant packaging vectors were tested in single-cycle replication assays. VLPs were produced in the absence or presence of 0.5 μ g of hA3G-HA plasmid. Infectivity, normalized to p19(MA) levels, is plotted relative to the value determined for wild-type vector in the absence of hA3G set at 100. Error bars indicate standard deviations of duplicate samples from at least two experiments. (C) 293T cells were cotransfected with the indicated wild-type or mutant HTLV-1 packaging vectors or without HTLV-1 expression plasmid (lane 1, mock). All samples were cotransfected with 1 μ g of hA3G expression plasmid. Cell and VLP extracts were immunoblotted as in Fig. 1.

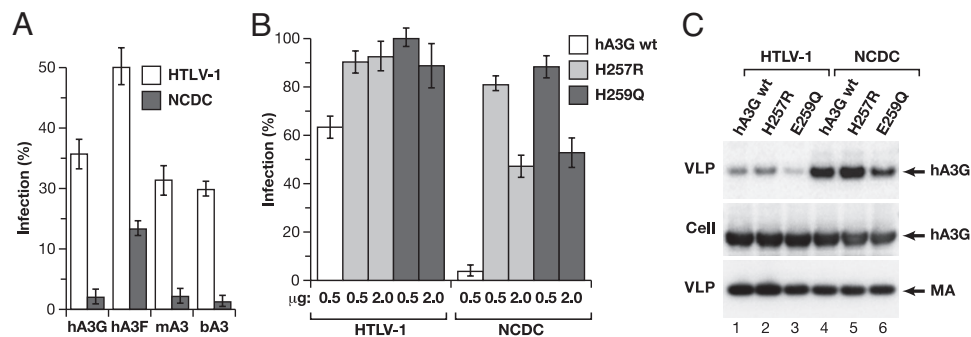


Fig. 5. Single-cycle replication of HTLV-1 vectors is inhibited by various APOBEC proteins and inhibition requires cytidine deaminase activity. (A) HTLV-1 or NCDC vectors were produced in the absence or presence of 1 μ g of the indicated APOBEC expression plasmids; hA3G, hA3F, mouse A3 (mA3), or bovine A3 (bA3). The data are plotted as the percent activity remaining in the presence of APOBEC relative to no-APOBEC controls. (B) HTLV-1 and NCDC vectors were produced in the presence of the indicated amounts of wild-type hA3G, H257R, or E259Q. The data are plotted as the percent activity remaining in single-cycle replication assays compared with no-hA3G controls. (C) Packaging of wild-type or mutated forms of hA3G into HTLV-1 or NCDC VLPs. 293T cells were cotransfected with HTLV-1 or NCDC expression vectors and 2 μ g of the indicated hA3G expression plasmids. Cell and VLP extracts were immunoblotted as in Fig. 1.

NCDC mutant. The mutated viral vectors were transfected without or with 0.5 μ g of hA3G-HA plasmid, and VLPs were tested in single-cycle replication assays (Fig. 4B). In the absence of hA3G, specific infectivity ranged between 60% above (LLL), about equal (EED), or 40% below wild type. In these experiments, cotransfection with 0.5 μ g of hA3G-HA plasmid inhibited wild-type HTLV-1 infectivity by \approx 20% and inhibited NCDC by \approx 80%. All of the site-directed mutants were more susceptible to hA3G compared with wild type. Cotransfection with 0.5 μ g of hA3G-HA plasmid inhibited replication of the LLL mutant by 50%; the EED mutant was inhibited by 54%; and the TIPE mutant was inhibited by 66% (Fig. 4B). The relative amounts of hA3G that were incorporated into VLPs produced by the mutated viral vectors were higher than wild-type VLPs (Fig. 4C), and the amounts of VLP-associated hA3G correlated with the antiviral effect. Furthermore, wild-type and mutated viruses packaged equivalent amounts of viral RNA (data not shown). In summary, site-directed mutagenesis indicates that the cluster of acidic amino acids and the LLLDL motif contribute to the ability of the C-terminal region of HTLV-1 NC to resist hA3G.

To determine whether HTLV-1 resistance to hA3G mediated by the C terminus of NC extends to other APOBEC proteins, we tested human A3F (hA3F), mouse A3 (mA3), and bovine A3 (bA3) proteins against wild-type or NCDC HTLV-1 vectors. In all cases, NCDC was much more sensitive to APOBEC 3 compared with wild-type HTLV-1 vector. In general, hA3F was less inhibitory than hA3G, whereas mA3 and bA3 were slightly more active than hA3G (Fig. 5A). These results indicate that the C terminus of HTLV-1 NC acts to resist the APOBEC 3 proteins in a general manner and also suggest that these APOBEC proteins are packaged into HTLV-1 VLPs by a common mechanism.

To determine whether cytidine deaminase activity of hA3G is required for the inhibition of HTLV-1 infection, we mutated conserved amino acids in the second zinc coordination motif of hA3G, which is essential for enzymatic activity. The H257R and E259Q mutations were previously shown to diminish anti-HIV-1 activity of hA3G (11, 16, 18). hA3G, H257R, and E259Q were tested as inhibitors in single-cycle replication assays with either HTLV-1 or NCDC vectors (Fig. 5B). With HTLV-1 vectors, 0.5 μ g of hA3G plasmid inhibited replication by \approx 40%, whereas cotransfection with 0.5 μ g of either H257R or E259Q plasmids had no significant inhibitory effect. Increasing the amounts of H257R or E259Q plasmids to 2 μ g did not result in significant inhibition. With NCDC vectors, cotransfection with 0.5 μ g of hA3G inhibited replication by $>$ 95% and 0.5 μ g of either H257R

or E259Q resulted in 10% to 20% inhibition (Fig. 5B). Increasing the amounts of H257R and E259Q plasmids to 2 μ g resulted in \approx 50% inhibition of NCDC replication. Both H257R and E259Q were incorporated into HTLV-1 and NCDC VLPs, although E259Q was packaged less efficiently than wild-type hA3G or H257R (Fig. 5C). As before, NCDC VLPs contained more of the wild-type or mutated hA3G protein than HTLV-1 VLPs. The second Zn-coordination motif, and hence the cytidine deaminase activity of hA3G, is required to achieve complete inhibition of HTLV-1 infectivity.

Discussion

Although HTLV-1 and HIV-1 both infect human T lymphocytes, the viruses differ in many ways and have evolved different strategies to circumvent cellular restriction mechanisms. HTLV-1 provides the first clear example of a virus that evades hA3G by a cis-acting exclusion mechanism. In contrast, other retroviruses evade hA3G by expressing transacting factors, such as HIV-1 Vif (23–28) or primate foamy virus Bet proteins (8). A previous study indicated that HTLV-1 resists G-to-A hypermutation elicited by hA3G (10). Two other groups reported that HTLV-1 was weakly susceptible to hA3G inhibition (11, 12), but they did not address the basis for this apparent resistance. The resistance of HTLV-1 to hA3G restriction and the ability of the virus to prevent hA3G packaging are not absolute. As shown here and before (11, 12), hA3G can be detected in HTLV-1 particles when it is expressed at high levels. Nonetheless, by quantifying the relative levels of hA3G and Gag, we showed that hA3G is packaged much less efficiently into HTLV-1 compared with HIV Δ vif VLPs. We also found that elements in the C terminus of HTLV-1 NC inhibit hA3G packaging into HTLV-1 particles. When these elements were deleted or mutated, the virus was more susceptible to hA3G inhibition and incorporated more hA3G into viral particles than wild-type virus.

HTLV NC proteins are different from those expressed by all other retroviruses in that they contain a 35-aa extension on the C-terminal side of the zinc finger domains. By comparison, NC proteins from HIV-1 and the closely related deltaretrovirus, bovine leukemia virus, have only 6-aa extensions. Site-directed mutations in this region of HTLV-1 NC indicated that two highly conserved motifs were responsible for hA3G resistance, which consisted of a small cluster of acidic amino acids and an adjacent leucine-rich motif (LLL Δ DL). Mutations in either of these elements resulted in increased hA3G incorporation into

fragment from the HTLV-1 *gag* gene (30). Hybridization was quantified on a Storm phosphorimager (Applied Biosystems, Foster City, CA).

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