The Interaction between HIV-1 Gag and APOBEC3G*

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APOBEC3G, a member of an RNA/DNA cytidine deaminase superfamily, has been identified as a cellular inhibitor of HIV-1 infectivity, possibly through the dC to dU deamination of the first minus strand cDNA synthesized during reverse transcription. Virions incorporate APOBEC3G during viral assembly in non-permissive cells, and this incorporation is inhibited by the viral protein Vif. The mechanism of APOBEC3G incorporation into HIV-1 is examined in this report. In the absence of Vif, cytoplasmic APOBEC3G becomes membrane-bound in cells expressing HIV-1 Gag, and its incorporation into Gag viral-like particles (VLPs) is proportional to the amount of APOBEC3G expressed in the cell. The expression of Vif, or mutant Gag unable to bind to membrane, prevents the APOBEC3G association with membrane. HIV-1 Gag alone among viral proteins is sufficient for packaging of APOBEC3G into Gag VLPs, and this incorporation requires the presence of Gag nucleocapsid. The presence of amino acids 104–156 in APOBEC3G, located in the linker region between two zinc coordination motifs, is also required for its incorporation into Gag VLPs. Evidence against an RNA bridge facilitating the Gag/APOBEC3G interaction includes data indicating that 1) the incorporation of APOBEC3G occurs independently of viral genomic RNA, 2) a Gag/APOBEC3G complex is immunoprecipitated from cell lysate after RNase treatment, and 3) the zinc coordination motif, rather than the regions flanking this motif, have been implicated in RNA binding in another family member, APOBEC1.

Vif† (virus infectivity factor) is a 190–240-amino acid protein that is encoded by all of the lentiviruses except for equine infectious anemia virus (1–12). Vif is required for HIV-1 to replicate in certain “non-permissive” cell types, such as primary T lymphocytes, macrophages, and some of T-cell lines, including H9, but is not required in other “permissive” cell types, such as SupT1 and Jurkat cells (3, 5, 11). The ability of Vif-negative viruses to replicate in target cells is determined by the cell producing the virus (5, 12). Thus, Vif-deficient viruses produced from non-permissive cells are impaired in their ability to replicate in target cells.

Non-permissive cells have been found to contain a protein called APOBEC3G (also known as CEM-15), which prevents HIV-1 replication in the absence of Vif (13). APOBEC3G belongs to an APOBEC superfamily containing at least 10 members, which share a cytidine deaminase motif (14). These include APOBEC1 and activation-induced cytidine deaminase (AID), which have been shown to deaminate C in RNA (14) and DNA (15), respectively. It is not known if APOBEC3G can edit RNA, but several reports suggest that the anti-HIV-1 activity of this protein stems from its ability to form dU by deaminating dC in the first minus strand cDNA produced during HIV-1 reverse transcription (16–19). Vif-negative HIV-1 produced in non-permissive cells package APOBEC3G during assembly while Vif-positive virions do not (13, 16). cDNA synthesis is low in the target cell infected with Vif-negative viruses, and the minus strand cDNA made contains 1–2% of the cytosines mutated to uracil. This could allow for cDNA degradation by the DNA repair system. The coding strand found in double-stranded cDNA also contains an increase in G to A mutations that could also contribute to the anti-viral activity of APOBEC3G through mutant coding regions for viral proteins. Vif is able to bind to APOBEC3G (20) and can reduce both the cellular expression of APOBEC3G and its incorporation into virions (21). The reduction in cellular expression has been attributed to both inhibition of APOBEC3G translation and its degradation in the cytoplasm by Vif (22), and recent evidence suggests that Vif interacts with cytoplasmic APOBEC3G as part of a Vif-Cul5-SCF complex, resulting in the ubiquination of APOBEC3G and its degradation (23).

Enzymes similar to the human APOBEC superfamily are also encoded by the mouse and African green monkey (20), and a mouse gene on chromosome 15 (murine CEM15) shows amino acid similarity and structural homology with human APOBEC3G (13, 24). Vif is not present in the simple retrovirus MuLV, and Vif from HIV-1 is unable to prevent encapsidation of murine APOBEC into HIV-1, whose packaging results in severe inhibition of HIV-1 replication (20). Interestingly, while murine APOBEC is incorporated into murine leukemia virus, it appears to have little effect upon the replication of this virus (16, 18, 20). On the other hand, the human APOBEC3G can inhibit the infectivity of different retroviruses including murine leukemia virus, and simian immunodeficiency virus, and equine infectious anemia virus (16, 18), although at lower efficiency than for HIV-1.

The mechanism by which APOBEC3G is incorporated into Vif-negative HIV-1 is not clear. In this report, we present data that the incorporation of APOBEC3G into HIV-1 requires sequences found between the two zinc coordination motifs found

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§ The abbreviations used are: Vif, viral infectivity factor; HIV-1, human immunodeficiency virus type 1; BH10P–, HIV-1 containing an inactive viral protease; Gag, HIV-1 precursor protein containing sequences coding for HIV-1 structural proteins; MA, matrix; CA, capsid; NC, nucleocapsid; p6, p6 protein; VLP, viral-like particle; HA, hemagglutinin epitope.
in this protein (amino acids 104–156) and the nucleocapsid sequence in Gag. HIV-1 Gag alone among viral proteins is sufficient to package APOBEC3G into Gag viral-like particles (VLPs). Evidence is also presented that suggest that an RNA bridge between these two molecules is not involved in facilitating the Gag/APOBEC3G interaction.

EXPERIMENTAL PROCEDURES

Plasmid Construction—SVC21BH10.P is a simian virus 40-based vector that contains full-length wild-type HIV-1 proviral DNA containing an inactivating viral protease (D25G) and was a gift from E. Cohen, University of Toronto. SVC21BH10.FS contains mutations at the frameshift. Modified-Eagle medium (Amersham Biosciences), Western blots were probed with monoclonal antibodies that are specifically reactive with HIV-1 capsid (Zepo Metros Inc.), HA (Santa Cruz Biotechnology Inc.), and β-actin (Sigma) or with Vif-specific polyclonal antiserum number 2221 (National Institutes of Health AIDS Research and Reference Reagent Program). Detection of proteins was performed by enhanced chemiluminescence (PerkinElmer Life Sciences), using as secondary antibodies anti-mouse (for capsid and β-actin) and anti-rabbit (for HA and Vif), both obtained from Amersham Biosciences. Bands in Western blots were quantitated using UN-SCAN-IT gelTM automated digitizing system.

Immunoprecipitation Assay—293T cells from 100-mm plates were collected 48 h post-transfection and lysed in 500 μl of Tnt buffer (20 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% Triton X-100). Insoluble material was pelleted at 1800 g for 30 min. The supernatant was used as the source of immunoprecipitated Gag/APOBEC3G complexes. Equal amounts of protein were incubated with 30 μl of HA-specific antibody for 16 h at 4 °C, followed by the addition of protein A-Sepharose (Amersham Biosciences) for 2 h. For a Western blot of different cell lysates, 500 μg of lysis protein was used for immunoprecipitation from each lysate, while for different nuclease experiments on the same lysate sample, ~200 μg of lysis protein was used for immunoprecipitation. Lysate protein was determined by the Bio-Rad assay. The immunoprecipitate was then washed three times with Tnt buffer and twice with phosphate-buffered saline. After the final supernatant was removed, 30 μl of 2% SDS sample buffer (120 mM Tris-HCl pH 6.8, 20% glycerol, 4% SDS, 2% β-mercaptoethanol, and 0.02% bromphenol blue) was added, and the precipitate was then boiled for 5 min to release the precipitated proteins. After microcentrifugation, the resulting supernatant was analyzed using Western blots. In the DNase and RNase treatment assay, the cell lysates were pretreated with 20 μg of DNase or RNase before the immunoprecipitation, as described previously (29).

Subcellular Fractionation and Sucrose Floatation Assay—Cells were lysed 48 h post-transfection at 4 °C by Dounce homogenization in 1.0 ml hypotonic TE buffer (20 mM Tris-HCl, pH 7.4, 1 mM EDTA, 0.01% β-mercaptoethanol), supplemented with protease inhibitors mixture (Complete,” Roche Applied Science). The cell homogenate was then sedimented at 1500 g for 30 min to remove nuclei and unbroken cells. After the supernatant was removed, 0.5 ml of the resulting supernatant (SI) was mixed into 2 ml of final 73% sucrose. 7 ml of 65% sucrose in TNE were layered on top of the 73% sucrose, and 1.5 ml of 10% sucrose was layered on top of the 65% sucrose. The gradients were then centrifuged at 100,000 g for 1 h in a Beckman SW 41 rotor at 4 °C. 2-ml fractions were collected and diluted with 10 ml of TNE, and each fraction was centrifuged at 100,000 g at 4 °C for 1 h. The pellets from each fraction were dissolved in SDS sample buffer and analyzed by SDS-PAGE and Western blotting.

RESULTS

Incorporation of APOBEC3G into Gag VLPs—293T cells were co-transfected with a plasmid coding for human APOBEC3G containing a C-terminal HA tag and plasmid containing wild-type or mutant HIV-1 proviral DNA. BH10.Vif— and BH10.P—Vif— both contain a stop codon immediately after the initiation ATG codon of the Vif reading frame, and BH10P— contains an inactive viral protease. hGag contains a humanized HIV-1 Gag gene (i.e. codon usage optimized for translation in mammalian cells (27)), and only wild-type HIV-1 Gag and Gag VLPs are produced (25). The cell lysates of transfected cells were analyzed by Western blots (Fig. 1A), using anti-HA (top panel), anti-β-actin (middle panel), and anti-Vif (bottom panel) antibodies as probes. Vif is detected only in cells transfected with BH10. In cells producing virions or Gag VLPs...
lacking Vif, APOBEC3G is strongly expressed, while in cells producing BH10, very little APOBEC3G is seen in the cytoplasm. The viruses produced from these cells were analyzed by Western blotting (Fig. 1B), using anti-HA (top panel) and anti-CAp24 (bottom panel). While no APOBEC3G is seen in wild-type BH10, it is found in virions not expressing Vif. These results also indicate that Gag alone is sufficient among the viral proteins for facilitating APOBEC3G incorporation. Our results also confirm previous observations of a diminished presence of APOBEC3G in both the cytoplasm and in virions in the presence of Vif expression, and this has been shown to be due to the Vif-induced polyubiquination of APOBEC3G and subsequent degradation by the proteasome (22, 23, 30–32).

As well as lacking coding sequences downstream of Gag, the RNA coding for hGag has the 5′ RU5 and leader sequence of the viral RNA replaced with a cytomegalovirus promoter. Therefore, it is not expected that hGag VLPs will specifically package this RNA, which lacks viral packaging signals. This suggests that APOBEC3G incorporation into these particles occurs independently of viral genomic RNA packaging. To further confirm this, total RNA was extracted from cells co-transfected with APOBEC3G and either BH10.P-Vif− or hGag and from the virions produced from these cells. Viral mRNA in the cells and virions were quantified by dot blot, using a 32P-labeled DNA probe specific for the p6 coding sequence, which is present in both BH10.P-Vif− and hGag RNA. The ratios for viral RNA:β-actin in the cytoplasm, and viral RNA:Gag in virions, is presented graphically in Fig. 1C. Although cytoplasmic expression of viral genomic RNA is strong in cells expressing hGag (top panel, Fig. 1C), the genomic RNA/Gag in hGag VLPs is reduced to ~15% of that found in BH10.P−Vif− (bottom panel, Fig. 1C). This reduced incorporation of viral RNA does not, however, affect APOBEC3G incorporation into hGag VLPs (B), indicating that APOBEC3G incorporation into virions occurs independently of viral RNA incorporation.

The Nucleocapsid Sequence within Gag Is Required for the Viral Packaging of APOBEC3G—A series of Gag deletion constructs were used to identify the motif within Gag involved in the incorporation of APOBEC3G into virions. These constructs are shown in Fig. 2A. 293T cells were co-transfected with APOBEC3G and wild-type or mutant Gag constructs, and cells were lysed in radioimmune precipitation assay buffer. Western blots of cell lysates (Fig. 2B) were probed with anti-CA (upper panel) or anti-HA (lower panel). The first lane represents cells transfected with hGag alone. All Gag mutants were expressed at similar levels in the cytoplasm except for the 378–500 construct. This Gag has NC, p1, and p6 deleted from the C terminus and is expressed 2–3-fold higher than full-length Gag.

Most of these mutant Gag molecules are impaired in their ability to form extracellular particles due to the absence of membrane- or RNA-binding regions. We have therefore investigated the interaction between APOBEC3G and mutant Gag species using immunoprecipitation to detect cellular complexes. The presence of both Gag and APOBEC3G in the cell lysate was first analyzed by Western blots probed with anti-CA (Fig. 2B, upper panel) and anti-HA (Fig. 2B, lower panel). The Gag:apoBEC3G ratios, listed at the bottom of C, normalized to the hGag:apoBEC3G ratio, are similar for all mutant Gag species expressed, except for Δ378–500, which shows a higher expression of Gag. APOBEC3G in each cell lysate was then immunoprecipitated by anti-HA, and the presence of both Gag and APOBEC3G in the immunoprecipitate was analyzed by Western blotting using anti-CA (Fig. 2C, upper panel) and anti-HA (Fig. 2C, lower panel). The Gag:apoBEC3G ratios, listed at the bottom of C, normalized to the hGag:apoBEC3G ratio, indicate no change in the association of Gag with APOBEC3G with removal of the N-terminal matrix domain (MA) sequences (Δ1–132) and a small decrease (12%) with removal of the C-terminal p1/p6 sequences (Δ433–500). However, a C-terminal deletion of Gag, which also included NC (Δ378–500), resulted in a >95% reduction in the interaction of Gag with APOBEC3G, even though the expression of this mutant Gag is greater in the cell lysate than seen for hGag (Fig. 2B). A larger C-terminal Gag deletion (Δ284–500), in which p2 and the C-terminal region of capsid (including the MHR domain) have been further removed, also prevented interaction
with APOBEC3G. These data suggest that nucleocapsid sequences within Gag are responsible for the interaction between APOBEC3G and Gag. The small decrease in the Gag:APOBEC3G ratio found with removal of the p1/p6 sequences might reflect an altered conformation affecting the neighboring NC-binding site in Gag.

Both Gag nucleocapsid (33) and members of the APOBEC family, including APOBEC3G (14), can bind to RNA, so that the interaction demonstrated between Gag and APOBEC3G could be mediated by an RNA bridge. However, the data in Fig. 2D suggest that an RNA bridge is not likely. 293T cells were co-transfected with BH10.P−.Vif− and APOBEC3G, and the cell lysates were subjected to RNase or DNase treatment, followed by immunoprecipitation with either anti-IN or anti-HA, respectively. The immunoprecipitates were analyzed by Western blotting, using anti-CA to detect the presence of Gag in the immunoprecipitate from RNase digestion by the two proteins.

The requirement for nucleocapsid sequence is further shown in Fig. 3, in which the nucleocapsid sequence in HIV-1 has been replaced with a yeast leucine zipper domain to allow for protein/protein interactions (plasmid ZWt-p6.Vif−). It has previously been shown that the parental plasmid, ZWt-p6, can efficiently produce extracellular viruses (26). Another mutant, BH10.FS−.Vif−, in which frameshift sequence had been changed to produce only Gag, was used as a control. 293T cells were co-transfected with APOBEC3G and mutant HIV-1 plasmids, and expression of APOBEC3G in cells were analyzed by Western blotting, probed with anti-HA, anti-CA, and anti-β-actin (Fig. 3A). The results show that similar amounts of APOBEC3G were efficiently produced in all the cells transfected with Vif− constructs (Fig. 3A, upper panel, lanes 2, 4, and 6), whereas cellular APOBEC3G was severely reduced if the viral constructs produced Vif (Fig. 3A, upper panel, lanes 1, 3, and 5). The absence or presence of Vif had no effect upon cellular Gag levels (Fig. 3A, middle panel). The ability of the viruses to package APOBEC3G was then assessed by Western blots of viral lysates probed with anti-CA (Fig. 3B, lower panel) or anti-HA (Fig. 3B, upper panel). The results show that BH10.FS−.Vif− can package APOBEC3G as efficiently as BH10.P−. On the other hand, the ability of ZWt-p6.Vif− to incorporate APOBEC3G is reduced 90% compared with BH10.FS−.Vif−. These data demonstrate that while the leucine zipper motif can functionally replace nucleocapsid for Gag multimerization and virus assembly, it cannot replace its ability to facilitate APOBEC3G incorporation.
**Fig. 3. The ability of APOBEC3G to be incorporated into wild-type or mutant HIV-1.** 293T cells were co-transfected with APOBEC3G expression vector and different plasmids containing wild-type or mutant HIV-1 proviral DNA. The plasmids used are listed along the top of each panel and described under “Experimental Procedures.” A, Western blots of cell lysates were probed with either anti-HA (upper), anti-CA (middle), or anti-β-actin (bottom). B, Western blots of cell lysates of Gag VLPs produced from transfected cells were probed with either anti-HA (upper) or anti-CA (bottom).

Sequences in APOBEC3G Required for Its Incorporation into Gag VLPs—293T cells were co-transfected with hGag and a plasmid coding for wild-type or N- or C-terminal-deleted APOBEC3G tagged with HA. These constructs are shown graphically in Fig. 4A. APOBEC3G has sequence homology with APOBEC1 and contains two or one active site regions, respectively (H-X-E-[O]34–36-P-P-X-C) containing a zinc coordination motif. The cytoplasmic expression and viral incorporation of the different APOBEC3G variants was determined by Western blots probed with anti-HA and anti-β-actin for cells (Fig. 4B) or anti-HA and anti-CA for viruses (Fig. 4C). The mutant APOBEC3G/β-actin ratio in the cell lysates, or APOBEC3G/Gag ratio in the viral lysates, are normalized to a ratio of 1.0 for wild-type APOBEC3G and are listed at the bottom of each panel. As shown in Fig. 4C, deletion of the N-terminal 104 amino acids or the C-terminal 157–384 amino acids does not affect the ability of APOBEC3G to be packaged into Gag VLPs, whereas the deletion of the N-terminal 156 amino acids abolishes its incorporation into viruses. This result indicates that amino acids 104–156, found in the N-terminal portion of a linker sequence between the two zinc coordination motifs in APOBEC3G, are required for its incorporation into Gag VLPs.

All C-terminal APOBEC3G deletions shown in Fig. 4 show reduced expression in the cell lysate (10–20% of wild-type (Fig. 4B)). This may be due to intracellular degradation, since it has been reported that N-terminal fragments of APOBEC3G are inherently unstable (34). Interestingly, the viral content of these N-terminal fragments is >60% of wild-type APOBEC3G, i.e. does not reflect their low cytoplasmic expression. Thus, the removal of the C-terminal regions of APOBEC3G appears to result in a significant decrease in its concentration in the total cell lysate without a similar quantitative decrease in its incorporation into Gag VLPs. While this might suggest that the decreased APOBEC3G pools are not the source of viral APOBEC3G, there is as yet no evidence for the existence of separate cellular APOBEC3G pools. Thus, the floatation gradients of post-nuclear supernatant, as shown in Fig. 5, indicate that almost all cytoplasmic APOBEC3G interacts with Gag and moves to the membrane, and other reports (16, 22) have indicated that cellular APOBEC3G is primarily cytoplasmic. An alternative explanation that the C-terminally truncated APOBEC3G interacts with Gag more efficiently than wild-type Gag is also not likely, since, as shown in Fig. 6, increasing concentrations of wild-type APOBEC3G in the cytoplasm interact efficiently with Gag. We therefore have no ready explanation for this phenomenon.

Effect of Gag Expression upon the Intracellular Distribution of APOBEC3G—293T cells were transfected with the plasmid coding for APOBEC3G alone or co-transfected with this plasmid and plasmids coding for mutant forms of hGag in the presence or absence of Vif. Transfected cells were lysed in hypotonic buffer, and after a low speed centrifugation to remove broken cells and nuclei, the post-nuclear supernatant was resolved on sucrose gradients into membrane-free and membrane-bound protein, as described previously (35). Gradient fractions were analyzed by Western blots, probed with anti-HA or anti-CA antibody. As shown in Fig. 5A, in the absence of Gag, >90% APOBEC3G is present near the bottom of the gradient, i.e. in the cytoplasmic fraction (lanes 5 and 6). However, in the presence of Gag (Fig. 5B), >90% of APOBEC3G is localized in the membrane-bound protein near the top of the gradient at the 10%/65% sucrose interface, reflecting a similar intracellular distribution for Gag (35). If Vif is also expressed, the APOBEC3G remains in the cytoplasm at reduced levels (Fig. 5C). When cells express both APOBEC3G and the mutant Gag species, ZWt-p6.Vif−, the majority of APOBEC3G remains in the cytoplasm even though most Gag is found at membrane (Fig. 5D). When cells are transfected with a mutant Gag that can no longer bind to membrane (Δ1–132), but that retains the ability to bind to APOBEC3G, the APOBEC3G remains in the cytoplasm (Fig. 5E). These data indicate that binding to Gag transports most cytoplasmic APOBEC3G to the membrane during viral assembly. This interaction is efficient, since when cells are co-transfected with the hGag plasmid and increasing amounts of the plasmid expressing APOBEC3G, the amount of APOBEC3G incorporated into viruses is proportional to the amount of APOBEC3G expressed in the cell (Fig. 6).
DISCUSSION

In this work, we have shown that Gag alone among viral proteins is sufficient for the incorporation of APOBEC3G, and deletion analysis shows that Gag nucleocapsid and amino acids 104–156 in APOBEC3G are required for the Gag/APOBEC3G interaction. Fig. 2C shows that the cytoplasmic interaction between Gag and APOBEC3G requires NC sequences. The requirement for Gag nucleocapsid suggests a direct interaction of this Gag domain with APOBEC3G but could also reflect a requirement for either Gag multimerization or for an RNA bridge binding the two proteins. The fact that the Gag/APOBEC3G interaction is still detected after RNase A treatment (Fig. 2D) suggests that Gag multimerization is not required for the interaction. Furthermore, Gag multimerization is not sufficient for the incorporation of APOBEC3G into viral particles. Thus, experiments with ZWt-p6.Vif/H11002, a virus in which the nucleocapsid sequence has been replaced with a yeast leucine zipper responsible for facilitating protein interactions, show that the resulting extracellular Gag particles produced do not incorporate APOBEC3G (Fig. 3B), i.e. the presence of NC is still required. This indicates that while the incorporation of APOBEC3G into Gag VLPs is proportional to its expression in the cell (Fig. 6), APOBEC3G is not randomly incorporated into Gag VLPs or virions. The simple production of viral particles does not ensure a random incorporation of APOBEC3G. On the other hand, the fact that APOBEC3G is incorporated into virions with diverse Gag sequences, including HIV-1, murine leukemia virus, simian immunodeficiency virus, and equine infectious anemia virus (16, 18), suggests some common property of Gag NC other than sequence similarity is required. This feature could be common structural motifs, or it could be their common ability to bind RNA.

However, the data presented here, while not eliminating the existence of an RNA bridge facilitating the interaction between Gag and APOBEC3G, does not favor the prime importance of such a bridge. The RNA producing hGag does not contain viral genomic RNA packaging signals. The hGag VLPs produced, while containing only 14% as much viral genomic RNA as virions containing wild-type Gag (Fig. 1C), do efficiently package APOBEC3G (Fig. 1B). This indicates that APOBEC3G packaging occurs independently of HIV-1 viral genomic RNA and supports an earlier finding that used a UV cross-linking assay to demonstrate that APOBEC3G bound specifically to apoB mRNA and UA-rich RNA but not to HIV-1 RNA (14). A unique role for cellular RNA in facilitating an APOBEC3G/Gag interaction is also not supported by the data. The ability to immunoprecipitate a cytoplasmic Gag/APOBEC3G complex is only slightly diminished upon prior treatment with RNase A (10–14% decrease), while the immunoprecipitation of a Gag/GagPol complex is completely inhibited by a similar RNase A treatment (Fig. 2D). However, we cannot eliminate the possibility that RNA bridging Gag and APOBEC3G is not protected from RNase digestion by these proteins.

Although the RNA-binding region(s) within APOBEC3G are not known, they have been mapped in the related family member APOBEC1 to its single zinc coordination motif (36, 37). APOBEC3G binds to zinc in vitro and has an RNA binding capacity similar to APOBEC1 (14). Amino acids 104–156 in APOBEC3G are required for the incorporation of this molecule into Gag VLPs yet lay outside either zinc coordination motif,
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Fig. 5. Distribution of APOBEC3G between cytoplasm and membrane. 2 μg of APOBEC3G expression vector were transfected into 293T cells or co-transfected with 2 μg of plasmids coding for wild-type or mutant hGag. Cells were lysed hypotonically in TE buffer, and the post-nuclear supernatant was resolved by the sucrose floatation assay into membrane-bound (J) and membrane-free (B) protein, as described under "Experimental Procedures." The left side of A–E show Western blots of gradient fractions probed with anti-HA, while the right side of each panel presents these blots, as well as blots probed with anti-CA, graphically showing the percentage of analyzed protein in each gradient fraction. □ and □ represent APOBEC3G and Gag, respectively. A, cells are transfected with the plasmid coding for APOBEC3G and plasmid(s) coding for hGag (B), hGag and Vif (C), the mutant Gag ZWt-p6.Vif (D), and the Δ1–132 hGag (E). I and B at the top of each panel represent interface and bottom fraction in the discontinuous sucrose gradient, respectively.

Fig. 6. Incorporation of APOBEC3G into Gag VLPs is proportional to its cellular expression. 293T cell were co-transfected with 2 μg of hGag and various amounts of plasmid coding APOBEC3G. Western blots of cell lysate or Gag VLP lysates probed for APOBEC3G with anti-HA are shown in the upper and lower blot, respectively. Bands in Western blots were quantitated, and the right panel plots the relative intensities of APOBEC3G expressed in the cell or APOBEC3G incorporated into Gag VLPs, which does not support a major role for RNA in the Gag/APOBEC3G interaction. There also does not appear to be any local cluster of basic amino acids within amino acids 101–156, which could contribute to the non-specific binding of RNA. We observe little or no effect on APOBEC3G incorporation into virions with the removal of either zinc coordination motif (Fig. 4C).

The data presented in the middle panel in Fig. 3A do not show a difference in Gag levels in Vif+ or Vif− cells expressing APOBEC3G, i.e., while the cellular expression of APOBEC3G is decreased in Vif− cells, Gag does not decrease. In fact, while the presence of Vif in non-permissive cells alters the cytoplasmic distribution of APOBEC3G, it does not alter the cytoplasmic distribution of Gag. This is shown in Fig. 5, A–C. APOBEC3G in the post-nuclear supernatant is found primarily in the cytoplasm of non-permissive cells (Fig. 5A). In cells also expressing Gag, almost all of it is carried to the membrane in the absence of Vif (Fig. 5B), but wild-type Gag does not carry APOBEC3G to the membrane in the presence of Vif (Fig. 5C). It can also be seen that the cellular distribution of Gag between membrane and cytoplasm is unaltered whether Vif is present or not. The ability of Gag to alter the cytoplasmic distribution of APOBEC3G depends upon the ability of Gag to interact with either cell APOBEC3G (Fig. 5D, in which the mutant Gag species ZWt-p6.Vif is expressed) or with membrane (Fig. 5E, in which the Δ1–132 mutant Gag species, which lacks membrane-binding sequences, is expressed).

The data in Figs. 3 and 5 suggest that little, if any, Gag is associated with the Vif/APOBEC3G complex. Although immunofluorescence studies showed a co-localization of Gag and Vif in the cell (38), co-sedimentation studies indicated an interaction of Vif only with some early viral assembly intermediates, and the presence of Vif in mature virions remains controversial (39–46). In insect cell infected with baculovirus expressing Gag and Vif, it was estimated that there were 70 Vif molecules per 2000 Gag molecules in extracellular Gag particles or one molecule of Vif for every 30 molecules of Gag (47). If single Gag molecules bound to Vif at this same ratio within an APOBEC3G/Vif/Gag complex destined for degradation in the proteasome, this would account for only 3.5% of Gag molecules produced, and a change in Gag distribution in the cell would not be detectable by our Western blot assay.

Alternatively, the formation of an APOBEC3G/Vif/Gag complex may be prevented by overlapping binding sites. While the ability to co-immunoprecipitate Gag and Vif from cell lysates has met with varying degrees of success (48, 49), the in vitro interaction between Vif and Gag has been used to map interacting sites on these two molecules (47). These results indicate...
that the Vif-binding sites on Gag include the C-terminal of NC (including the second zinc finger), the spacer peptide sp2, and the N-terminal region of p6. Since NC is involved in binding to both Vif and APOBEC3G, the latter two molecules might compete for binding to Gag. Similarly, the APOBEC3G-binding sites for Vif and Gag have been estimated to include amino acids 54–124 for Vif (34) and amino acids 104–156 for Gag, as reported herein. The lack of formation of a Gag/Vif/APOBEC3G complex could therefore also be due competitive binding between Gag and Vif for sites on APOBEC3G or to conformational restraints preventing both molecules binding to APOBEC3G.

Most cytidine deaminases act as homodimers or homotetramers (50, 51). It has been reported for APOBEC1 that small N- (10 amino acids) or C- (10 amino acids) terminal deletions reduce RNA editing, RNA binding, and homodimerization activities (51). Similarly, it has been reported for APOBEC3G that N- and C-terminal deletions that do not eliminate either active site still destroy enzyme activity and that this is due to inhibition of APOBEC3G dimerization (52). We show here that larger N- and C-terminal deletions of APOBEC3G can still be packaged into HIV-1 (Fig. 4), which suggests that neither APOBEC3G dimerization nor its binding to RNA is required for this process.

It is not clear if the deoxycytidine deaminase activity of APOBEC3G is the sole determinant in inhibiting HIV-1 replication. For example, while two reports have indicated that mutations in either active site result in similar losses of both deoxycytidine deaminase activity and anti-viral activity (16, 17), a more recent paper reports that mutations in either active site inhibit deoxycytidine deaminase activity to different extents but have the same anti-viral activity (52). This latter observation implies that deoxycytidine deaminase activity of APOBEC3G may not be the sole determinant of anti-viral activity. It is possible that the interaction of APOBEC3G with nucleosapld might result in the inhibition of viral functions associated with nucleosapld. For example, Gag nucleosapld sequences facilitate trRNA<sub>5'–3'</sub>-annealing to viral genomic RNA (53), which could explain the observation that deproteinized viral RNA (which contains primer trRNA<sub>5'–3'</sub>-annealed to viral genomic RNA) extracted from Vif-negative HIV-1 produced in non-permissive cells shows a decreased ability to support reverse transcription in vitro compared with the same RNA extracted from similar virions produced in permissive cells (8). Alternatively, this observation might reflect the presence in non-permissive cells of other anti-HIV-1 factors yet to be discovered.

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The Interaction between HIV-1 Gag and APOBEC3G
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