JOURNAL OF VIROLOGY, July 2005, p. 8201–8207 Vol. 79, No. 13
0022-538X/05/$08.00+0 doi:10.1128/JVI.79.13.8201–8207.2005

Differential Sensitivity of Murine Leukemia Virus to APOBEC3-Mediated Inhibition Is Governed by Virion Exclusion

Brian P. Doehle, Alexandra Schäfer, Heather L. Wiegand, Hal P. Bogerd, and Bryan R. Cullen*

Center for Virology and Department of Molecular Genetics and Microbiology, Duke University Medical Center, Durham, North Carolina 27710

Received 20 December 2004/Accepted 16 March 2005

While members of the APOBEC3 family of human intrinsic resistance factors are able to restrict the replication of Vif-deficient forms of human immunodeficiency virus type 1 (HIV-1), they are unable to block replication of wild-type HIV-1 due to the action of Vif, which induces their degradation. In contrast, HIV-1 Vif is unable to block inhibition mediated by APOBEC3 proteins expressed by several heterologous species, including mice. Here, we have asked whether the simple retrovirus murine leukemia virus (MLV) is sensitive to restriction by the cognate murine or heterologous, human APOBEC3 proteins. We demonstrate that MLV is highly sensitive to inhibition by human APOBEC3G and APOBEC3B but resistant to inhibition by murine APOBEC3 or by other human APOBEC3 proteins, including APOBEC3F. This sensitivity fully correlates with the ability of these proteins to be packaged into MLV virion particles: i.e., human APOBEC3G and APOBEC3B are packaged while murine APOBEC3 and human APOBEC3F are excluded. Moreover, this packaging in turn correlates with the differential ability of these APOBEC3 proteins to bind MLV Gag. Together, these data suggest that MLV Gag has evolved to avoid binding, and hence virion packaging, of the cognate murine APOBEC3 protein but that MLV infectivity is still restricted by certain heterologous APOBEC3 proteins that retain this ability. Moreover, these results suggest that APOBEC3 proteins may help prevent the zoonotic infection of humans by simple retroviruses and provide a mechanism for how simple retroviruses can avoid inhibition by APOBEC3 family members.

Intrinsic immunity to retroviral infection has recently emerged as a significant determinant of the species tropism of primate lentiviruses (2). One important defense against retroviral infection is provided by the APOBEC3 family of proteins, which consists of up to seven distinct gene products in humans and other primates but of only a single gene in mice. The prototype of this family of intrinsic defense factors, human APOBEC3G (hA3G), was initially identified as a critical target for the human immunodeficiency type 1 (HIV-1) auxiliary protein Vif (21). Specifically, cells that express hA3G are nonpermissive for replication of HIV-1 variants lacking a functional Vif gene. In the absence of Vif, hA3G is packaged into progeny HIV-1 virions due to a specific interaction with the nucleocapsid (NC) domain of the Gag polyprotein (1, 6, 14, 19, 28). After infection of target cells, hA3G interferes with HIV-1 replication by massively editing deoxycytidine residues to deoxyuridine on the nascent DNA minus strand (10, 15, 26, 29). This is thought to result in degradation of HIV-1 reverse transcripts or in the integration of lethally hypermutated proviruses (2). In wild-type HIV-1-infected cells, in contrast, the viral Vif protein directly interacts with hA3G and targets this protein for degradation by the proteasome, thus preventing virion incorporation (8, 11, 17, 18, 22, 23, 27). More recently, a second human APOBEC3 family member, APOBEC3F (hA3F), has been shown to also inhibit infection by Vif-deficient, but not wild-type, HIV-1 virions produced in its presence (3, 13, 24, 30). hA3F, which is extensively coexpressed with hA3G in vivo, appears to function by a mechanism that is closely analogous to that of hA3G. Data suggesting that human APOBEC3B (hA3B) either does (3) or does not (25) inhibit HIV-1 infectivity have also been reported, while APOBEC3A (hA3A) and APOBEC3C (hA3C) are not inhibitory to HIV-1 (3, 24, 25).

Although HIV-1 Vif is able to overcome the inhibition of virion infectivity mediated by hA3G and hA3F, HIV-1 Vif is inactive against several simian variants of hA3G and also cannot rescue the potent inhibition of wild-type HIV-1 infectivity seen upon expression of murine APOBEC3 (mA3) (4, 16, 20). Similarly, the Vif protein encoded by African green monkey simian immunodeficiency virus (SIV) is inactive against both hA3G and mA3 (4, 16, 20). Together, these observations have led to the suggestion that APOBEC3 proteins could play an important role in determining the species tropism of primate immunodeficiency viruses (2).

While APOBEC3 proteins can strongly influence the replication of HIV-1 and other lentiviruses, their role in regulating the replication of other retroviruses has only begun to be explored. In this context, it is interesting to speculate as to the effect of APOBEC3 protein expression on the infectivity of simple retroviruses, such as murine leukemia virus (MLV), that do not encode a Vif ortholog or indeed any auxiliary proteins. Although Mariani et al. (16) have reported that hA3G does not influence the infectivity of MLV virions produced in its presence, several other groups have reported that hA3G is a potent inhibitor of MLV infectivity (3, 12, 15).

* Corresponding author. Mailing address: Duke University Medical Center, Box 3025, Durham, NC 27710. Phone: (919) 684-3369. Fax: (919) 681-8979. E-mail: culle002@mc.duke.edu.

Interestingly, however, two groups have now reported that MLV infectivity is not reduced upon expression of levels of mA3 that can effectively inhibit HIV-1. Recently, Kobayashi et al. (12) have reported that this difference correlates with the ability of MLV to package hA3G, but not mA3, into MLV virion particles. However, this result disagrees with earlier data from the Landau laboratory (16) arguing that MLV efficiently packages both hA3G and mA3 into progeny virions.

In this article, we have addressed the mechanism underlying MLV sensitivity to specific APOBEC3 family members. We report that MLV virion infectivity is strongly inhibited by expression of hA3G and hA3B in *trans* but is largely unaffected by expression of the cognate mA3 protein or of several other human APOBEC3 proteins, including hA3F. Inhibition correlated with the specific packaging of hA3G and hA3B, but not mA3 or hA3F, into MLV virions, which in turn correlated with the ability of hA3G and hA3B, but not mA3 or hA3F, to bind the MLV Gag protein specifically. These data argue that the MLV Gag protein can distinguish between different APOBEC3 family members and suggest that MLV Gag has evolved to exclude the mA3 protein from MLV virion particles. However, MLV Gag retains the ability to bind, and package, the human APOBEC3 proteins hA3G and hA3B, and therefore MLV would likely be unable to replicate in human cells that express these proteins. These results suggest that APOBEC3 proteins may play an important role in determining the species tropism of simple retroviruses and provide an explanation for how simple animal retroviruses like MLV can avoid restriction by the APOBEC3 proteins expressed in their normal host species.

MATERIALS AND METHODS

Construction of molecular clones. The MLV proviral construct pNCS has been described previously (9). The MLV-based luciferase expression construct pFB-Luc was obtained from Stratagene. The HIV-1 proviral constructs pNL-HXB-LUC-ΔVif and pNL4-3ΔVifΔEnv have been previously described (4, 24). The vesicular stomatitis virus G expression plasmid pHIT/G (19) was used for pseudotyping MLV in infectivity assays. pcDNA3-based expression plasmids for hemagglutinin (HA)-tagged hA3A, hA3C, hA3F, hA3G, mA3 and β-arrestin 2 (βARR2) have been described previously (4, 15, 24). A plasmid expressing hA3B was constructed similarly by PCR amplification of a full-length hA3B cDNA from a cDNA preparation derived from the human cell line K562. Additional versions of all the HA-tagged plasmids were constructed in the pK plasmid backbone (5) by cleaving the pcDNA3-based plasmids with HindIII and XhoI and then moving the excised cDNA into the corresponding sites present in the pK parental plasmid. Plasmids expressing full-length HIV-1 or MLV Gag (pK-HIV-GAG-GST, pK-MLV-GAG-GST) fused to a carboxy-terminal glutathione *S*-transferase (GST) moiety were derived in the same pK background by sequential insertion of the GST gene as a BamHI/NotI fragment, followed by insertion of a codon-optimized form of HIV-1 Gag (19), or the wild-type MLV *gag* gene, as a HindIII/BamHI fragment. Derivatives of pK-MLV-GAG-GST either lacking the carboxy-terminal nucleocapsid (NC) domain or containing only the MLV NC domain were prepared using standard techniques. A plasmid expressing nonfused GST was also constructed (pK-GST).

Cell culture and virion infectivity assays. 293T cells were cultured in 10% fetal bovine serum in Dulbecco's modified Eagle's medium and transfected using the calcium phosphate method. HIV-1 infectivity assays were performed as described previously (4). MLV infectivity assays were performed by cotransfecting 293T cells with 1 μg of the MLV proviral plasmid pNCS, 1 μg of pFB-Luc, and 250 ng of pHIT/G. An additional 500 ng of plasmid DNA (hA3G or mA3 with the parental pcDNA3 plasmid as filler) was added for titration experiments. All remaining infectivity assays used 125 ng of an APOBEC3 expression construct. Forty-four hours posttransfection, virus-containing supernatant media were filtered through a 0.45-μm-pore-size syringe tip filter (Whatman) and added to recipient cells. Twenty-four hours postinfection, cell lysates were harvested and assayed for luciferase activity as previously described (4, 24).

Virion packaging of APOBEC3 proteins was analyzed largely as previously described (4, 24). Briefly, 293T cells were transfected with either pNL4-3ΔVifΔEnv or pNCS and an APOBEC3 expression plasmid. Forty-four hours later, virus-containing media were collected, filtered, and layered onto a 20% sucrose cushion. Virus was pelleted by centrifugation at 35,000 rpm for 1.5 h at 4°C in a Beckman SW41 rotor. Pellets were then lysed and analyzed by Western blotting.

Western blot analysis. Cell lysates, virion lysates, and immunoprecipitates were subjected to sodium dodecyl sulfate (SDS) gel electrophoresis and then transferred to a nitrocellulose membrane. Membranes were probed with a mouse monoclonal antibody specific for the HIV-1 capsid protein (7), a mouse monoclonal antibody specific for the HA epitope tag (Covance), a goat polyclonal antiserum specific for the MLV capsid protein (a gift of Stephen Goff), or a mouse monoclonal antibody specific for GST (Santa Cruz). Reactive proteins were detected with the Lumi-Light Western blotting substrate (Roche) as previously described (4).

mRNA expression analysis. Analysis of the tissue expression pattern of mA3 mRNA was performed using semiquantitative reverse transcription-PCR (RT-PCR) (24). Total RNA was isolated from a variety of mouse tissues and cell lines using TRIzol reagent (Invitrogen) according to the manufacturers' instructions. Peripheral blood lymphocytes (PBLs) were isolated from whole blood using Lymphocyte separation media (Cellgro) following the manufacturers' instructions. Purified T cells were a gift from Garnett Kelsoe. BW5147 is a mouse T-cell lymphoma line. All RNA preparations were treated with RQ1 RNase-free DNase (Promega) to remove any genomic DNA contamination. Ten micrograms of treated total RNA was subjected to reverse transcription using the StrataScript First Strand synthesis system (Stratagene) using the provided oligo(dT) primers. PCR was then performed using gene-specific primers for 30 cycles of PCR under standard conditions.

Coimmunoprecipitation assays. To detect the binding of APOBEC3 proteins to HIV-1 or MLV Gag, 293T cells were cotransfected with 1.5 μg of a plasmid expressing a GAG-GST fusion protein, or GST as a control, together with 1.5 μg of a plasmid expressing an HA-tagged APOBEC3 protein, or HA-tagged β-ARR2 as a control. Forty-eight hours posttransfection, cells were harvested and lysed in 250 μl of binding buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 5% glycerol, 1% Triton X-100). Fifty microliters of the lysate was retained for analysis of input protein expression levels. The remaining lysate was incubated with 75 μl of glutathione Sepharose 4B beads (Amersham) for 1 h at 4°C. The beads were then washed three times with binding buffer, and bound proteins were removed by heating in the presence of SDS. To determine whether RNA was required for binding, the same protocol was used except that the lysate was incubated with 50 μg/ml of RNase A (QIAGEN) for 15 min at 37°C prior to addition of the glutathione beads.

RESULTS

Murine APOBEC3 mRNA is widely expressed in vivo. One possible mechanism by which simple retroviruses such as MLV could avoid restriction by APOBEC3 proteins is to replicate in tissues that do not express these proteins. In the case of humans, hA3G and hA3F have been shown to be widely expressed while hA3B expression appears more limited (3, 24, 25). To examine the tissue expression profile of mA3, we performed a semiquantitative RT-PCR analysis of mA3 mRNA expression in murine tissues. These data (Fig. 1) documented mA3 mRNA expression in a range of murine tissues, including T cells, a known target for infection by MLV. Indeed, MLV gained its name from its ability to induce T-cell leukemia in infected animals. The only tissues examined where mA3 expression appeared fairly low were liver (Fig. 1) and skeletal muscle (data not shown). Together, these data argue that mA3 mRNA is expressed in tissues that are targets for MLV replication in vivo.

MLV infectivity is not affected by mA3 expression. Given that the *mA3* gene is widely expressed in vivo, is MLV in fact sensitive to inhibition by mA3 or by the heterologous hA3G protein? To address this question, we cotransfected cells with the full-length wild-type MLV proviral clone pNCS (9) together with an MLV-based viral vector, pFB-Luc, encoding the

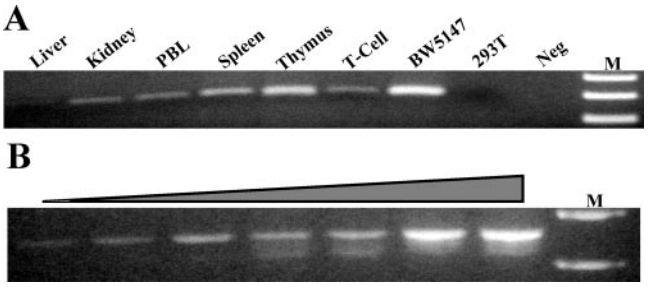

FIG. 1. Expression of mA3 mRNA in a variety of cell lines and murine tissues. (A) RT-PCR analysis using total RNA preparations from a variety of mouse tissues and cell lines. BW5147 is a murine T-cell lymphoma cell line, while 293T cells are of human origin and function as a negative control. Neg, no cDNA added; M, marker. (B) This RT-PCR amplification of mA3 mRNA was performed in parallel using increasing amounts of BW5147 cDNA under the same conditions as in panel A.

luciferase indicator gene. We also cotransfected increasing levels of expression plasmids encoding hA3G or mA3. In parallel, we also transfected 293T cells with the previously described, Vif-deficient HIV-1 indicator construct pNL-HXB-LucΔVif (4) and plasmids expressing either mA3 or hA3G. As shown in Fig. 2A, MLV infectivity was inhibited ~7-fold by cotransfection of 125 ng of hA3G and this inhibition increased to ~25-fold when 500 ng of hA3G was cotransfected. In contrast, cotransfection of an mA3 expression plasmid had no significant effect on MLV infectivity. Western analysis (Fig. 2C) demonstrated that hA3G did not inhibit the synthesis of MLV structural proteins in the cotransfected cells and that the level of expression of mA3 and hA3G was both comparable and proportional to the level of the transfected APOBEC3 expression plasmid.

The lack of inhibition of MLV infectivity by the mA3 protein was not due to the fact that the mA3 protein was inactive, as the same levels of mA3 potently inhibited the infectivity of HIV-1 virions (Fig. 2B) while not affecting the production of HIV-1 virion particles, as determined by enzyme-linked immunosorbent assay of the supernatant media for HIV-1 capsid production (data not shown). We therefore conclude that the infectivity of MLV virions is unaffected by levels of mA3 that effectively inhibit HIV-1 virion infectivity.

To extend this analysis, we asked whether MLV virion infectivity would also be affected by the expression of other human APOBEC3 proteins that have been reported to inhibit the infectivity of Vif-deficient HIV-1 and/or SIV (3, 13, 24, 25, 30) (Fig. 3). In fact, MLV infectivity was strongly inhibited by not only hA3G but also hA3B. In contrast hA3F, which is a potent inhibitor of Vif-deficient HIV-1 (3, 13, 24, 30), and hA3C, which has been reported to inhibit Vif-deficient SIV

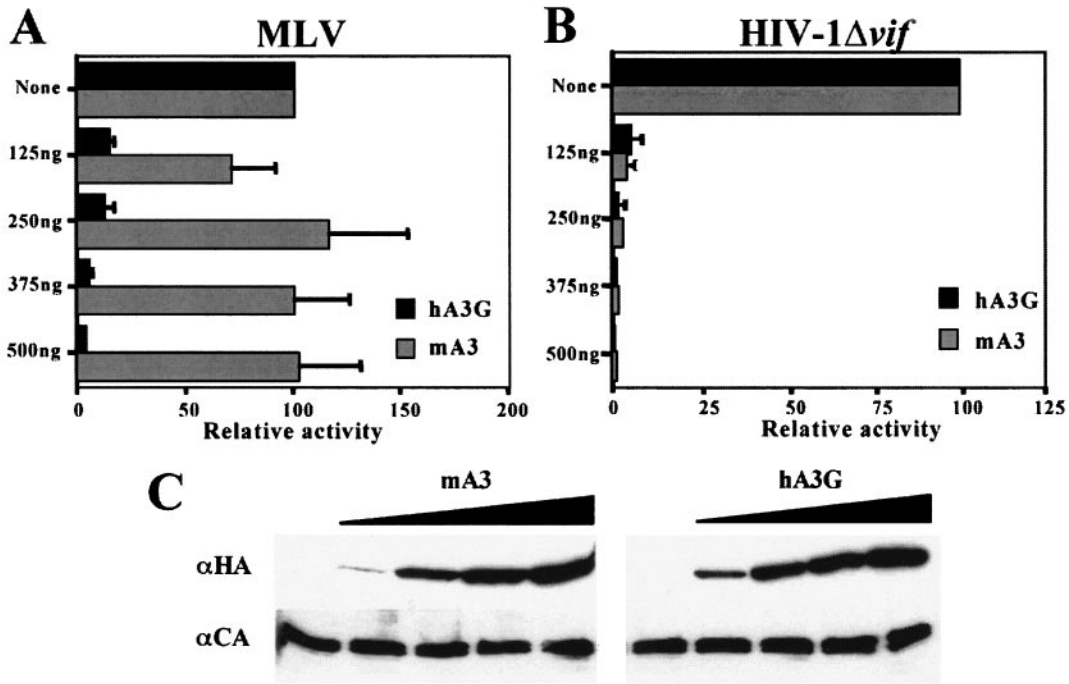

FIG. 2. Comparison of the abilities of hA3G and mA3 to inhibit Vif-deficient HIV-1 or wild-type MLV infectivity. (A) 293T cells were transfected with the full-length MLV proviral clone pNCS together with the MLV-based luciferase expression vector pFB-Luc. Increasing levels of plasmids encoding mA3 or hA3G were cotransfected, with the parental pcDNA3 plasmid acting as a control (None) and used to maintain a constant level of DNA. Supernatant virions were collected and used to infect naïve 293T cells at 44 h after transfection. Induced luciferase levels were analyzed 24 h after infection. Data are presented as a percentage of the level of luciferase activity detected in cells infected with virions derived from cells that did not express an exogenous APOBEC3 protein. The average of three experiments with standard deviation is indicated. (B) Same as panel A, except that the 293T cells were transfected with the Vif-deficient HIV-1 luciferase indicator proviral clone pNL-HXB-LUCΔVIF in place of pNCS and pFB-Luc. (C) Western blot of the virus-producing 293T cells analyzed in panel A using antisera specific for the HA epitope tag present on the APOBEC3 proteins (αHA) or using an anti-MLV capsid antiserum (αCA).

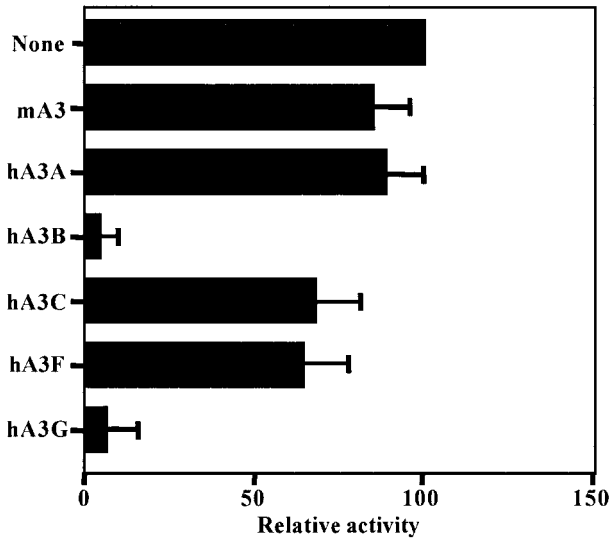

FIG. 3. hA3B, but not hA3F, inhibits MLV infectivity. One hundred twenty-five nanograms of each of the indicated APOBEC3 expression plasmids, or the pcDNA3 parental plasmid (None), was cotransfected into 293T cells along with pNCS and pFB-Luc, and the effect on MLV infectivity was analyzed as described in the legend to Fig. 2.

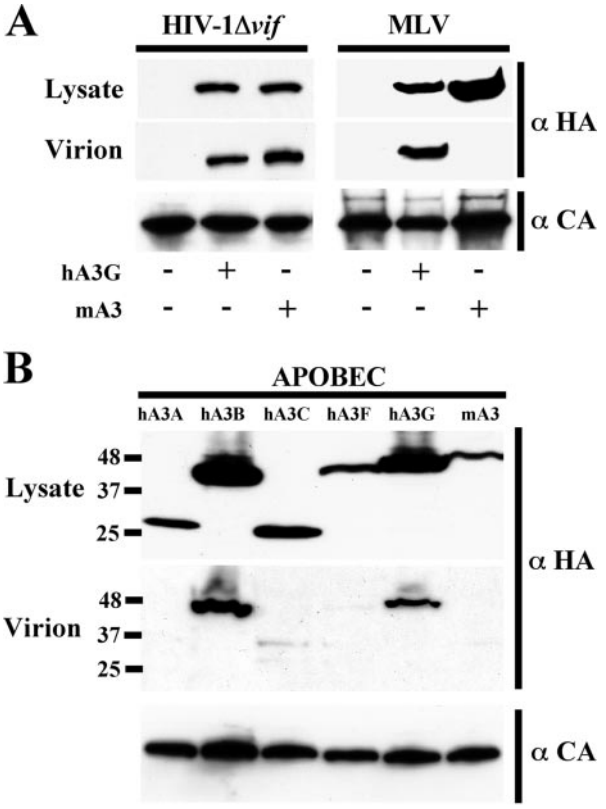

FIG. 4. Specific packaging of APOBEC3 proteins into HIV-1 and MLV virions. (A) Analysis of hA3G and mA3 packaging into either Vif-deficient HIV-1 or wild-type MLV virions. A total of 1.5 μg of either pNL4-3-Luc-ΔEnvΔVif or pNCS was transfected into 293T cells together with a plasmid expressing either hA3G-HA or mA3-HA, or the parental plasmid pcDNA3. Producer cell lysates and virion lysates were then subjected to Western analysis using an antibody specific for the HA epitope tag (αHA). Virion lysates were also probed with an antibody specific for the CA protein of either HIV-1 or MLV (αCA), as a loading control. (B) Specific packaging of hA3B but not hA3F into MLV virions. This MLV virion packaging experiment was performed as described for panel A using a range of APOBEC3 expression plasmids.

infectivity (25), did not markedly reduce MLV virion infectivity, although a reproducible, weak effect was noted (Fig. 3).

MLV virions do not package the mA3 protein. We next asked whether MLV would package the hA3G and mA3 protein into progeny virions. For this purpose, we used centrifugation through a sucrose cushion to collect Vif-deficient HIV-1 and wild-type MLV virions produced in the presence or absence of HA-tagged hA3G or mA3. We then performed a Western analysis on the lysed virions and producer cells using antisera specific for the HA-tag or the HIV-1 or MLV capsid protein. As shown in Fig. 4A, and as previously reported (16, 24), Vif-deficient HIV-1 virions packaged both the hA3G and mA3 proteins, consistent with their sensitivity to both hA3G and mA3. In contrast, MLV virions efficiently incorporated the hA3G protein but did not incorporate detectable levels of mA3.

The data presented in Fig. 4A suggest that the sensitivity of MLV to inhibition by hA3G but not mA3 correlates with the incorporation of the former, but not the latter, into progeny virions. To explore whether this correlation could be extended to other human APOBEC3 proteins, we asked whether hA3B, a potent inhibitor of MLV infectivity (Fig. 3), and hA3F and hA3C, which fail to inhibit MLV, could also package into MLV virions. As shown in Fig. 4B, we observed efficient packaging of hA3B and hA3G but little or no packaging of hA3F, hA3C, mA3, or hA3A. Therefore, the sensitivity of MLV to specific APOBEC3 proteins indeed correlates with virion incorporation.

Murine APOBEC3 fails to bind MLV Gag in vivo. In the case of hA3G, virion incorporation has been shown to be due to a specific interaction between hA3G and the NC domain of Gag, a process that also requires the presence of apparently nonspecific, i.e., nonviral, RNA (1, 6, 14, 19, 28). To test whether the MLV Gag protein interacts with specific APOBEC3 proteins in vivo, we coexpressed full-length HIV-1 or MLV Gag fused to GST, as well as HA epitope-tagged forms of hA3G, hA3F, hA3B and mA3, in 293T cells. After 48 h, cell lysates were incubated with glutathione Sepharose beads to recover protein complexes. After extensive washing, proteins bound to the beads were analyzed by Western blotting using antibodies specific for GST or the HA tag. As shown in Fig. 5A and B, none of the APOBEC3 proteins bound to GST while hA3G, hA3F, hA3B, and mA3 all bound to the HIV-1 Gag-GST fusion protein, as expected. In contrast, while hA3G and hA3B interacted specifically with the MLV Gag-GST fusion protein, no binding of mA3 or hA3F to MLV Gag was detected (Fig. 5A and B). Therefore, the incorporation of APOBEC proteins into MLV virions (Fig. 4) correlates with their ability to bind MLV Gag specifically (Fig. 5A and B).

Binding of hA3G by MLV Gag is mediated by the NC domain and requires RNA. As noted above, incorporation of hA3G into HIV-1 virion particles is mediated by the NC domain of HIV-1 Gag (1, 6, 14, 19, 28). MLV Gag also contains an ~10-kDa NC domain, which is located at the carboxy terminus of the Gag polyprotein. To test whether the NC domain of MLV Gag is also necessary and sufficient for specific binding

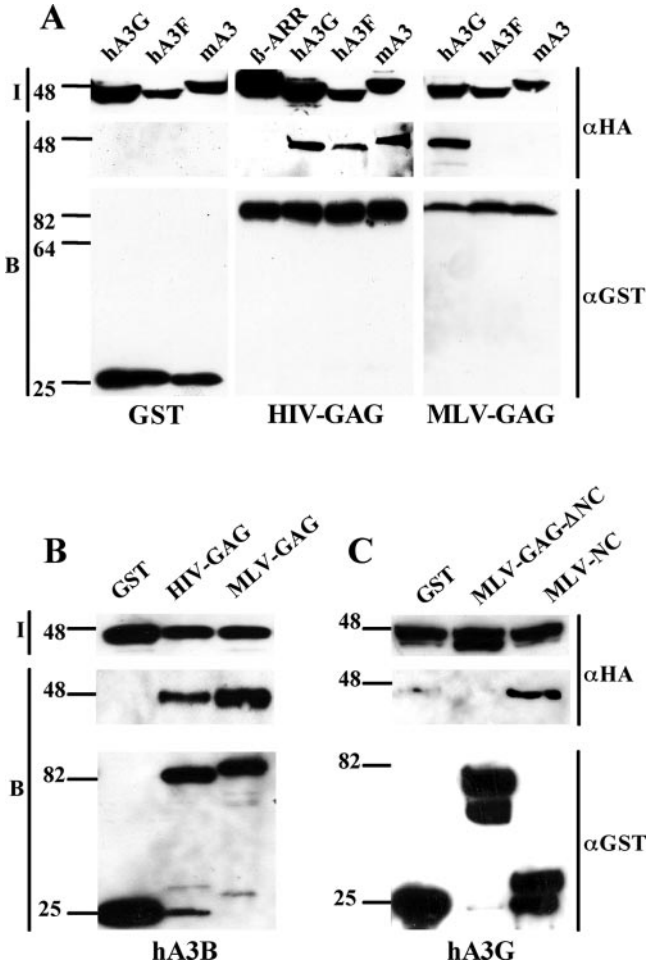

FIG. 5. The MLV Gag protein binds to hA3G and hA3B, but not to mA3 or hA3F. (A) 293T cells were cotransfected with plasmids expressing wild-type GST, or GST fused to the full-length HIV-1 or MLV Gag protein, together with plasmids expressing HA-epitope-tagged forms of hA3G, hA3F, or mA3 or the cytoplasmic β-ARR2 protein as a negative control. After cell lysis, GST proteins were collected by incubation with glutathione Sepharose beads and coprecipitated proteins were detected by Western analysis using an antibody specific for the HA-epitope tag (αHA). Controls include Western analysis of the bound beads using a GST-specific antiserum (αGST) and Western analysis of the input cell lysate, prior to incubation with the glutathione beads, using an HA-tag-specific antiserum. (B) Similar to panel A, except that binding of hA3B-HA to GST or to GST fused to full-length HIV-1 or MLV Gag was analyzed. (C) Similar to panel A, except that the binding of hA3G-HA to fusion proteins consisting of GST linked to the MLV Gag NC domain (MLV-NC), or to the MLV Gag polyprotein precisely lacking the carboxy-terminal NC domain (MLV-GAG-ΔNC), was analyzed. I, input proteins; B, bound proteins.

to hA3G in vivo, we expressed fusion proteins consisting of GST linked either to the MLV Gag NC domain alone or to a deletion mutant of MLV Gag (GAGΔNC) precisely lacking the carboxy-terminal NC domain but retaining all other MLV Gag domains, including matrix, p12, and capsid. As shown in Fig. 5C, we detected a specific interaction of the MLV NC-GST fusion protein with hA3G while the MLV GAGΔNC-GST fusion protein failed to bind hA3G detectably in vivo We therefore conclude that hA3G binding to MLV Gag, like hA3G binding to HIV-1 Gag, is mediated by the NC domain.

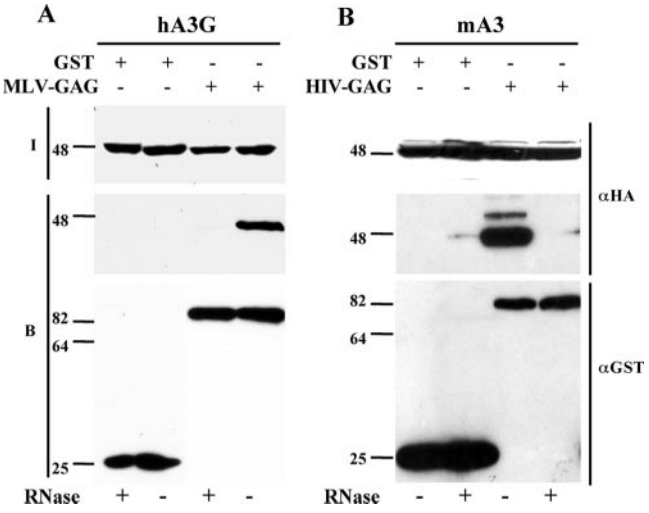

FIG. 6. Binding of hA3G by MLV Gag, and of mA3 by HIV-1 Gag, requires RNA. This analysis was performed as described in Fig. 5, except that the lysates were incubated for 15 min at 37°C, either in the presence or in the absence of RNase A, prior to binding to the glutathione Sepharose beads.

A key function for the Gag NC domain in the retroviral life cycle is to bind to the viral RNA genome and to recruit it into progeny virion particles. Interestingly, the interaction of hA3G with the HIV-1 Gag NC domain requires the presence of RNA, although nonspecific(i.e., cellular) RNA appears to function as well as viral RNA (19, 28). The role of this RNA in mediating the HIV-1 NC-hA3G interaction remains unknown, and it remains possible that it simply serves as a bridge between two proteins that can bind RNA nonspecifically.

To test whether RNA also plays a role in mediating hA3G binding to MLV Gag, we subjected lysates of cells coexpressing MLV-Gag-GST and hA3G-HA to extensive digestion with RNase A prior to addition of the glutathione beads. As shown in Fig. 6A, RNase A digestion indeed blocked the ability of hA3G to interact with the MLV Gag protein. Similarly, as shown in Fig. 6B, RNase A digestion also blocked the ability of HIV-1 Gag to bind the mA3 protein. Therefore, RNA may be a general requirement for Gag binding by APOBEC3 proteins. These data, together with the finding that MLV Gag does not interact with either hA3F or mA3, even though these bind to HIV-1 Gag at readily detectable levels (Fig. 5A), strongly argue against the hypothesis that the RNA is facilitating the Gag-APOBEC interaction by acting as a simple bridge and instead indicate that specific protein-protein interactions play a critical role in complex formation.

DISCUSSION

Although the role of the human APOBEC3 protein family as intrinsic antiretroviral resistance factors was first defined on the basis of the ability of hA3G, and subsequently hA3F, to block the infectivity of Vif-deficient HIV-1 (3, 13, 21, 24, 30), it seems unlikely that the APOBEC3 proteins originally evolved to restrict infection of HIV-1 and other lentiviruses, given that these have only very recently crossed over into the human population. Moreover, the fact that rodents also en-

code an APOBEC3 protein that is active in restricting HIV-1 infectivity (16), even though no rodent lentiviruses are known, suggests that the APOBEC3 proteins may have originally evolved to prevent the replication of other retroviral species. As the majority of retroviruses are of the simple class (i.e., do not encode Vif orthologues or any other auxiliary proteins), one obvious possibility is that the APOBEC3 proteins originally evolved to control simple retroviruses such as MLV. However, in that case, how is MLV able to replicate in mice?

Here, we demonstrate that MLV is highly sensitive to restriction by the heterologous human APOBEC3 proteins hA3G and hA3B but is resistant to inhibition by the cognate mA3 protein and by the human proteins hA3A, hA3C, and hA3F (Fig. 2 and 3). This pattern of sensitivity correlates with the finding that MLV virions package hA3G and hA3B but exclude mA3, hA3A, hA3C, and hA3F (Fig. 4). This in turn appears to be due to the fact that MLV Gag specifically interacts with hA3G and hA3B, but not mA3 or hA3F, in vivo (Fig. 5A and B). Therefore, we propose that MLV has evolved to selectively exclude the cognate mA3 protein from virion particles, thus neutralizing this intrinsic restriction pathway in its normal host species. In contrast, MLV remains fully susceptible to two human APOBEC3 proteins (i.e., hA3G and hA3B) and we hypothesize that the presence of these gene products would tend to prevent the crossover of MLV from rodents to humans as a zoonotic infection. Therefore, our data are consistent with the hypothesis that the APOBEC3 proteins may have evolved to prevent the productive infection of cells by simple and/or complex retroviruses derived from heterologous species. Unfortunately, in the case of HIV-1 and HIV-2, the chimpanzee and sooty mangabey SIV strains were able to overcome this intrinsic resistance barrier due, at least in part, to the fact that these viruses encode Vif proteins that are able to induce the degradation of not only simian but also human APOBEC3G and APOBEC3F (4, 16, 20).

An interesting question that remains unresolved is how MLV Gag distinguishes between hA3G and hA3B on the one hand and mA3 and hA3F on the other (Fig. 4 and 5). Comparison of the amino acid sequences of these proteins provides no obvious clues, as the three human proteins are all from 50% to 59% identical to each other. (hA3G and hA3F are the most dissimilar, while hA3B and hA3F are the most similar.) Moreover, all three human APOBEC3 proteins are from 32% to 34% identical to mA3 at the amino acid level. It is interesting to note, however, that mA3, like hA3F, prefers to edit the deoxycytidine, marked by an asterisk in the viral DNA target sequence TTC*, while hA3G prefers to edit CCC* (12, 13, 24). Perhaps these distinct consensus editing sequence preferences correlate with MLV Gag binding specificity.

In the case of HIV-1, hA3G inclusion into virions is mediated by the NC domain of the Gag polyprotein and is dependent on the presence of cellular RNA (1, 6, 14, 19, 28). The role of the RNA has remained unclear. However, the RNA may facilitate the formation of a specific hA3G/NC complex by, for example, inducing an appropriate conformation in one or both of these RNA binding proteins (26). Alternately, one could propose that the RNA simply serves as a bridge and that no specific protein-protein interaction is required.

In this article, we demonstrate that the NC domain of MLV Gag is both necessary and sufficient for specific binding to hA3G in vivo (Fig. 5C) and that the interaction of full-length MLV Gag with hA3G is RNA dependent (Fig. 6A). While these characteristics are therefore shared between HIV-1 and MLV Gag (1, 6, 14, 19, 28), MLV Gag differs in that it is unable to bind to two APOBEC3 proteins (i.e., mA3 and hA3F) that are fully able to interact with HIV-1 Gag (Fig. 5A). Moreover, we demonstrate that formation of the HIV-1 Gag/mA3 protein complex is again RNA dependent (Fig. 6B). Together these data clearly demonstrate that RNA is not simply acting to nonspecifically bridge retroviral Gag and host APOBEC3 proteins but rather argue that formation of the Gag/APOBEC3/RNA ternary complex is dependent on specific protein-protein interactions. How this complex is formed and how the RNA participates in complex formation remain critical issues for future investigation.

ACKNOWLEDGMENTS

The authors thank Stephen Goff for the gift of MLV constructs and antisera and Garnett Kelsoe for purified mouse T cells.

This work was supported by grants AI57099 and AI65301 from the National Institute of Allergy and Infectious Diseases.

REFERENCES

1. **Alce, T. M., and W. Popik.** 2004. APOBEC3G is incorporated into virus-like particles by a direct interaction with HIV-1 Gag nucleocapsid protein. J. Biol. Chem. **279:**34083–34086.
2. **Bieniasz, P. D.** 2004. Intrinsic immunity: a front-line defense against viral attack. Nat. Immunol. **11:**1109–1115.
3. **Bishop, K. N., R. K. Holmes, A. M. Sheehy, N. O. Davidson, S.-J. Cho, and M. H. Malim.** 2004. Cytidine deamination of retroviral DNA by diverse APOBEC proteins. Curr. Biol. **14:**1392–1396.
4. **Bogerd, H. P., B. P. Doehle, H. L. Wiegand, and B. R. Cullen.** 2004. A single amino acid difference in the host APOBEC3G protein controls the primate species specificity of HIV-1 Vif. Proc. Natl. Acad. Sci. USA **101:**3770–3774.
5. **Brownawell, A. M., and I. G. Macara.** 2002. Exportin-5, a novel karyopherin, mediates nuclear export of double-stranded RNA binding proteins. J. Cell Biol. **156:**53–64.
6. **Cen, S., F. Guo, M. Niu, J. Saadatmand, J. Deflassieux, and L. Kleiman.** 2004. The interaction between HIV-1 Gag and APOBEC3G. J. Biol. Chem. **279:**33177–33184.
7. **Chesebro, B., K. Wehrly, J. Nishio, and S. Perryman.** 1992. Macrophage-tropic human immunodeficiency virus isolates from different patients exhibit unusual V3 envelope sequence homogeneity in comparison with T-cell-tropic isolates: definition of critical amino acids involved in cell tropism. J. Virol. **66:**6547–6554.
8. **Conticello, S. G., R. S. Harris, and M. S. Neuberger.** 2003. The Vif protein of HIV triggers degradation of the human antiretroviral DNA deaminase APOBEC3G. Curr. Biol. **13:**2009–2013.
9. **Gao, G., and S. P. Goff.** 1998. Replication defect of Moloney murine leukemia virus with a mutant reverse transcriptase that can incorporate ribonucleotides and deoxyribonucleotides. J. Virol. **72:**5905–5911.
10. **Harris, R. S., K. N. Bishop, A. M. Sheehy, H. M. Craig, S. K. Petersen-Mahrt, I. N. Watt, M. S. Neuberger, and M. H. Malim.** 2003. DNA deamination mediates innate immunity to retroviral infection. Cell **113:**803–809.
11. **Kao, S., M. A. Khan, E. Miyagi, R. Plishka, A. Buckler-White, and K. Strebel.** 2003. The human immunodeficiency virus type 1 Vif protein reduces intracellular expression and inhibits packaging of APOBEC3G (CEM15), a cellular inhibitor of virus infectivity. J. Virol. **77:**11398–11407.
12. **Kobayashi, M., A. Takaori-Kondo, K. Shindo, A. Abudu, K. Fukunaga, and T. Uchiyama.** 2004. APOBEC3G targets specific virus species. J. Virol. **78:**8238–8244.
13. **Liddament, M. T., W. L. Brown, A. J. Schumacher, and R. S. Harris.** 2004. APOBEC3F properties and hypermutation preferences indicate activity against HIV-1 *in vivo.* Curr. Biol. **14:**1385–1391.
14. **Luo, K., B. Liu, Z. Xiao, Y. Yu, X. Yu, R. Gorelick, and X.-F. Yu.** 2004. Amino-terminal region of the human immunodeficiency virus type 1 nucleocapsid is required for human APOBEC3G packaging. J. Virol. **78:**11841–11852.
15. **Mangeat, B., P. Turelli, G. Caron, M. Friedli, L. Perrin, and D. Trono.** 2003. Broad antiretroviral defence by human APOBEC3G through lethal editing of nascent reverse transcripts. Nature **424:**99–103.
16. **Mariani, R., D. Chen, B. Schröfelbauer, F. Navarro, R. König, B. Bollman, C. Münk, H. Nymark-McMahon, and N. R. Landau.** 2003. Species-specific exclusion of APOBEC3G from HIV-1 virions by Vif. Cell **114:**21–31.

17. **Marin, M., K. M. Rose, S. L. Kozak, and D. Kabat.** 2003. HIV-1 Vif protein binds the editing enzyme APOBEC3G and induces its degradation. Nat. Med. **9:**1398–1403.
18. **Mehle, A., B. Strack, P. Ancuta, C. Zhang, M. N. McPike, and D. Gabuzda.** 2004. Vif overcomes the innate antiviral activity of APOBEC3G by promoting its degradation in the ubiquitin-proteasome pathway. J. Biol. Chem. **279:**7792–7798.
19. **Schäfer, A., H. P. Bogerd, and B. R. Cullen.** 2004. Specific packaging of APOBEC3G into HIV-1 virions is mediated by the nucleocapsid domain of the gag polyprotein precursor. Virology **328:**163–168.
20. **Schröfelbauer, B., D. Chen, and N. R. Landau.** 2004. A single amino acid of APOBEC3G controls its species-specific interaction with virion infectivity factor (Vif). Proc. Natl. Acad. Sci. USA **101:**3927–3932.
21. **Sheehy, A. M., N. C. Gaddis, J. D. Choi, and M. H. Malim.** 2002. Isolation of a human gene that inhibits HIV-1 infection and is suppressed by the viral Vif protein. Nature **418:**646–650.
22. **Sheehy, A. M., N. C. Gaddis, and M. H. Malim.** 2003. The antiretroviral enzyme APOBEC3G is degraded by the proteasome in response to HIV-1 Vif. Nat. Med. **9:**1404–1407.
23. **Stopak, K., C. de Noronha, W. Yonemoto, and W. C. Greene.** 2003. HIV-1 Vif blocks the antiviral activity of APOBEC3G by impairing both its translation and intracellular stability. Mol. Cell **12:**591–601.
24. **Wiegand, H. L., B. P. Doehle, H. P. Bogerd, and B. R. Cullen.** 2004. A second human antiretroviral factor, APOBEC3F, is suppressed by the HIV-1 and HIV-2 Vif proteins. EMBO J. **23:**2451–2458.
25. **Yu, Q., D. Chen, R. König, R. Mariani, D. Unutmaz, and N. R. Landau.** 2004. APOBEC3B and APOBEC3C are potent inhibitors of simian immunodeficiency virus replication. J. Biol. Chem. **279:**53379–53386.
26. **Yu, Q., R. König, S. Pillai, K. Chiles, M. Kearney, S. Palmer, D. Richman, J. M. Coffin, and N. R. Landau.** 2004. Single-strand specificity of APOBEC3G accounts for minus-strand deamination of the HIV genome. Nat. Struct. Mol. Biol. **11:**435–442.
27. **Yu, X., Y. Yu, B. Liu, K. Luo, W. Kong, P. Mao, and X.-F. Yu.** 2003. Induction of APOBEC3G ubiquitination and degradation by an HIV-1 Vif-Cul5-SCF complex. Science **302:**1056–1060.
28. **Zennou, V., D. Perez-Caballero, H. Göttlinger, and P. D. Bieniasz.** 2004. APOBEC3G incorporation into human immunodeficiency virus type 1 particles. J. Virol. **78:**12058–12061.
29. **Zhang, H., B. Yang, R. J. Pomerantz, C. Zhang, S. C. Arunachalam, and L. Gao.** 2003. The cytidine deaminase CEM15 induces hypermutation in newly synthesized HIV-1 DNA. Nature **424:**94–98.
30. **Zheng, Y.-H., D. Irwin, T. Kurosu, K. Tokunaga, T. Sata, and B. M. Peterlin.** 2004. Human APOBEC3F is another host factor that blocks human immunodeficiency virus type 1 replication. J. Virol. **78:**6073–6076.