Inhibition of HIV-1 Infection and Replication by Enhancing Viral Incorporation of Innate Anti-HIV-1 Protein A3G

A NON-PATHOGENIC Nef MUTANT-BASED ANTI-HIV STRATEGY

Linden A. Green, Ying Liu, and Johnny J. He

From the Department of Microbiology and Immunology, Center for AIDS Research, and Walther Oncology Center, Indiana University School of Medicine and Walther Cancer Institute, Indianapolis, Indiana 46206

APOBEC3G (A3G) is a cellular protein that has been identified as an innate anti-human immunodeficiency virus type 1 (HIV-1) factor. One of the major functions of HIV-1 virion infectivity protein (Vif) is to target A3G for ubiquitination/proteasome-mediated degradation and, as a result, evade the host innate defense mechanism. Thus, we wished to devise a strategy to restore the anti-HIV activity of A3G by actively targeting it into HIV-1 virions and countering HIV-1 Vif-targeted degradation. In the current study we performed a series of proof-of-concept experiments for this strategy using as a delivery vehicle of A3G, a derivate of non-pathogenic Nef mutant Nef7 that is capable of being efficiently incorporated into HIV-1 virions. We demonstrate that the Nef7.A3G fusion protein retains several important properties of Nef7; that is, the higher virion incorporation efficiency, no PAK-2 (p21-activated kinase 2) activation, and no CD4 and major histocompatibility complex class I down-regulation. Meanwhile, we show that virion incorporated Nef7.A3G possesses the anti-HIV infectivity function of A3G. Moreover, we show that virus-like particle-mediated inverse fusion delivery of Nef7.A3G into HIV-infected CD4+ T lymphocytes leads to potent inhibition of HIV-1 replication in these cells. Taken together, these results indicate that Nef7.A3G can effectively restrict HIV infection and replication by restoring the virion incorporation of A3G, even in the presence of Vif.

Received for publication, August 26, 2008, and in revised form, March 18, 2009 Published, JBC Papers in Press, March 26, 2009 DOI 10.1074/jbc.M806631200

© 2009 by The American Society for Biochemistry and Molecular Biology, Inc. Printed in the U.S.A.
A3G-derived Anti-HIV Therapeutic Strategy

**EXPERIMENTAL PROCEDURES**

**Cell Lines and Transfections**—293T and HeLa cells were purchased from American Tissue Culture Collection (ATCC, Manassas, VA). U87.CD4.CCR5 and U87.CD4.CXCR4 cells were obtained from NIH AIDS Reagent Program, which were generously donated by Dr. D. Littman. These cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 100 μg/ml penicillin and streptomycin, and 2 mM glutamine at 37 °C with 5% CO₂. 293T cells were transfected by using the standard calcium phosphate precipitation method. HeLa cells were transfected using the Lipofectamine 2000 system (Invitrogen). pcDNA3 was used throughout the studies to normalize the amounts of DNA for all transfections.

**Plasmids**—pcDNA3.1-APOBEC3G-HA and psPAX2 plasmids were obtained from NIH AIDS Research Reagent Program and were donated by Dr. W. C. Greene and Dr. D. Trono, respectively. pHLa-A2 plasmid was a kind gift from Dr. C. Toloukhian. HIV.env⁻ nef⁻ plasmid was generously provided by Dr. M. Emmerman. HIV.env⁻ vif⁻ plasmid has been previously described (34). HIV-Luc, VSV-G, HXB2.env, YU2.env, and 293T cells were prepared as previously described (35). Briefly, 293T cells were transfected with plasmids HIV-Luc or HIV-luc, VSV-G, HXB2.env, or YU2.env and Nef or each of the Nef derivatives by the calcium phosphate precipitation method. Cell culture supernatants were collected 72 h after transfection, filtered, and saved as progeny viruses. Progeny viruses were assayed for RT activity. Viruses with an equal level of RT activity were incubated with target cells at 37 °C for 2 h followed by repeated washes with fresh medium to remove the remaining viruses. Infected cells were cultured further in fresh complete medium for 48 h. The cells were analyzed for viral infectivity, as measured by the luc activity assay. For infection, the amount of VSV-G-pseudotyped viruses used was only one-tenth that of YU2.env and HXB2.env-pseudotyped viruses.

**Analysis of Nef Virion Incorporation and Intracellular Delivery**—293T cells were transfected with plasmids HIV.env⁻ nef⁻ or HIV.env⁻ vif⁻ and Nef or each of the Nef derivatives. In some experiments VSV-G, HXB2.env, or YU2.env plasmid was also included. Cell culture supernatants were collected 72 h after transfection, cleared of cell debris, and used as progeny viruses. Progeny viruses were assayed for RT activity as previously described (35). Viruses with an equal level of RT activity were lysed in radioimmune precipitation assay buffer (1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS in phosphate-buffered saline (PBS)), electrophoresed on 12% SDS-PAGE gel, and analyzed by immunoblotting. The blots were probed first with an anti-Myc, anti-HA (Santa Cruz Biotechnologies Inc., Santa Cruz, CA), or anti-HIV p24 antibody (NIH AIDS Reagent Program), then with appropriate horseradish peroxidase-conjugated secondary antibodies and were visualized with a homemade ECL system. Relative levels of protein incorporation into virions were determined by densitometric scanning of the blots and calculated using HIV-1 p24 as an internal standard. To determine delivery of virion-incorporated Nef-GFP fusion proteins, HIV.env⁻ nef⁻ or HIV.env⁻ vif⁻ virions containing Nef-GFP or Nef7-GFP were prepared by transfection as stated above. The viruses were used to infect 293T cells via spinoculation as described (32). Briefly, the viruses were allowed to adsorb onto cells by 1 h of centrifugation at 150 × g at room temperature followed by incubation at 37 °C for 3 h. Cells were then trypsinized to remove cell-surface-bound viruses and analyzed for GFP-positive cells using a FACS Calibur II.

**Preparation of Cell Lysates, Immunoprecipitation/Western Blot Analysis, and in Vitro Kinase Assay**—Cells were washed twice with ice-cold PBS. Cells were pelleted and lysed in radioimmune precipitation assay buffer and 1× protease inhibitor mixture (Roche Diagnostics), and cell lysates were obtained by centrifugation and removal of the cell debris. For immunopre-
Using two-tailed Student’s t test, we determined whether virion incorporation of WT Nef, NefV153L, NefE177G, or Nef7 was significantly different from that of WT Nef. Compared with WT Nef, NefE7 showed 9-fold higher virion incorporation (Fig. 1B). The WT Nef value was set as 1.

**RESULTS**

**Enhanced Nef7 Incorporation into HIV-1 Virions**—To test our hypothesis that A3G fusion to Nef7 (Nef7.A3G) can restore the virion incorporation of A3G and inactivate HIV-1 infectivity in the presence of HIV-1 Vif expression, we first decided to ascertain the high virion incorporation property of Nef7. We constructed Nef and Nef7 mutants in which V152L and E177G point mutations were introduced and compared their ability to be incorporated into HIV-1 virions. We then transfected 293T cells with an env- and nef-defective HIV-1 (HIV.env- nef-“) and wild-type Nef (WT Nef) or Nef 7. We also constructed and transfected two single point Nef7 mutants, V153L and E177G, in these experiments. Viruses were collected 72 h post-transfection and assayed for RT activity. Viruses with equal levels of RT activity were pelleted and analyzed using a Western blot (WB) assay with an antibody against the Myc epitope that was added to the C terminus of all Nefs and an antibody against HIV-1 p24 antigen (A). The relative (Rel.) level of virion protein from three independent experiments was determined by densitometry and normalized to the virion p24 level (B). The WT Nef value was set as 1.

**Efficient Delivery of Virion Nef7 into Cells**—We next determined whether virion incorporated Nef would be efficiently delivered into target cells. We constructed Nef-GFP and Nef7-GFP fusion plasmids and transfected them into 293T cells along with HIV.env- nef-“ and VSV-G to produce GFP-labeled VSV-G-pseudotyped HIV-1. GFP expression in trans and incorporation into HIV-1 virions would allow accurate quantitation of the delivery efficiency of virion proteins into host cells. Thus,
we infected fresh 293T cells with these newly pseudotyped viruses containing an equal level of RT activity by spinoculation and analyzed the percentage of GFP+/H11001 cells in each infection (Fig. 2A). In agreement with the virion incorporation, about 5–6-fold more GFP+/H11001 cells were detected in the infections by viruses carrying Nef7-GFP than those of viruses carrying WT Nef-GFP (Fig. 2B), suggesting that virion Nef7 protein can be delivered into target cells through infection.

Higher Virion Incorporation of the Nef7.A3G Fusion Protein—
Next, we determined whether Nef7 in the form of fusion with A3G (Nef7.A3G) retained the higher virion incorporation property of Nef7. We constructed the Nef7.A3G fusion expression plasmid. We then prepared HIV.env−/nef− virus carrying WT Nef, Nef7, or A3G, as described above and compared Nef7 and Nef7.A3G for their incorporation by Western blot analysis. We also included WT Nef and A3G as controls in these experiments. We performed Western blot analysis to ensure a comparable expression level of all proteins (Fig. 3A). Quantification of virion protein incorporation showed that Nef7 and Nef7.A3G had comparable levels in HIV-1 virions (Fig. 3B). As Nef is a virion protein, it was detected in the virus blot. On the other hand, Vif expression greatly diminishes encapsidation of A3G in the virus particles, and as a result, only a very faint band of A3G appeared in the virus blot. These results indicate that Nef7.A3G fusion does not alter the higher incorporation of Nef7 into HIV-1 virions.

No PAK-2 Activation by the Nef7.A3G Fusion Protein—
Activation of PAK-2 is an important mechanism whereby Nef exerts its pathogenic function on the host (39). Nef7 does not activate PAK-2 (31). Therefore, we determined whether the Nef7.A3G fusion led to PAK-2 reactivation. We transfected 293T cells with HIV.env−/nef− virus carrying WT Nef, Nef7, or A3G, as described above and compared Nef7 and Nef7.A3G for their incorporation by Western blot analysis. We also included WT Nef and A3G as controls in these experiments. We performed Western blot analysis to ensure a comparable expression level of all proteins (Fig. 3A). Quantification of virion protein incorporation showed that Nef7 and Nef7.A3G had comparable levels in HIV-1 virions (Fig. 3B). As Nef is a virion protein, it was detected in the virus blot. On the other hand, Vif expression greatly diminishes encapsidation of A3G in the virus particles, and as a result, only a very faint band of A3G appeared in the virus blot. These results indicate that Nef7.A3G fusion does not alter the higher incorporation of Nef7 into HIV-1 virions.

No PAK-2 Activation by the Nef7.A3G Fusion Protein—
Activation of PAK-2 is an important mechanism whereby Nef exerts its pathogenic function on the host (39). Nef7 does not activate PAK-2 (31). Therefore, we determined whether the Nef7.A3G fusion led to PAK-2 reactivation. We transfected 293T cells with HIV.env−/nef− virus carrying WT Nef, Nef7, or A3G, as described above and compared Nef7 and Nef7.A3G for their incorporation by Western blot analysis. We also included WT Nef and A3G as controls in these experiments. We performed Western blot analysis to ensure a comparable expression level of all proteins (Fig. 3A). Quantification of virion protein incorporation showed that Nef7 and Nef7.A3G had comparable levels in HIV-1 virions (Fig. 3B). As Nef is a virion protein, it was detected in the virus blot. On the other hand, Vif expression greatly diminishes encapsidation of A3G in the virus particles, and as a result, only a very faint band of A3G appeared in the virus blot. These results indicate that Nef7.A3G fusion does not alter the higher incorporation of Nef7 into HIV-1 virions.

No PAK-2 Activation by the Nef7.A3G Fusion Protein—
Activation of PAK-2 is an important mechanism whereby Nef exerts its pathogenic function on the host (39). Nef7 does not activate PAK-2 (31). Therefore, we determined whether the Nef7.A3G fusion led to PAK-2 reactivation. We transfected 293T cells with HIV.env−/nef− virus carrying WT Nef, Nef7, or A3G, as described above and compared Nef7 and Nef7.A3G for their incorporation by Western blot analysis. We also included WT Nef and A3G as controls in these experiments. We performed Western blot analysis to ensure a comparable expression level of all proteins (Fig. 3A). Quantification of virion protein incorporation showed that Nef7 and Nef7.A3G had comparable levels in HIV-1 virions (Fig. 3B). As Nef is a virion protein, it was detected in the virus blot. On the other hand, Vif expression greatly diminishes encapsidation of A3G in the virus particles, and as a result, only a very faint band of A3G appeared in the virus blot. These results indicate that Nef7.A3G fusion does not alter the higher incorporation of Nef7 into HIV-1 virions.
Both CD4 and MHC I down-regulation (31). We then attempted to confirm the diminished pathogenic phenotype of Nef7 and to determine whether this activity was present in the Nef7.A3G fusion protein by taking advantage of a system that was previously validated to study Nef-mediated MHC I down-regulation (41). We adapted this system to determine the relationship between cell surface expression of CD4 and MHC I and expression of Nef or its derivatives. We transfected HeLa cells with plasmids expressing CD4, GFP, and each of the Nefs using Lipofectamine. After 24 h we performed cell surface immunofluorescence staining using an anti-CD antibody and a phycoerythrin-conjugated secondary antibody followed by fluorescence-activated cell sorter for CD4+ cells among all GFP+ cells. We also performed similar experiments with MHC I (Fig. 5B). As expected, WT Nef down-regulated both CD4 and MHC I expression (Fig. 5, C and D). In contrast, Nef7 and Nef7.A3G showed no significant changes in the surface expression of these two receptors. These results show that both Nef7 and Nef7.A3G do not alter the cell surface expression of CD4 and MHC I.

**Impaired HIV-1 Infectivity by Nef7.A3G Virion Incorporation**—Nef7.A3G was detected in HIV-1 virions in the context of HIV-1 Vif expression (Fig. 3). It did not activate PAK-2 (Fig. 4) or down-regulate CD4 and MHC I expression (Fig. 5). These findings led us to determine the effect of Nef7.A3G on the HIV-1 infectivity. We transfected 293T cells with HIV-Luc or HIVΔvif, VSV-G, and Nef or each of the Nef derivatives to produce VSV-G-pseudotyped HIV-Luc viruses carrying each of the Nefs. HIVΔvif has no functional env or vif gene, whereas HIV-Luc contains no functional HIV-1 env or nef gene and has the Luc reporter gene inserted at the 5′ end of the nonfunctional nef gene; it allows in trans complementation of other viral envelopes to determine the viral tropism and only single round infection for accurate determination of HIV-1 entry (35). VSV-G envelope was used to facilitate HIV-1 infection of cells that do not usually express HIV-1 receptor CD4 and chemokine receptors CXCR4 or CCR5. To ensure the specificity of Nef7.A3G, we also constructed and included two Nef7.A3G point mutants as controls, Nef7.A3G/D128K and Nef7.A3G/E259Q, in these experiments. Both point mutations were made in the A3G portion of the fusion protein. D128K mutation does not bind to HIV-1 Vif protein and renders the A3G protein Vif-resistant (6, 9), whereas E259Q mutation within the second deaminase domain leads to enzymatically inactive A3G (42). In addition, we also included A3G alone in the transfection, and we performed all infections with both HIV-Luc. We collected viruses from transfected cells, harvested these transfected cells for cell lysates, and performed Western blot analysis using an anti-HA antibody on the cell lysate and virus of both the HIV-Luc (Fig. 6A) and HIVΔvif (Fig. 6B) transfections. As expected, due to differential sensitivity to Vif-mediated degradation, A3G had the lowest level in the cells, whereas Nef7-A3G(D128K) had the highest level in the cells. The expression levels of both Nef7.A3G and Nef7.A3G(E259Q) were found to be between A3G and Nef7.A3G(D128K), suggesting that Nef fusion had made A3G less sensitive to Vif-mediated degradation. As a result, Nef7.A3G(D128K) was incorporated slightly more into viruses than Nef7, Nef7.A3G, or Nef7A3G(E259Q). Compared with the above findings, lack

**Down-regulation of CD4 and MHC I by Nef7.A3G**—Another key function of Nef is the down-regulation of cell surface expression of various membrane proteins, the most well known being CD4 and MHC I (40). Nef7 is reported to be defective in both CD4 and MHC I down-regulation (31). We then
of Vif expression (and, therefore, Vif-mediated degradation) resulted in a similar level of detection of A3G and the three A3G fusion proteins in both the cells and the viruses. On the other hand, lack of Vif expression did not alter the fact that Nef7 was always detected in virions much more than its wild-type counterpart Nef. Taken together, these results indicate that Nef7-A3G is partially Vif-resistant but not to the same extent as Nef7-A3G(D128K), possibly due to structural constraints on the binding of Vif to the fusion protein. However, the extent to which the levels of Nef7-A3G and Nef7-A3G(D128K) differ between cells and viruses suggests that the slight resistance of Nef7-A3G to Vif is not solely responsible for its higher virion incorporation. Meanwhile, we used virus collected from the HIV-Luc transfections to infect fresh 293T cells. As expected, viruses carrying WT Nef and Nef7 and HIV-Luc viruses collected from A3G-transfected cells showed a level of infectivity similar to that of pcDNA3 control viruses, as measured by the Luc activity (Fig. 6C). Compared with viruses carrying WT Nef or Nef7, HIV-Luc viruses carrying Nef7.A3G showed a marked decrease in infectivity. Moreover, the infectivity of the viruses carrying the Vif-resistant D128K mutant was further decreased over that of its counterpart Nef7.A3G, whereas the viruses carrying the nonfunctional A3G mutant E259Q showed little inhibition. To ensure the functionality of all the A3G derivatives and the non-functionality of the E259Q mutant, we constructed HIV-Luc.vif−/H11002, which contains a stop codon at the beginning of the vif gene, rendering it nonfunctional, and included it as a control in the infectivity experiments. The HIV-Luc.vif− viruses containing Nef7.A3G, Nef7.A3G(D128K) and A3G showed complete inhibition of infection, similar to that observed in the HIV-Luc viruses containing the Vif-resistant Nef7.A3G(D128K) mutant. On the other hand, the inactive Nef7.A3G(E259Q) mutant had little effect on infectivity regardless of Vif expression. These results show that although Nef7.A3G virion incorporation does not restrict HIV-1 infection to the same extent as A3G does in the absence of Vif, as
demonstrated by the Nef7.A3G(D128K) mutation, it does significantly impair HIV-1 infectivity, even in the presence of Vif.

To further corroborate these findings, we prepared HXB2.env- and YU2.env-pseudotyped HIV-Luc reporter viruses carrying WT Nef, Nef7, Nef7.A3G, or A3G, used HXB2.env pseudotyped viruses to infect U87.CD4.CXCR4 cells or YU2.env-pseudotyped viruses to infect U87.CD4.CCR5 cells, and determined the infectivity. Similar to the results obtained using VSV-G-pseudotyped HIV-Luc viruses, Nef7.A3G-containing viruses showed a significantly lower level of viral infection than all others (Fig. 7). Moreover, we also infected U87.CD4.CXCR4 and U87.CD4.CCR5 cells with VSV-G-pseudotyped viruses carrying the respective proteins and obtained similar results (data not shown).

**Block of HIV-1 Replication by Nef7.A3G-containing VLPs**

CD4+ T lymphocytes are natural target cells for HIV-1 infection. Thus, we next determined the feasibility of using Nef7.A3G to target and inhibit HIV-1 replication in Jurkat cells, a CD4+ human T lymphocyte cell line commonly used for HIV-1 infection. To ensure efficient delivery of Nef7.A3G into HIV-1-infected cells, we took advantage of the VLPs-based inverse fusion strategy (43). We transfected 293T cells with an HIV-based packaging vector psPAX2 (36), Nef7.A3G, CD4, and CXCR4 and collected the cell culture supernatants as the VLPs. We also included WT Nef, Nef7, and A3G to produce control VLPs. CD4 and CXCR4 expression and presentation on the outer viral membrane allows recognition and binding to the gp120 that is expressed on the cell surface of HIV-1-infected cells, resulting in fusion of VLPs with these cells and delivery of virion components within VLPs into these cells (43). We infected Jurkat cells with replication-competent HIV-1 HXB2 viruses and exposed the infected cells to each of the VLPs. We then monitored HIV-1 infection and replication in these cells. Notably, treatment of HIV-infected Jurkat cells with VLPs that were derived from Nef7.A3G transfection gave rise to little viral replication (Fig. 8). In contrast, treatment of VLPs derived from cells expressing WT Nef, Nef7, and A3G all showed similar viral replication kinetics to that of VLPs from pcDNA3 control. Based on the number of cells that were inoculated with HXB2 virus (10^6) and the estimated initial infection efficiency (20–25%), the antiviral activity of the Nef7.A3G-containing VLP was in the range of 2–2.5 x 10^5-fold. These results suggest that Nef7.A3G-containing VLPs expressing CD4 and CXCR4 can successfully target HIV-infected cells and inhibit virus replication in HIV-1-infected Jurkat cells.

**DISCUSSION**

In this study we demonstrate that Nef7.A3G incorporated into HIV-1 virions (Fig. 3). We further show that the Nef7.A3G fusion protein fails to activate PAK2 (Fig. 4) or to down-regulate CD4 or MHC I (Fig. 5). Importantly, we show that the Nef7.A3G fusion protein retains the anti-HIV activity of A3G even in the context of HIV-1 Vif expression (Figs. 6 and 7). Furthermore, we show that Nef7.A3G-containing VLPs potently inhibit HIV-1 replication (Fig. 8). Nef7 has been previously shown to incorporate into viruses at an estimated 1100
molecules per virion, as compared with the estimated 10 molecules of WT Nef per virion, although this difference may not translate linearly to detection on Western blots, and the study did not determine fold incorporation by densitometry analysis of the Western blots (32). Using densitometry analysis, our results showed higher virus incorporation of Nef7 than WT Nef by about 9-fold. Our results also show that Nef7.A3G maintains the higher virion incorporation property of Nef7. A3G by itself was used as a control and, as expected, failed to encapsidate efficiently. A very recent study has shown that fusion of A3A, a Vif-resistant member of the APOBEC3 family that does not restrict HIV-1 infectivity, to the HIV-1 protein Vpr resulted in the alteration of its virion localization from the matrix to the viral core, which granted Vpr.A3A anti-HIV activity (44). A second very recent study showed that fusion of A3G to a virion-targeting peptide derived from Vpr (R88-A3G) resulted in restriction of HIV-1 infectivity (45). This fusion protein is susceptible to Vif-mediated degradation, and the higher virion incorporation is due to increased targeting to the virion by the remaining intracellular R88-A3G. Compared with Vpr, Nef7 is incorporated into virion at a higher level (32, 46). In addition, the higher level of virion incorporation of Nef7 as compared with WT Nef offers Nef7 an advantage over the native HIV-1 Nef protein for virion incorporation. In contrast, Vpr.A3A and R88-A3G are likely to be equally efficient as native HIV-1 Vpr protein in virion incorporation. This is very important when the anti-viral activity of these chimeras is evaluated in the context of HIV-1 infected cells. Furthermore, our results showed that Nef7.A3G had anti-HIV activity in both replication-defective single round HIV infection and the HIV-1 replication assay. However, whether Vpr.A3A would be an effective anti-HIV agent in the context of HIV-1 replication is not established.

Aside from its virion incorporation, a key property of Nef7 that allows it to be used as a carrier for therapeutic proteins is its relative non-toxicity. Although Nef is the most important pathogenic factor of HIV-1, Nef7 has been shown to be defective for a number of key Nef properties. One of the best known characteristics of Nef is its ability to activate the cellular kinase PAK-2, and disease progression in macaques has been associated with reversion of Nef mutants to a PAK-2-activating phenotype (47). Previous work has shown that Nef7 does not activate PAK-2 (31), and our data support that conclusion. In an in vitro kinase assay, both Nef7 and the Nef7.A3G fusion protein failed to activate PAK-2, confirming that Nef7 is less pathogenic than WT Nef and that fusion of A3G to Nef7 does not alter this phenotype. This loss of function does not appear to correlate with a loss of Nef7 binding to PAK-2, as PAK-2 is detected in immunoprecipitates of both Nef7 and Nef7.A3G. Likewise, the expression of Nef7 does not affect the intracellular levels of PAK-2. Taken together, these data suggest that the loss of PAK-2 activation in Nef7 is due to an inability of bound Nef7 to activate PAK-2. Our conclusion is in agreement with the study in which the Nef residues 85, 89, 187, 188, and 191 are found to be critical for Nef binding to PAK-2 (37), as none of these residues are affected in Nef7 as compared with WT Nef.

A second well known property of Nef, the down-regulation of surface molecules such as CD4 and MHC I, has also been shown to be defective in Nef7 (31). Our results show that in HeLa cells WT Nef down-regulates both CD4 and MHC I, with MHC I down-regulation being much more efficient. Both Nef7 and Nef7.A3G show no significant decrease in the surface expression of either CD4 or MHC I. Nef7 had previously been shown to down-regulate CD4 slightly (31) although much less effi-
A3G-derived Anti-HIV Therapeutic Strategy

Further characterization of the molecular mechanism responsible for Nef7’s higher virion incorporation and the structure-function relationship of Nef7 shall surely help fine-tune this strategy to minimize any residual pathogenic activity of Nef7 and to maximize the benefits of this novel strategic anti-HIV platform for its potential clinical translation.

Acknowledgments—We thank Dr. W. C. Green for pcDNA3.1-APOBEC3G-HA, Dr. D. Trono for psPAX2, Dr. C. Touloukian for pHLA-A2, Dr. M. Emmerman for HIV.env nef, Dr. J. Blum for anti-MHC I antibody, and Dr. D. Littman for U87.CD4.CXCR4 and U87.CD4.CCR5 cells.

REFERENCES

1. Simon, J. H., Gaddis, N. C., Foucher, R. A., and Malim, M. H. (1998) Nat. Med. 4, 1397–1400
2. Sheehy, A. M., Gaddis, N. C., Choi, J. D., and Malim, M. H. (2002) Nature 418, 646–650
3. Harris, R. S., Bishop, K. N., Sheehy, A. M., Craig, H. M., Petersen-Mahrt, S. K., Watt, I. N., Neuberger, M. S., and Malim, M. H. (2003) Cell 113, 803–809
4. Miyagi, E., Opi, S., Takeuchi, H., Khan, M., Goila-Gaur, R., Kao, S., and Strehbl, K. (2007) J. Virol. 81, 13346–13353
5. Mehle, A., Goncalves, J., Santa-Marta, M., McPike, M., and Gabuzda, D. (2004) Genes Dev. 18, 2861–2866
6. Huthoff, H., and Malim, M. H. (2007) J. Virol. 81, 3807–3815
7. Marin, M., Rose, K. M., Kozak, S. L., and Kabat, D. (2003) Nature Med. 9, 1398–1403
8. Sheehy, A. M., Gaddis, N. C., and Malim, M. H. (2003) Nat. Med. 9, 1404–1407
9. Zhang, L., Saadatmand, J., Li, X., Guo, F., Niu, M., Jiang, J., Kleiman, L., and Cen, S. (2008) Virology 370, 113–121
10. Wolf, D., Giese, S. I., Witte, V., Krautkramer, E., Trapp, S., Ghezzi, S. L., and Daniel, M. D., and A3G in the presence or absence of Vif and analyzed both cellular expression levels and virion incorporation. Nef7.A3G(D128K) had the highest level of intracellular expression followed by Nef7.A3G and Nef7.A3G(E259Q).
11. Giese, S. I., Woerz, L., Homann, S., Tilbroni, N., Geyer, M., and Fackler, O. T. (2006) Virology 355, 175–191
12. Cullen, B. R. (1998) Cell 93, 685–692
13. Mariani, R., Kirchhoff, F., Greenough, T. C., Sullivan, J. L., Desrosiers, R. C., and Skowronska, J. (1996) J. Virol. 70, 7752–7764
14. Jamieson, B. D., Cullen, B. R. (1998) J. Virol. 72, 6742–6748
15. Daniel, M. D., and Desrosiers, R. C. (1991) Cell 65, 651–662
16. Crotti, A., Neri, F., Corti, D., Ghezzi, S., Heltai, S., Baur, A., Poli, G., Santagostino, E., and Vicenzi, E. (2006) J. Virol. 80, 10663–10674
17. Swigut, T., Shohdy, N., and Skowronska, J. (2001) EMBO J. 20, 1593–1604
18. Koncz, S., Michel, N., Allespach, L., Fackler, O. T., and Keppler, O. T. (2006) J. Virol. 80, 11141–11152
19. Daniel, M. D., and Desrosiers, R. C. (1991) Cell 65, 651–662
20. Crotti, A., Neri, F., Corti, D., Ghezzi, S., Heltai, S., Baur, A., Poli, G., Santagostino, E., and Vicenzi, E. (2006) J. Virol. 80, 10663–10674
21. Swigut, T., Shohdy, N., and Skowronska, J. (2001) EMBO J. 20, 1593–1604
22. Koncz, S., Michel, N., Allespach, L., Fackler, O. T., and Keppler, O. T. (2006) J. Virol. 80, 11141–11152
23. Daniel, M. D., and Desrosiers, R. C. (1991) Cell 65, 651–662
24. Crotti, A., Neri, F., Corti, D., Ghezzi, S., Heltai, S., Baur, A., Poli, G., Santagostino, E., and Vicenzi, E. (2006) J. Virol. 80, 10663–10674
25. Crotti, A., Neri, F., Corti, D., Ghezzi, S., Heltai, S., Baur, A., Poli, G., Santagostino, E., and Vicenzi, E. (2006) J. Virol. 80, 10663–10674
26. Qi, M., and Aiken, C. (2008) Virology 373, 287–297
27. de Ronde, A., Klaver, B., Keulen, W., Smit, L., and Goudsmit, J. (1992) Virology 188, 391–395

This strategy has many potential uses aside from the Nef7.A3G fusion protein. Because of its high virion incorporation, Nef7 fusion proteins can be used to target several components of the HIV virion, such as the tRNA primer for reverse transcription. Nef7 fusion proteins could also be used for novel experimental procedures in the laboratory. For example, a Nef7.GFP fusion protein could be used to track viral cores at early stages of infection, and a Nef7.luciferase fusion protein could be used as a sensitive assay for measuring virus production. Moreover, Nef fusion proteins can be adapted to deliver anti-HIV therapeutic proteins or components directly into HIV or HIV-infected cells.
A3G-derived Anti-HIV Therapeutic Strategy

28. Welker, R., Harris, M., Cardel, B., and Krausslich, H. G. (1998) *J. Virol.* 72, 8833–8840
29. Chazal, N., Singer, G., Aiken, C., Hammarskjold, M. L., and Rekosh, D. (2001) *J. Virol.* 75, 4014–4018
30. D’Aloja, P., Olivetta, E., Bona, R., Nappi, F., Pedacchia, D., Pugliese, K., Ferrari, G., Verani, P., and Federico, M. (1998) *J. Virol.* 72, 4308–4319
31. D’Aloja, P., Santarcangelo, A. C., Arolf, S., Baur, A., and Federico, M. (2001) *J. Gen. Virol.* 82, 2735–2745
32. Peretti, S., Schiavoni, I., Pugliese, K., and Federico, M. (2005) *Mol. Ther.* 12, 1185–1196
33. Peretti, S., Schiavoni, I., Pugliese, K., and Federico, M. (2006) *Virology* 345, 115–126
34. Lewis, P., Hensel, M., and Emerman, M. (1992) *EMBO J.* 11, 3053–3058
35. He, J., Chen, Y., Farzan, M., Choe, H., Ohagen, A., Gartner, S., Busciglio, J., Yang, X., Hofmann, W., Newman, W., Mackay, C. R., Sodroski, J., and Gabuzda, D. (1997) *Nature* 385, 645–649
36. Dull, T., Zufferey, R., Kelly, M., Mandel, R. J., Nguyen, M., Trono, D., and Naldini, L. (1998) *J. Virol.* 72, 8463–8471
37. Agopian, K., Wei, B. L., Garcia, J. V., and Gabuzda, D. (2006) *J. Virol.* 80, 3050–3061
38. He, J., and Landau, N. R. (1995) *J. Virol.* 69, 4587–4592
39. Sawai, E. T., Khan, I. H., Montbriand, P. M., Peterlin, B. M., Cheng-Mayer, C., and Luciw, P. A. (1996) *Curr. Biol.* 6, 1519–1527
40. Roeth, J. F., and Collins, K. L. (2006) *Microbiol. Mol. Biol. Rev.* 70, 548–563
41. Lubben, N. B., Sahlender, D. A., Motley, A. M., Lehner, P. J., Benaroche, P., and Robinson, M. S. (2007) *Mol. Biol. Cell* 18, 3351–3365
42. Schumacher, A. J., Hake, G., Macduff, D. A., Brown, W. L., and Harris, R. S. (2008) *J. Virol.* 82, 2652–2660
43. Endres, M. J., Jaffer, S., Haggarty, B., Turner, J. D., Doran, B. J., O’Brien, P. J., Kolson, D. L., and Hoxie, J. A. (1997) *Science* 278, 1462–1464
44. Aguiar, R. S., Lovsin, N., Tanuri, A., and Peterlin, B. M. (2008) *J. Biol. Chem.* 283, 2518–2525
45. Ao, Z., Yu, Z., Wang, L., Zheng, Y., and Yao, X. (2008) *PLoS ONE* 3, e1995
46. Singh, S. P., Tungaturthi, P., Cartas, M., Tomkowicz, B., Rizvi, T. A., Khan, S. A., Kalyanaraman, V. S., and Srinivasan, A. (2001) *Virology* 283, 78–83
47. Khan, I. H., Sawai, E. T., Antonio, E., Weber, C. J., Mandell, C. P., Montbriand, P., and Luciw, P. A. (1998) *J. Virol.* 72, 5820–5830