37. Esnouf R, Ren J, Ross C, Jones Y, Stammers D, Stuart D. Mechanism of inhibition of HIV-1 reverse transcriptase by non-nucleoside inhibitors. *Nat Struct Biol* 1995; **2**:303–308.

38. Spence RA, Kati WM, Anderson KS, Johnson KA. Mechanism of inhibition of HIV-1 reverse transcriptase by nonnucleoside inhibitors. *Science* 1995; **267**:988–993.

39. Das K, Lewi PJ, Hughes SH, Arnold E. Crystallography and the design of anti-AIDS drugs: conformational flexibility and positional adaptability are important in the design of non-nucleoside HIV-1 reverse transcriptase inhibitors. *Prog Biophys Mol Biol* 2005; **88**:209–231.

40. Das K, Clark AD, Jr, Lewi PJ, *et al.* Roles of conformational and positional adaptability in structure-based design of TMC125-R165335 (etravirine) and related non-nucleoside reverse transcriptase inhibitors that are highly potent and effective against wild-type and drug-resistant HIV-1 variants. *J Med Chem* 2004; **47**:2550–2560.

41. Palmer S, Margot N, Gilbert H, Shaw N, Buckheit R, Jr, Miller M. Tenofovir, adefovir, and zidovudine susceptibilities of primary human immunodeficiency virus type 1 isolates with non-B-subtypes or nucleoside resistance. *AIDS Res Hum Retroviruses* 2001; **17**:1167–1173.

42. Srinivas RV, Fridland A. Antiviral activities of 9-*R*-2-phosphonomethoxypropyl adenine (PMPA) and bis(isopropyloxymethylcarbonyl)PMPA against various drug-resistant human immunodeficiency virus strains. *Antimicrob Agents Chemother* 1998; **42**:1484–1487.

43. Tuske S, Sarafianos SG, Clark AD, Jr, *et al.* Structures of HIV-1 RT-DNA complexes before and after incorporation of the anti-AIDS drug tenofovir. *Nat Struct Mol Biol* 2004; **11**:469–474.

44. Yoshimura K, Kato R, Yusa K, *et al.* JE-2147: a dipeptide protease inhibitor (PI) that potently inhibits multi-PI-resistant HIV-1. *Proc Natl Acad Sci U S A* 1999; **96**:8675–8680.

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SOCS1 is an inducible host factor during HIV-1 infection and regulates the intracellular trafficking and stability of HIV-1 Gag

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Human immunodeficiency virus type 1 (HIV-1) utilizes the macromolecular machinery of the infected host cell to produce progeny virus. The discovery of cellular factors that participate in HIV-1 replication pathways has provided further insight into the molecular basis of virus–host cell interactions. Here, we report that the suppressor of cytokine signaling 1 (SOCS1) is an inducible host factor during HIV-1 infection and regulates the late stages of the HIV-1 replication pathway. SOCS1 can directly bind to the matrix and nucleocapsid regions of the HIV-1 p55 Gag polyprotein and enhance its stability and trafficking, resulting in the efficient production of HIV-1 particles via an IFN signaling-independent mechanism. The depletion of SOCS1 by siRNA reduces both the targeted trafficking and assembly of HIV-1 Gag, resulting in its accumulation as perinuclear solid aggregates that are eventually subjected to lysosomal degradation. These results together indicate that SOCS1 is a crucial host factor that regulates the intracellular dynamism of HIV-1 Gag and could therefore be a potential new therapeutic target for AIDS and its related disorders.

AIDS | pathogenesis | drug target | lysozyme

Human immunodeficiency virus type 1 (HIV-1) infection is a multistep and multifactorial process mediated by a complex series of virus–host cell interactions (1, 2). The molecular interactions between host cell factors and HIV-1 are vital to our understanding of not only the nature of the resulting viral replication, but also the subsequent cytopathogenesis that occurs in the infected cells (3). The characterization of the genes in the host cells that are up- or down-regulated upon HIV-1 infection could therefore provide a further elucidation of virus–host cell interactions and identify putative molecular targets for the HIV-1 replication pathway (4).

The HIV-1 p55 Gag protein consists of four domains that are cleaved by the viral protease concomitantly with virus release. This action generates the mature Gag protein comprising the matrix (MA/p17), capsid (CA/p24), nucleocapsid (NC/p7), and p6 domains, in addition to two small spacer peptides, SP1 and SP2 (5, 6). The N-terminal portion of MA, which is myristoylated, facilitates the targeting of Gag to the plasma membrane (PM), whereas CA and NC promote Gag multimerization. p6 plays a central role in the release of HIV-1 particles from PM by interacting with the vacuolar sorting protein Tsg101 and AIP1/ALIX (7–9). Several recent studies have implicated the presence of host factors in the control of the intracellular trafficking of Gag. AP-3δ is a recently characterized endosomal adaptor protein that binds directly to the MA region of Gag and enhances its targeting to the multivesicular body (MVB) during the early stages of particle assembly (10). The *trans*-Golgi network (TGN)-associated protein hPOSH plays another role in Gag transport by facilitating the egress of Gag cargo vesicles from the TGN, where it assembles with envelope protein (Env) before transport to PM (11). Although the involvement of these host proteins in the regulation of intracellular Gag trafficking has been proposed, the detailed molecular mechanisms underlying this process are still not yet well characterized.

In our current work, we demonstrate that the suppressor of cytokine signaling 1 (SOCS1) directly binds HIV-1 Gag and facilitates the intracellular trafficking and stability of this protein, resulting in the efficient production of HIV-1 particles. These results indicate that SOCS1 is a crucial host factor for efficient HIV-1 production and could be an intriguing molecular target for future treatment of AIDS and related diseases.

Results

SOCS1 Is Induced upon HIV-1 Infection and Facilitates HIV-1 Replication via Posttranscriptional Mechanisms. We and others have shown that HIV-1 infection can alter cellular gene expression patterns, resulting in the modification of viral replication and impaired homeostasis in the host cells (4, 12). Hence, to elucidate further the genes and cellular pathways that participate in HIV-1 replication processes, we performed serial analysis of gene expression (SAGE) using either a HIV-1 or mock-infected human T cell line, MOLT-4 (12). Further detailed analysis of relatively low-abundance SAGE tags identified *SOCS1* as a preferentially up-regulated gene after HIV-1 infection. This finding was validated by both semiquantitative RT-PCR and immunoblotting analysis with anti-SOCS1 anti-

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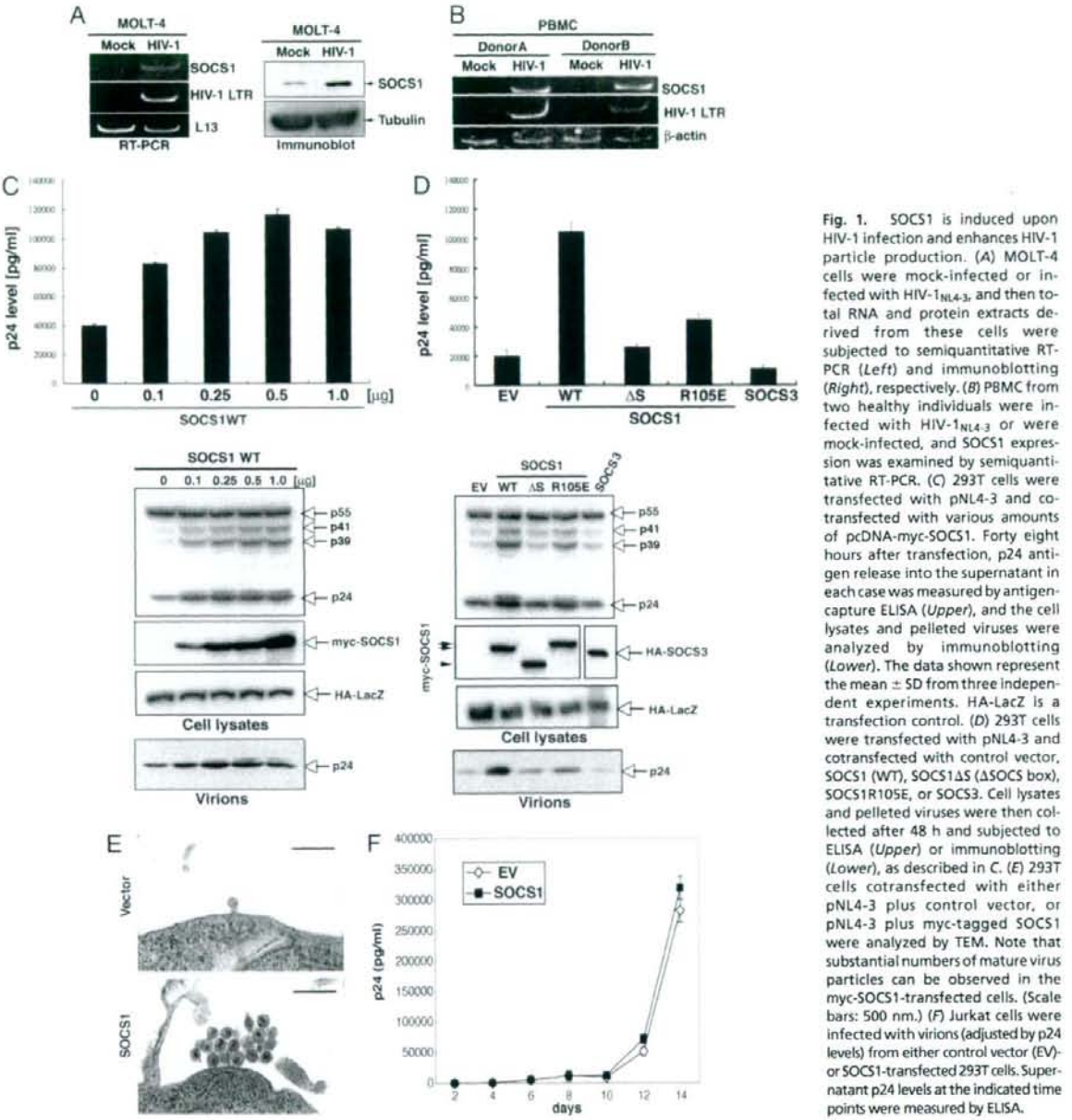

Fig. 1. SOCS1 is induced upon HIV-1 infection and enhances HIV-1 particle production. (*A*) MOLT-4 cells were mock-infected or infected with HIV-1_{NL4-3}, and then total RNA and protein extracts derived from these cells were subjected to semiquantitative RT-PCR (*Left*) and immunoblotting (*Right*), respectively. (*B*) PBMC from two healthy individuals were infected with HIV-1_{NL4-3} or were mock-infected, and SOCS1 expression was examined by semiquantitative RT-PCR. (*C*) 293T cells were transfected with pNL4-3 and cotransfected with various amounts of pcDNA-myc-SOCS1. Forty eight hours after transfection, p24 antigen release into the supernatant in each case was measured by antigen-capture ELISA (*Upper*), and the cell lysates and pelleted viruses were analyzed by immunoblotting (*Lower*). The data shown represent the mean ± SD from three independent experiments. HA-LacZ is a transfection control. (*D*) 293T cells were transfected with pNL4-3 and cotransfected with control vector, SOCS1 (WT), SOCS1ΔS (ΔSOCS box), SOCS1R105E, or SOCS3. Cell lysates and pelleted viruses were then collected after 48 h and subjected to ELISA (*Upper*) or immunoblotting (*Lower*), as described in *C*. (*E*) 293T cells cotransfected with either pNL4-3 plus control vector, or pNL4-3 plus myc-tagged SOCS1 were analyzed by TEM. Note that substantial numbers of mature virus particles can be observed in the myc-SOCS1-transfected cells. (Scale bars: 500 nm.) (*F*) Jurkat cells were infected with virions (adjusted by p24 levels) from either control vector (EV)- or SOCS1-transfected 293T cells. Supernatant p24 levels at the indicated time points were measured by ELISA.

bodies (Fig. 1*A*). In addition, *SOCS1* was found to be up-regulated also in peripheral blood mononuclear cells (PBMC) from two different individuals (following HIV infection, Fig. 1*B*).

Our initial findings that SOCS1 is induced upon HIV-1 infection prompted us to examine whether this gene product affects viral replication. We first cotransfected 293T cells with a HIV-1 infectious molecular clone, pNL4-3 (13), and also pcDNA-myc-SOCS1, and then monitored the virus production levels in the resulting supernatant. We then performed ELISA using an anti-p24 antibody and found that wild-type SOCS1 significantly increases the production of HIV-1 in the cell supernatant in a dose-dependent manner (Fig. 1*C Upper*). In contrast, neither the SH2 domain-defective mutant (R105E) nor the SOCS box deletion mutant (ΔS) of SOCS1 could promote virus production to the same levels as wild type, indicating that both domains are required for this enhancement (Fig. 1*D Upper*). Furthermore, another SOCS box protein, SOCS3, failed to augment HIV-1 replication in a parallel experiment (Fig. 1*D Upper*), indicating that the role of SOCS1 during HIV-1 replication is specific.

We next performed immunoblotting analysis using cell lysates and harvested virus particles in further parallel experiments (Fig. 1 *C* and *D Lower*). Consistent with our ELISA analysis, the expres-

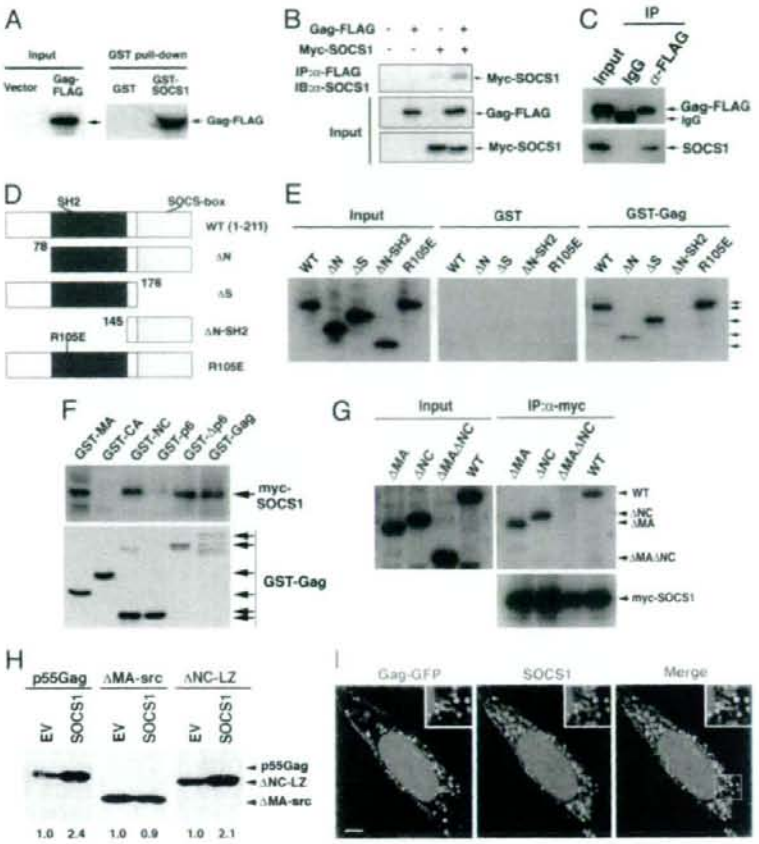

Fig. 2. SOCS1 interacts with HIV-1 Gag. (*A*) Extracts of 293T cells transfected with either empty vector or Gag-FLAG were subjected to pull-down analyses using glutathione–agarose beads with GST-SOCS1 in the presence of 10 ng/ml RNase followed by immunoblotting with anti-FLAG antibodies. (*B*) Extracts of 293T cells transiently expressing myc-SOCS1 and Gag-FLAG were subjected to immunoprecipitation (IP) with anti-FLAG monoclonal antibodies in the presence of 10 ng/ml RNase followed by immunoblotting (IB) analysis with either anti-FLAG or anti-myc polyclonal antibodies. (*C*) 293T cells were transiently transfected with Gag-FLAG, and cell lysates were then subjected to immunoprecipitation with anti-FLAG antibodies followed by immunoblotting with an antibody directed against endogenous SOCS1. (*D* and *E*) 293T cells expressing various myc-tagged SOCS1 mutants (schematically depicted in *D*) were analyzed by GST pull-down analysis with either GST or GST-Gag recombinant protein (*E*). (*F*) GST fusion proteins of the indicated regions of Gag were bound to glutathione beads and incubated with cell lysates from 293T cells expressing myc-SOCS1 in the presence of 10 ng/ml RNase followed by immunoblotting with anti-myc antibodies. (*G*) SOCS1 binds p55 Gag via either its MA or NC domains. 293T cells were transfected with myc-SOCS1 and cotransfected with Gag-FLAG, GagΔMA-FLAG, GagΔNC-FLAG, or GagΔMAΔNC-FLAG. At 24 h after transfection, cell lysates treated with 10 μg/ml RNase were subjected to coimmunoprecipitation with anti-myc monoclonal antibodies followed by immunoblotting with anti-FLAG or anti-myc polyclonal antibodies. (*H*) Functional interaction of SOCS1 with MA but not NC. 293T cells were transfected with wild-type Gag, ΔMA-*src*, or ΔNC-LZ (Z_{IL}-p6) and cotransfected with either control vector or SOCS1. Supernatant virus particles were then collected after 24 h and subjected to immunoblotting with anti-p24 antibody. Numerical values below the blots indicate fold induction of supernatant p55 signal intensities derived by densitometry. (*I*) Colocalization of SOCS1 with Gag. HeLa cells were transiently transfected with Gag-GFP. After 24 h, the cells were fixed, permeabilized, and immunostained with anti-SOCS1 polyclonal antibody followed by fluorescently labeled secondary antibodies before confocal microscopy. (Scale bar: 10 μm.)

sion of wild-type SOCS1, but neither its SH2 nor SOCS box mutant counterparts, resulted in a marked and dose-dependent increase in the level of intracellular Gag protein, particularly in the case of CA (p24) and intermediate cleavage products corresponding to MA-CA (p41) and CA-NC (p39). This increase was found to be accompanied by an enhanced level of HIV-1 particle production in the supernatant (Fig. 1 *C* and *D Lower*). These results together indicated that SOCS1 facilitates HIV-1 particle production in infected cells and that this role of SOCS1 requires the function of both its SH2 and SOCS box domains. For further details about SOCS1 interaction with MA and NC and SOCS1-enhanced particle production, see supporting information (SI) *Text*.

To examine the morphological aspects of HIV-1 particle production, transmission electron microscopy (TEM) was performed. 293T cells that had been cotransfected with pNL4-3, and either a control vector or a SOCS1 expression construct, were subjected to TEM analysis after fixation in glutaraldehyde. In SOCS1-transfected cells, a significantly increased number of mature virus particles was observed on the surfaces of PM compared with the control vector-transfected cells (Fig. 1*E*). There were also no obvious malformations of the virus particles in SOCS1-expressing cells, such as doublet formation or tethering to PM, which are characteristic of particle budding arrest (14) (Fig. 1*E*). Consistent with this observation, virions from SOCS1-transfected cells were found to be infectious as control viruses in Jurkat cells when the same amounts of virus were infected (Fig. 1*F*). These results together indicate that SOCS1 enhances mature and infectious HIV-1 particle formation.

To elucidate the specific step in HIV-1 production that is enhanced by SOCS1, we next performed gene reporter assays using either luciferase expression constructs under the control of wild-type HIV-LTR (pLTR-luc), or a full-length provirus vector (pNL4-3-luc) (15). Interestingly, SOCS1 overexpression was found not to affect the transcription of these reporter constructs (data not shown), indicating that SOCS1 enhances HIV-1 replication via posttranscriptional mechanisms during virus production.

SOCS1 Interacts with the HIV-1 Gag Protein. The results of our initial experiments indicated that SOCS1 enhances HIV-1 production via a posttranscriptional mechanism. We therefore next tested whether SOCS1 could bind directly to HIV-1 Gag. GST pull-down analysis using C-terminal FLAG-tagged p55 Gag (codon-optimized) and GST-fused SOCS1 revealed that p55 Gag undergoes specific coprecipitation with GST-SOCS1 (Fig. 2*A*). Furthermore, both ectopically expressed myc-tagged SOCS1 and endogenous SOCS1 were found to undergo coimmunoprecipitation with Gag-FLAG in 293T cells (Fig. 2 *B* and *C*). Additionally, GST pull-down analysis with various SOCS1 mutants, as depicted in Fig. 2*D*, further demonstrated that a mutant lacking the both N-terminal and SH2 domain (ΔN-SH2) could not bind

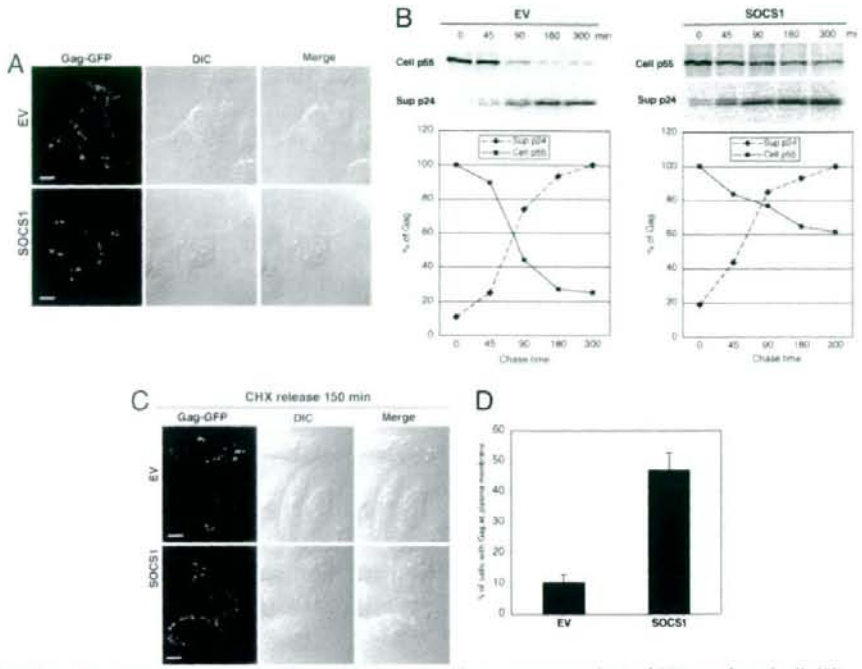

Fig. 3. SOCS1 enhances both the stability and trafficking of HIV-1 Gag. (*A*) HeLa cells cotransfected with pNL4-3 and either control vector (EV) or SOCS1 were immunostained with antibodies targeting anti-p24 (CA). Confocal microscopy with differential interference contrast (DIC) was then performed. (Scale bars: 10 μm.) (*B*) 293T cells were transfected with either a control empty vector (EV) (*Left*) or myc-SOCS1 (*Right*) and cotransfected with pNL4-3. After 48 h, cells were pulse-labeled with [^{35}S]methionine or [^{35}S]cysteine for 15 min and chased for the durations indicated. Cell lysates and pelleted supernatant virions were immunoprecipitated with anti-p24 antibodies followed by autoradiography. (*C* and *D*) HeLa cells seeded on poly-L-lysine-coated cover slides were transfected with either vector control or SOCS1. After 24 h, cells were again transfected with Gag-GFP for 3 h and then treated with 100 μg/ml CHX for 5 h to inhibit protein synthesis. This treatment was followed by incubation with fresh medium; then 150 min after the CHX release, cells were fixed and subjected to confocal microscopy (*C*). (Scale bars: 10 μm.) Cells with Gag protein on the plasma membrane were scored out of 200 transfected cells (*D*).

p55 Gag, whereas an N-terminal or a SOCS box deletion did not affect the binding of SOCS1 to Gag in 293T cells (Fig. 2*E*). This finding indicates that the SH2 domain is important for the interaction of SOCS1 with HIV-1 Gag. Interestingly, the R105E mutant of SOCS1, which disrupts the function of the SH2 domain, still binds Gag (Fig. 2*E*), indicating that the Gag–SOCS1 association is independent of the tyrosine phosphorylation of Gag, as is the case for both HPV-E7 and Vav (16, 17).

To elucidate the SOCS1-binding region of the Gag protein, GST pull-downs with various GST-fused Gag domain constructs were performed. SOCS1 was detected in glutathione bead precipitates with GST-wild-type Gag, GST-Δp6, GST-MA, and GST-NC, but not with other domain constructs (Fig. 2*F*), indicating that SOCS1 interacts with Gag via its MA and NC domains. Consistent with these results, the deletion of both the MA and NC domains of p55 Gag (ΔMAΔNC) completely abolishes its interaction with SOCS1 in coimmunoprecipitation experiments (Fig. 2*G*). Furthermore, *in vitro* analysis with purified proteins also demonstrated that SOCS1 can indeed interact with both the MA and NC regions of HIV-1 Gag in the absence of nucleic acids or other proteins (SI Fig. 5).

We next wished to determine the functional interaction domain in HIV-1 Gag through which SOCS1 functions in terms of virus-like particle production. To this end, we used a MA-deleted Gag mutant with an N-terminal myristoyl tag derived from src (ΔMA-src) (18) and also an NC-deleted Gag mutant with a GCN4 leucine zipper in place of NC, which we herein denote as ΔNC-LZ but which has been described as Z_{IL}-p6 (19). Both of these mutants have been shown still to assemble and bud (18, 19). We found that SOCS1 overexpression can still augment the particle formation of both wild-type Gag and ΔNC-LZ but not ΔMA-src (Fig. 2*H*), indicating that the functional interaction between SOCS1 and HIV-1 Gag is in fact mediated through MA.

To confirm further the direct interaction between SOCS1 and Gag in cells, we examined the intracellular localization of these two proteins. Confocal microscopy revealed that endogenous SOCS1 forms dotted filamentous structures in the cytoplasm and that Gag localizes in a very punctate pattern with SOCS1 from the perinuclear regions to the cell periphery (Fig. 2*I*). These data indicate that SOCS1 interacts with HIV-1 Gag in the cytoplasm during HIV-1 particle production.

SOCS1 Promotes both the Stability of Gag and Its Targeting to the Plasma Membrane. Because we had found from our initial data that SOCS1 increases HIV-1 particle production as a result of its direct interaction with intracellular Gag proteins, we next addressed whether SOCS1 positively regulates Gag stability and subsequent trafficking to PM. Our immunofluorescent analysis with the anti-p24 (CA) antibody initially revealed that SOCS1 overexpression increases the levels of Gag at PM when cotransfected with pNL4-3 at 48 h after transfection, although it was detected at PM in both control and SOCS1-expressing cells (Fig. 3*A*). Furthermore, the levels of cytoplasmic Gag were found to be much lower in the SOCS1-expressing cells compared with the control cells (Fig. 3*A*). These results indicate that SOCS1 enhances Gag trafficking to PM.

To examine next whether SOCS1 affects the stability and trafficking of newly synthesized Gag proteins, we performed pulse–chase analysis. This experiment revealed that SOCS1 significantly increases the stability of the intracellular p55 Gag polyprotein as well as the levels of p24 in the supernatant (Fig. 3*B*). Importantly, p24 was detectable at an earlier time point and reached maximum levels in a shorter period in the cell supernatant of SOCS1-transfected cells compared with control vector-transfected cells (Fig. 3*B*). This finding again suggests that SOCS1 facilitates the intracellular trafficking of newly synthesized Gag proteins to PM.

To confirm this hypothesis further, we performed cycloheximide (CHX) analysis with HeLa cells transfected using either vector control or SOCS1. After 24 h, cells were again transfected with Gag-GFP for 3 h and treated with CHX for 5 h to inhibit protein synthesis. Cells were then cultured in fresh medium without CHX for an additional 150 min and subjected to confocal microscopy. At

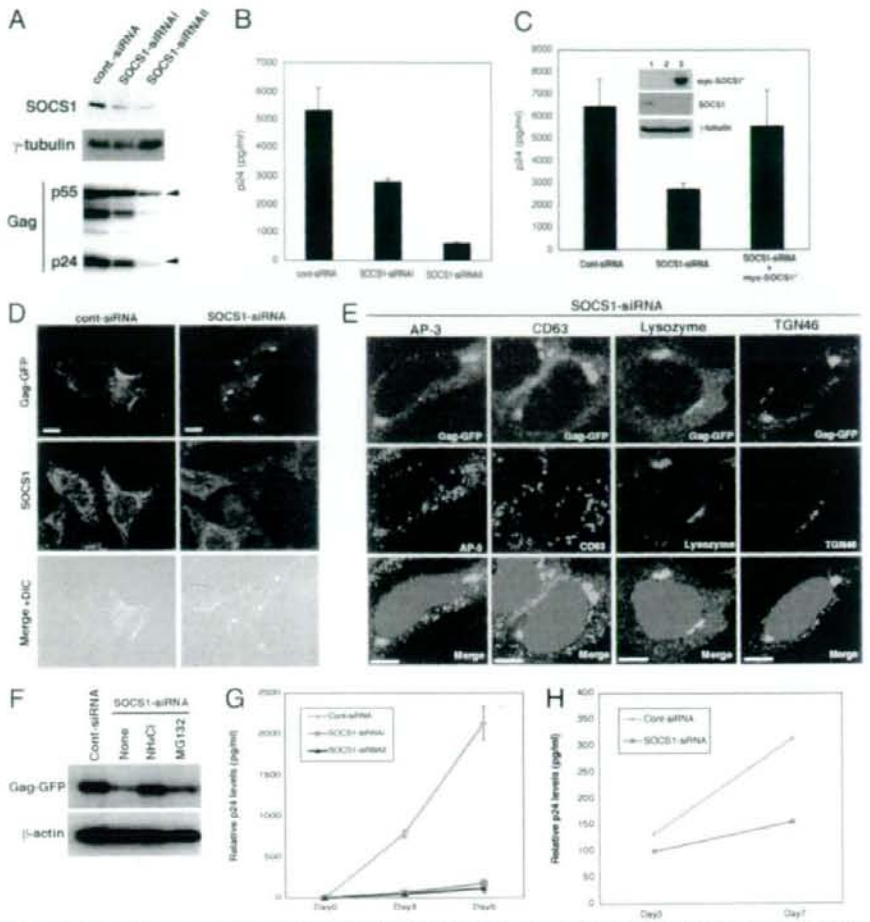

Fig. 4. The targeted inhibition of SOCS1 suppresses Gag trafficking and HIV-1 particle production and enhances Gag degradation in lysosomes. (*A* and *B*) 293T cells were transfected with either control siRNA or two different SOCS1-specific siRNAs (I or II) together with pNL4-3. At 48 h after transfection, cell lysates were subjected to immunoblotting analysis with the indicated antibodies (*A*). Cell supernatants were then subjected to ELISA analysis of p24 levels (*B*). (*C*) 293T cells were transfected with pNL4-3 and cotransfected with control-siRNA, SOCS1-siRNAI alone, or SOCS1-siRNAI plus siRNA-resistant myc-SOCS1 (myc-SOCS1*). After 48 h, cell supernatants were collected and subjected to p24 ELISA. (*Inset*) Immunoblots of the cell lysates. (*D*) HeLa cells were transfected with control or SOCS1-specific siRNA and cotransfected with GFP-Gag. At 48 h after transfection, the cells were subjected to confocal microscopy. (*E*) HeLa cells were transfected with Gag-GFP and SOCS1-siRNA constructs for 48 h. Cells were then fixed and subjected to immunofluorescent analysis with indicated antibodies followed by DAPI staining. (Scale bars: 10 mm.) (*F*) HeLa cells were transfected with Gag-GFP and cotransfected with either control-siRNA or SOCS1-siRNA. After 36 h, the cells were treated with a mock solution, 10 mM NH_4Cl or 10 μM MG132 for another 16 h. Cell were then harvested and subjected to immunoblotting analysis with anti-GFP or anti-β-actin antibodies. (*G*) Jurkat cells were infected with a retroviral vector encoding control (Cont) or two different SOCS1-specific siRNAs (I or II). After selection with puromycin, the cells were then infected with HIV-1_{NL4-3} (multiplicity of infection, 0.1), and p24 antigen levels in cell supernatant were measured by ELISA at the indicated time points. (*H*) Human primary CD4 T cells were separated from healthy donors and infected with lentivirus vectors encoding either control- or SOCS1-siRNAI. The cells were then infected with HIV-1_{NL4-3} (multiplicity of infection, 0.1), and p24 antigen levels in cell supernatant were measured by ELISA at the indicated time points.

this time point, Gag-GFP was found to localize predominantly in a perinuclear region in the control cells (Fig. 3*C*), whereas almost half of the SOCS1-transfected cells exhibited Gag-GFP localization on PM (Fig. 3*D*). These results again indicate that SOCS1 efficiently enhances the trafficking of newly synthesized Gag protein to PM.

The Targeted Disruption of SOCS1 Inhibits Gag Trafficking and HIV-1 Particle Production. To delineate further the role of SOCS1 in the trafficking of Gag and in subsequent HIV-1 particle production, we depleted cellular SOCS1 by siRNA. The significant depletion of SOCS1 expression by two different SOCS1-specific siRNA constructs was confirmed by immunoblotting analysis (Fig. 4 *A* and *B*). Significantly, in cells cotransfected with pNL4-3 and SOCS1-specific siRNAs, both HIV-1 particle release and the levels of intracellular Gag protein are significantly decreased compared with the control cells (Fig. 4 *A* and *B*). Furthermore, the effects of SOCS1-siRNA on the inhibition of HIV-1 particle production was diminished by reexpression with a codon-optimized SOCS1 construct that is resistant to these siRNAs (Fig. 4*C*), indicating that the SOCS1 siRNA suppression of HIV-1 particle production depends on the availability of endogenous SOCS1.

Consistent with these observations, immunofluorescent analysis further revealed that the expression of SOCS1-siRNA dramatically inhibits Gag trafficking such that Gag proteins accumulate in the perinuclear regions as large solid aggregates, as has been reported (20) (Fig. 4*D*). This finding indicates that SOCS1 plays an essential role in the Gag trafficking from perinuclear clusters to PM. Interestingly, these discrete perinuclear clusters of Gag were found to colocalize with lysosome markers, lysozyme, and partly with AP-3, but neither with the late endosome MVB marker CD63 nor the *trans*-Golgi marker TGN46, indicating that Gag is targeted for degradation by lysosomes when the function of SOCS1 is inhibited (Fig. 4*E*). In support of this notion, the levels of intracellular Gag were found to be significantly increased by treatment with a lysosome inhibitor NH_4Cl but not by a proteasome inhibitor MG132 in SOCS1-siRNA cells (Fig. 4*F*), further indicating that the perinuclear clusters of Gag will undergo lysosomal degradation rather than proteasomal degradation when optimal Gag transport to PM is suppressed by the inhibition of SOCS1.

We next addressed whether targeted SOCS1 inhibition would affect HIV-1 particle production in human T cells. The effect of SOCS1 depletion was clearly evident in both HIV-1_{NL4-3}-infected

Jurkat cells and human primary CD4+ T cells, which demonstrated pronounced decreases in virus particle production in SOCS1-siRNA-expressed cells compared with the controls (Fig. 4 *G* and *H*). These results together indicate that the specific inhibition of SOCS1 suppresses the optimal trafficking of Gag to PM, resulting in the degradation of Gag in lysosomes, which in turn leads to the efficient and reproducible inhibition of HIV-1 particle production in various types of human cells.

Discussion

In this work, we report that SOCS1 is an inducible host factor during HIV-1 infection and plays a key role in the late stages of the viral replication pathway via an IFN-independent mechanism (SI Fig. 6). These results represent evidence that SOCS1 is a potent host factor that facilitates HIV-1 particle production via posttranscriptional mechanisms.

SOCS1 has been shown to be a suppressor of several cytokine signaling pathways, and like all SOCS family members it has a central SH2 domain and a conserved C-terminal domain known as the SOCS box (21, 22). Structure–function analyses have further demonstrated that the SOCS1 SH2 domain is required for the efficient binding of its substrates (23, 24). Indeed, our current analyses have also revealed that the SH2 domain of SOCS1 is required for its interaction with the HIV-1 Gag protein. We have shown from our present data that the SOCS box is also required for SOCS1 to function during HIV-1 particle production.

The SOCS box-mediated function of SOCS1 is chiefly exerted via its ubiquitin ligase activity (21, 25). Biochemical binding studies have shown that the SOCS box of SOCS1 interacts with the elongin BC complex, a component of the ubiquitin/proteasome pathway that forms an E3 ligase with Cul2 (or Cul5) and Rbx-1 (21, 26, 27). We show from our current experiments that the SOCS box is required for HIV-1 particle production, indicating the involvement of the ubiquitin/proteasome pathway. However, it is still unknown whether SOCS1 promotes the ubiquitination of Gag and, if so, whether the mono- or poly-ubiquitination of Gag would affect its trafficking and protein stability. Further studies will be necessary to clarify the biological significance of Gag ubiquitination.

Perlman and Resh (20) recently reported that newly synthesized Gag first appears to be diffusely distributed in the cytoplasm, accumulates in perinuclear clusters, passes transiently through a MVB-like compartment, and then traffics to PM. Consistent with these observations, our current work also shows that Gag is accumulated at perinuclear clusters as solid aggregates when its targeting to PM is impaired because of the SOCS1 inhibition.

Another aspect of SOCS1 function during HIV-1 infection was proposed recently. Song *et al.* (28) reported that SOCS1-silenced dendritic cells broadly induce the enhancement of HIV-1 Env-specific CD8+ cytotoxic T lymphocytes and CD4+ T helper cells as well as an antibody response. The induction of the SOCS1 gene in HIV-1 infected cells might therefore disrupt a specific intracellular immune response to HIV-1 in infected host cells.

Based on the strong evidence that we present in our current work that SOCS1 positively regulates the late stages of HIV replication, we conclude that SOCS1 is likely to be a valuable therapeutic target not only for future treatments of AIDS and related diseases, but also for a postexposure prophylaxis against disease in HIV-1-infected individuals.

Materials and Methods

Antibodies and Fluorescent Reagents. Antibodies and fluorescent reagents were obtained from the following sources. Anti-CD63, anti-AP-3, anti-myc (A-14), and anti-SOCS1 (H-93) were from Santa Cruz Biotechnology. Anti-SOCS1 was from Zymed Laboratories. Anti-FLAG (M2) and anti-HA (12CA5) were from Sigma and Roche Diagnostics, respectively. Anti-HIV-p24 (Dako; Cytomation), anti-STAT1, and anti-phospho-STAT1 (Y701) were from BD Transduction Laboratories. Sheep polyclonal anti-TGN46 was from GeneTex.

Plasmid Constructs. Expression constructs for SOCS1 have been described in ref. 29. GST fusion constructs with specific regions derived from the codon-optimized *gag* were generated (MA, CA, NC, p6, Δp6, full-length Gag) by cloning into pGEX-2T (GE Healthcare Bio-Sciences) as described in ref. 30. For retrovirus-mediated siRNA expression, pSUPER.retro.puro vector was digested, as described in ref. 31, with the following sequences: SOCS1-siRNAI, TCGAGCTGCTGGAGCACTA; SOCS1-siRNAII, GGCCAGAACCTTCCTCCTCTT; control siRNA, TCGTATGTTGTGTGGAATT.

Electron Microscopy. Transfected 293T cells were fixed with 2.5% glutaraldehyde and subjected to TEM, as described (14, 32).

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1. Sorin M, Kalpana GV (2006) *Curr HIV Res* 4:117–130.
2. Freed EO (2004) *Trends Microbiol* 12:170–177.
3. Peterlin BM, Trono D (2003) *Nat Rev Immunol* 3:97–107.
4. Trkola A (2004) *Curr Opin Microbiol* 7:555–559.
5. Freed EO (1998) *Virology* 251:1–15.
6. Adamson CS, Jones IM (2004) *Rev Med Virol* 14:107–121.
7. VerPlank L, Bouamr F, LaGrassa TJ, Agresta B, Kikonyogo A, Leis J, Carter CA (2001) *Proc Natl Acad Sci USA* 98:7724–7729.
8. Garrus JE, von Schwedler UK, Pornillos OW, Morham SG, Zavitz KH, Wang HE, Wettstein DA, Stray KM, Cote M, Rich RL, *et al.* (2001) *Cell* 107:55–65.
9. Strack B, Calistri A, Craig S, Popova E, Gottlinger HG (2003) *Cell* 114:689–699.
10. Dong X, Li H, Derdowski A, Ding L, Burnett A, Chen X, Peters TR, Dermody TS, Woodruff E, Wang JJ, *et al.* (2005) *Cell* 120:663–674.
11. Alroy I, Tuvia S, Greener T, Gordon D, Barr HM, Taglicht D, Mandil-Levin R, Ben-Avraham D, Konforty D, Nir A, *et al.* (2005) *Proc Natl Acad Sci USA* 102:1478–1483.
12. Ryo A, Suzuki Y, Ichiyama K, Wakatsuki T, Kondoh N, Hada A, Yamamoto M, Yamamoto N (1999) *FEBS Lett* 462:182–186.
13. Adachi A, Gendelman HE, Koenig S, Folks T, Willey R, Rabson A, Martin MA (1986) *J Virol* 59:284–291.
14. Demirov DG, Ono A, Orenstein JM, Freed EO (2002) *Proc Natl Acad Sci USA* 99:955–960.
15. Chang TL, Mosoian A, Pine R, Klotman ME, Moore JP (2002) *J Virol* 76:569–581.
16. De Sepulveda P, Okkenhaug K, Rose JL, Hawley RG, Dubreuil P, Rottapel R (1999) *EMBO J* 18:904–915.
17. Kamio M, Yoshida T, Ogata H, Douchi T, Nagata Y, Inoue M, Hasegawa M, Yonemitsu Y, Yoshimura A (2004) *Oncogene* 23:3107–3115.
18. Gallina A, Mantoan G, Rindi G, Milanesi G (1994) *Biochem Biophys Res Commun* 204:1031–1038.
19. Accola MA, Strack B, Gottlinger HG (2000) *J Virol* 74:5395–5402.
20. Perlman M, Resh MD (2006) *Traffic* 7:731–745.
21. Alexander WS (2002) *Nat Rev Immunol* 2:410–416.
22. Marine JC, Topham DJ, McKay C, Wang D, Parganas E, Stravopodis D, Yoshimura A, Ihle JN (1999) *Cell* 98:609–616.
23. Narazaki M, Fujimoto M, Matsumoto T, Morita Y, Saito H, Kajita T, Yoshizaki K, Naka T, Kishimoto T (1998) *Proc Natl Acad Sci USA* 95:13130–13134.
24. Yasukawa H, Misawa H, Sakamoto H, Masuhara M, Sasaki A, Wakioka T, Ohtsuka S, Imaizumi T, Matsuda T, Ihle JN, *et al.* (1999) *EMBO J* 18:1309–1320.
25. Tyers M, Rottapel R (1999) *Proc Natl Acad Sci USA* 96:12230–12232.
26. Kamizono S, Hanada T, Yasukawa H, Minoguchi S, Kato R, Minoguchi M, Hattori K, Hatakeyama S, Yada M, Morita S, *et al.* (2001) *J Biol Chem* 276:12530–12538.
27. Kamura T, Burian D, Yan Q, Schmidt SL, Lane WS, Querido E, Branton PE, Shilatifard A, Conaway RC, Conaway JW (2001) *J Biol Chem* 276:29748–29753.
28. Song XT, Evel-Kabler K, Rollins L, Aldrich M, Gao F, Huang XF, Chen SY (2006) *PLoS Med* 3:e11.
29. Ryo A, Suizu F, Yoshida Y, Perrem K, Liou YC, Wulf G, Rottapel R, Yamaoka S, Lu KP (2003) *Mol Cell* 12:1413–1426.
30. Morikawa Y, Kishi T, Zhang WH, Nermut MV, Hockley DJ, Jones IM (1995) *J Virol* 69:4519–4523.
31. Ryo A, Uemura H, Ishiguro H, Saitoh T, Yamaguchi A, Perrem K, Kubota Y, Lu KP, Aoki I (2005) *Clin Cancer Res* 11:7523–7531.
32. Nagashima Y, Nishihira H, Miyagi Y, Tanaka Y, Sasaki Y, Nishi T, Imaizumi K, Aoki I, Misugi K (1996) *Cancer* 77:799–804.

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The antiretroviral potency of APOBEC1 deaminase from small animal species

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ABSTRACT

Although the role of the APOBEC3-dependent retroelement restriction system as an intrinsic immune defense against human immunodeficiency virus type1 (HIV-1) infection is becoming clear, only the rat ortholog of mammalian APOBEC1s (A1) thus far has been shown to possess antiviral activity. Here, we cloned A1 cDNAs from small animal species, and showed that similar to rat A1, both wild-type and Δ*vif* HIV-1 infection was inhibited by mouse and hamster A1 (4- to 10-fold), whereas human A1 had negligible effects. Moreover, rabbit A1 significantly reduced the infectivity of both HIV-1 virions (>300-fold), as well as that of SIVmac, SIVagm, FIV and murine leukemia virus. Immunoblot analysis showed that A1s were efficiently incorporated into the HIV-1 virion, and their packaging is mediated through an interaction with the nucleocapsid Gag domain. Interestingly, there was a clear accumulation of particular C-T changes in the genomic RNAs of HIV-1 produced in their presence, with few G-A changes in the proviral DNA. Together, these data reveal that A1 may function as a defense mechanism, regulating retroelements in a wide range of mammalian species.

INTRODUCTION

It is now clear that the scope of intracellular defense mechanisms against retroviral infections extend beyond the conventional innate and acquired immune responses, involving a series of dominant inhibitory activities that influence retroviral tropism. Two major restriction factors identified thus far are the early block owing to Fv1 and TRIM5α that target incoming retroviral capsids, and cytidine deaminases, such as APOBEC3 (A3) that function at the late phase to hypermutate retroviral genome (1). The A3 proteins have been shown to inhibit the infectivity potential and mobility of a broad and growing number of exogenous retroviruses as well as endogenous retroelements (2,3). A3 edits deoxycytidine (dC) to deoxyuridine (dU) on nascent DNA minus strands during reverse transcription, but the mechanisms underlying the inhibitory effect on retroviruses are not fully understood.

The A3 encoded by mouse genome was found to be about ~30% identical to the human APOBEC3G (hA3G), initially identified as a critical target for the human immunodeficiency virus type1 (HIV-1) auxiliary protein Vif (4,5). Subsequently, anti-HIV activity of A3 was found to be maintained across diverse mammalian species, such as murine, cat and artiodactyls (cattle, pigs and sheep) in spite of extensive amino acid sequence divergence, and regardless of whether lentiviruses infect the species (5–8). However, the interaction of Vif with A3 molecules is species specific, and this Vif-resistant inhibition of HIV-1 by orthologous A3 proteins appears to contribute to restrict cells from nonprimate mammalian species to support productive HIV-1 replication. Thus, the removal of the A3-mediated block will be required for the development of a small animal model in which HIV-1 replicates efficiently.

Although the role of the A3-dependent retroelement restriction system as an intrinsic resistance mechanism is becoming clear, less well understood is mammalian APOBEC1 (A1), the catalytic component of a complex that deaminates apolipoprotein B mRNA in gastrointestinal tissues (9,10). It has been also shown that A1 exhibit potent DNA mutator activity in an *Escherichia coli* assay (11). Rodent A1s share ~70% amino acid sequence identity with human A1, but only rat homolog of A1 was shown to restrict HIV-1 independent of Vif (12,13). To address whether A1 orthologs are involved in an innate

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pathway of restriction of retrovirus infection, A1 cDNAs from small animal species were cloned, and expressed in order to examine their abilities to influence the infectivities of retroviral virions. Our studies show that several A1s from small animal species were efficiently incorporated into the HIV-1 virion via interaction with the nucleocapsid (NC) Gag domain, and suppressed HIV-1 replication in a cytidine deaminase dependent as well as independent manner. Interestingly, there was a clear accumulation of particular C-T changes in the genomic RNAs produced in the presence of rabbit A1, with few G-A changes in the proviral DNA. Moreover, the local mutational preferences on HIV-1 genomic RNA were found to be similar to those observed in apoB mRNA. Importantly, mutation of the catalytic residue Glu63 significantly reduced antiviral activity, and diminished G-A or C-T changes. Further, these deaminases also inhibited simian immunodeficiency virus (SIV)mac, SIVagm and feline immunodeficiency virus (FIV) infections and to a lesser extent murine leukemia virus (MLV). Together, these data reveal that, unlike their human counterparts, A1 in a wide range of mammalian species may function as a defense mechanism regulating retroelements.

MATERIALS AND METHODS

Molecular cloning of A1s

Primary tissues were prepared from small intestines, which had been removed aseptically from euthanatized ferret (*Mustela putorius furo*), rabbit (Kbt: NZW), hamster (Slc: Syrian) and mouse (C57BL/6N), respectively. Total RNA was prepared using TRIzol reagent (Invitrogen, Carlsbad, CA) and the synthesis of first strand cDNA was carried out with High Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA) using a random primer. cDNA from human small intestine and rat (Sprague-Dawley) liver was purchased from BD Biosciences Clontech, Palo Alto, CA (BD™ Marathon-Ready cDNA, Cat. #639326 and #639413, respectively). The cDNA encoding the entire open reading frame of the A1 were amplified using primer sets designated as seen in Table S1, based on A1 sequences from GeneBank except for ferret. Taq polymerase-amplified PCR products were cloned into pCR-Blunt (Invitrogen) vector and sequenced. The primary PCR product was subsequently reamplified by using oligonucleotides containing *Eco*RV and *Not*I cloning sites. Antisense primers encoded the hemagglutinin (HA)-epitope sequence YPYDVPDYA. Amplicons were cleaved at the restriction sites and ligated to similarly cleaved pCAGGS vector (14), yielding HA tagged A1 expression vectors. pCAGGS-based expression plasmids for HA-tagged hA3G has been described elsewhere (15). PCR products generated and digested as described above were also inserted into pcDNA 3.1/Zeo (Invitrogen).

Generation of catalytic site-mutated rabbit A1 expression vectors

Rabbit A1 catalytically inactive mutants E63A and E63Q were constructed with QuickChange® XL Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) using oligonucleotide primers (Table S1), and inserted into pCAGGS vector.

Amino acid alignments and phylogenetic analysis

Protein sequences of full-length mammalian A1s were aligned with Clustal W software. Phylogenetic trees were reconstructed using the neighbor-joining method with 2000 bootstrap replications. MEGA 3.1 was used for phylogenetic analysis. The GenBank accession numbers of the A1 sequences used in these comparisons were rat (NM012907), mouse (NM031159), human (NM001644), opossum (NM001032982), rabbit (U10695), orangutan (AH013823), chimpanzee (XM001164661), rhesus monkey (XM001112583), cattle (XM594173), dog (XM543826), hamster (AF176577) and horse (XM001493159). The sequence of A1s reported in this article has been deposited in the GenBank data base (accession number AB425821).

Viral preparation and infectivity assay

293T, GP293, HeLa, Caco-2 and *Mus dunni* tail fibroblasts (MDTF) cells were maintained in DMEM supplemented with 10% fetal calf serum (Gibco, Grand Island, NY). VSV-G pseudotyped HIV-1-based luciferase reporter virus stocks were produced in 293T cells by cotransfection of wild-type or *Δvif* pNL4-3 Luc E^-R^- (5), together with pVSV-G and one of several expression vectors encoding APOBEC proteins, which are HA-epitope tagged or a control empty vector using Effectene® (Qiagen, Hilden, Germany). Culture supernatants were harvested, filtered and frozen in aliquots. The p24 content of the viruses was determined in ELISA kits (ZeptoMetrix, Buffalo, NY). Target fresh 293T cells were infected with 0.5–1.5 ng equivalent of luciferase reporter viruses and cultured for 48 h. Infected cells were lyzed, and each lysate was assayed for luciferase activity as previously described (16). Single-round SIVmac and SIVagm luciferase reporter virus stocks with or without Vif were produced as VSV-G pseudotypes in 293T cells by cotransfection of pSIV Luc E^-R^- or pSIV Luc E^-R^- *Δvif* (5) and an expression vectors for APOBECs or a mock vector. The p27 content of the viruses was determined in ELISA kits (ZeptoMetrix). Target fresh 293T cells were infected for with 4.0–80 ng equivalent of luciferase reporter viruses and cultured for 48 h. Infected cells were lyzed and each lysate was assayed for luciferase activity.

To generate FIV-GFP virus, 293T cells were transfected with pFIV-H1/copGFP and pFIV-34N (System Bioscience, Mountain View, CA), pVSV-G and APOBEC expression vector or an empty vector. Culture supernatants were filtered and centrifuged at 40 000*g* for 1 h. Target cells were infected with FIV-GFP viruses, equivalent to 8 ng of reverse transcriptase estimated by using Reverse Transcriptase Assay (Roche, Basel, Switzerland) and the infectivity was measured by flow cytometry at 48 h postinfection. Single-round MLV reporter virus stocks were produced as VSV-G pseudotypes in GP293 cells expressing Moloney MLV *gag* and *pol* genes (17) by cotransfection of pFB-Luc or pFB-hrGFP (Stratagene) together with expression vectors for APOBECs or a mock vector. Virus-containing supernatants were normalized for equal MLV p30 CA content estimated by Western analysis. Target MDTF cells were infected with equivalent amounts of MLV reporter viruses and cultured for 48 h. Values are presented as the percent

infectivity relative to the value of the wild-type virus without the expression of APOBECs.

Editing of HIV-1 proviral DNA and genomic RNA

The cells were harvested 48 h postinfection, and total DNA was isolated using the QIAamp DNA Blood Mini Kit (Qiagen). A 408 bp of the HIV-1 *pol* region was amplified with the high-fidelity DNA polymerase (Takara, Ohtsu, Japan) subsequent to the digestion with *DpnI*. Amplified fragments were subsequently gel purified, then cloned into pCR-Blunt vector (Invitrogen) and sequenced. The viral genomic RNA in cell-free virions was purified using QIAamp viral RNA Mini Kit (Qiagen) and converted to cDNA *in vitro* using a High Capacity cDNA Archive kit with random primers, sebsequent to the treatment with DNase. The *pol* region was amplified by PCR, cloned and sequenced as described above.

Western blot analysis

The encapsidation of APOBEC proteins into HIV-1 or MLV virions were detected by pelleting the supernatant of 293T cells transfected with viral DNA and HA-epitope tagged forms of the APOBEC expression vector through a 20% sucrose cushion. The pellets were solubilized in 50 µl of 1% Triton-containing buffer and the equivalent p24 of each solubilized virions was subjected to immunoblot analysis. Cell lysates, virion lysates and immunoprecipitates were subjected to SDS–PAGE, and then transferred to a PVDF membrane (Millipore, Bedford, MA). The membranes were probed with the anti-HA epitope (HA.11; Covance, Princeton, NJ) or anti-β-actin (AC-74; Sigma, Saint Louis, MO) antibody. A monoclonal antibody recognizing the p24 CA (18) and a goat anti-MLV p30 serum (ViroMed Biosafety Labs, Camden, NJ) was used for detection of HIV-1 and MLV CA, respectively. Reactive proteins were detected using biotin-conjugated rabbit immunoglobulin (Sigma), streptoavidin-conjugated peroxidase (Sigma) and developed using Chemi-Lumi One (Nacalai Tesque, Kyoto, Japan). The plasmids expressing HIV-1 Gag or the deletion mutants were described previously (22). Bands in western blots were quantified on a VersaDoc 5000 imager (BIO-RAD, Hercules, CA).

Nucleotide sequence accession numbers

The sequences determined in this study have been submitted to GenBank and assigned accession no. AB425821.

RESULTS

Molecular cloning and phylogenetic analysis of mammalian A1s

We cloned, sequenced and compared the predicted amino acid sequences of A1 cDNAs from primate (human), carnivora (ferret), lagomorphs (rabbit) and rodents (hamster, rat, mouse). Sequence analysis revealed that the clones obtained encoded genes that were identical to the GenBank sequences of A1s (rabbit;U10695, hamster; AF176577, rat;NM012907 and mouse;NM031159), except for one amino acid residue (M80I) substitution in human A1 (NM001644). Results showed that human A1 cDNA encodes an open reading frame of 236 amino acids that has a 78.0% amino acid sequence identity with ferret, 75.8% with rabbit and 70–72% with rodent orthologs. A short C-terminal extension was found in human and rabbit as previously reported (19), and also in ferrets but not in rodents. In particular, the active site motif, designated as His-X-Glu-$(X)_{23-28}$-Pro-Cys-X_2-Cys (X can be any amino acid) (20), was well conserved (Figure 1A). Phlyogenetic tree analyses revealed that the rabbit A1 gene is related to primate A1 genes, while A1s from rodents form a single separate cluster (Figure 1B).

A1s from small animal species inhibit HIV-1 virion infectivity

To examine the anti-HIV activities of A1s from these small animal species, single-round infectivity assay with VSV-G pseudotyped wild-type and *Δvif* HIV-1 luciferase reporter viruses (5) produced in the presence of the influenza HA epitope-tagged A1 proteins was performed. hA3G was used as a control in these experiments. To examine the effect on HIV-1 infectivity quantitatively, virions were normalized based on p24 content. Virus-induced intracellular luciferase activity, which is directly proportional to the infectivity of the virus, was measured 48 h after infection and calculated relative to the APOBEC-negative control virus (Figure 2A, Mock). As expected, hA3G caused a modest decrease in infectivity of the wild-type and a more pronounced decrease in infectivity of the *Δvif* HIV-1virus (Figure 2A). A1 from human intestine did not showed any antiviral activity, while, in agreement with previous observations (12,13), A1 from rat caused a relatively small (2- to 3-fold) decrease in infectivity of the *Δvif* HIV-1virus and a >5-fold decrease in infectivity of the wild-type virus (Figure 2A). In contrast with previous findings however (5,12,13), the A1s from rodents, such as hamster and mouse were equally active against wild-type and *Δvif* HIV-1 viruses, reducing their infectivity >10-fold in some experiments. A modest reduction in infectivity of wild-type and *Δvif* viruses was also found with A1 from ferrets, but of more interest are the findings with A1 from rabbits. Over 100-fold decrease in the *Δvif* HIV-1 virus and a more pronounced 300-fold decrease in infectivity of the wild-type virus was seen with this cytidine deaminase from rabbits. Identical results were obtained when rabbit A1 was expressed with an alternate expression vector, pcDNA3.1 (Figure S1), ruling out an effect specific to the pCAGGS construct used in these experiments. Dose titration studies showed that as little as 0.05 µg of rabbit A1 was sufficient to achieve significant inhibition against HIV-1 (Figure 2B), 10- to 20-fold more potent than APOBECs from other small animal species. For further confirmation, the anti-HIV activity of A1s from small animal species was examined with the use of a HIV-1-based the green fluorescent protein (GFP)-expressing reporter virus. As shown in Figure S2, the results obtained with the HIV-GFP vector are consistent with those seen using the HIV-Luc vector. These multiple lines of investigations indicate that, similar to hA3G, A1s from small animal species can function on HIV-1.

The cytidine deaminase domains of APOBEC proteins contains an active site with conserved consensus motifs in

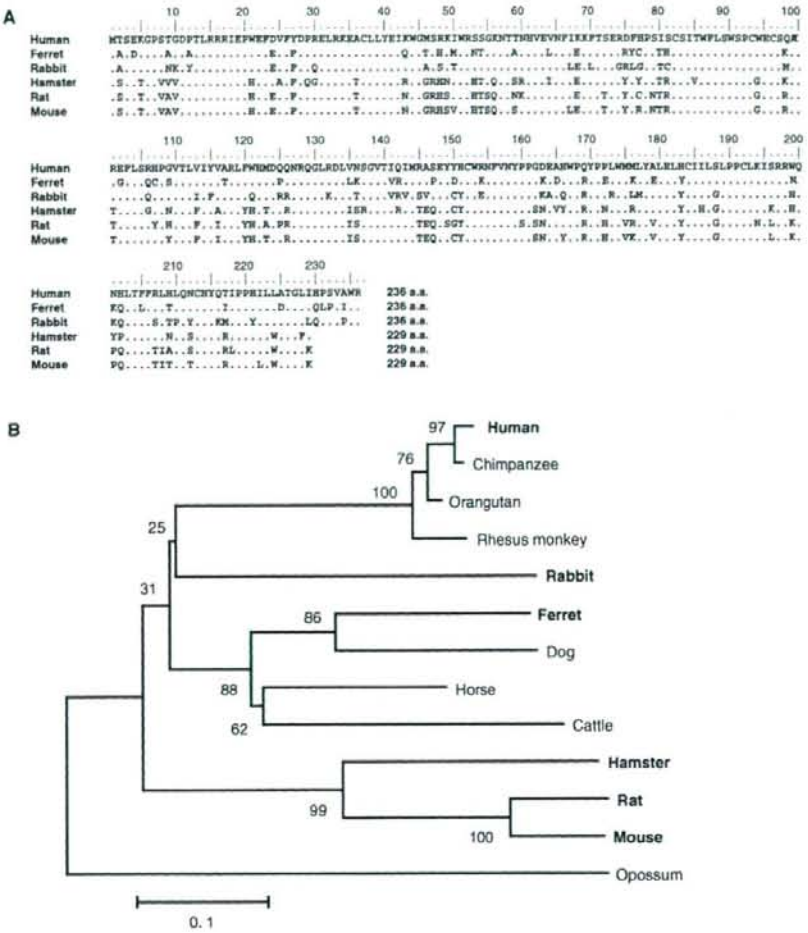

Figure 1. Alignment of the amino acid sequence and phylogenetic analysis of mammalian A1 proteins. (**A**) Amino acid sequence alignment of A1 from human, ferret, rabbit, hamstar, rat and mouse. The predicted amino acid sequences of these cloned A1 molecules are aligned with Clustal W software to the previously identified sequences of mammalian A1s. The numbers are amino acid residue positions. (**B**) Phylogenetic analysis of the protein sequences of full-length mammalian A1s. The tree was reconstructed by neighbor-joining method with protein *p*-distances using MEGA 3.1. Shown interior nodes are bootstrap percentages derived from 2000 replications. Branch lengths represent the number of substitution per site. Opossum is a separate group. The species used for further experiments in this study are indicated in bold letters.

which the His–Cys–Cys residues coordinate a Zn^{2+} ion, and the Glu residue serving an essential role in catalysis as a proton shuttle (21). Therefore, we generated the catalytic site mutant forms of rabbit A1 in which the critical Glu-63 of active site was changed to Ala or Gln (E63A, E63Q), and examined their antiviral activity. Results showed that the ability of the mutant proteins to restrict the infectivity of wild-type and *Δvif* viruses were severely, but not completely impaired (Figure 2C), suggesting the existence of an albeit weak deaminase-independent restriction mechanism by A1.

A1s are packaged into HIV-1 virions

It is now well established that human and murine A3s inhibit HIV-1 infectivity by being packaged into progeny virions. Therefore, we verified whether A1 proteins (which are epitope tagged) would indeed be selectively packaged into HIV-1 virions. For these studies, transfected cells were harvested, and proteins in whole-cell lysates were analyzed by western blotting with anti-HA, anti-HIV-1 CA p24 and anti-β-actin monoclonal antibodies with the β-actin blot serving as a loading control. Data showed comparable amounts of A1 proteins and HIV-1 Gag precursor protein (p55) expression, but minor effects on the processing efficiency into p24 CA with some A1 proteins (e.g. human and rabbit, Figure 2D). Cell-free virus was concentrated by pelleting through a 20% sucrose cushion, and then equivalent amount of solubilized virions was subjected into the western blotting. A1 proteins from small animal species were

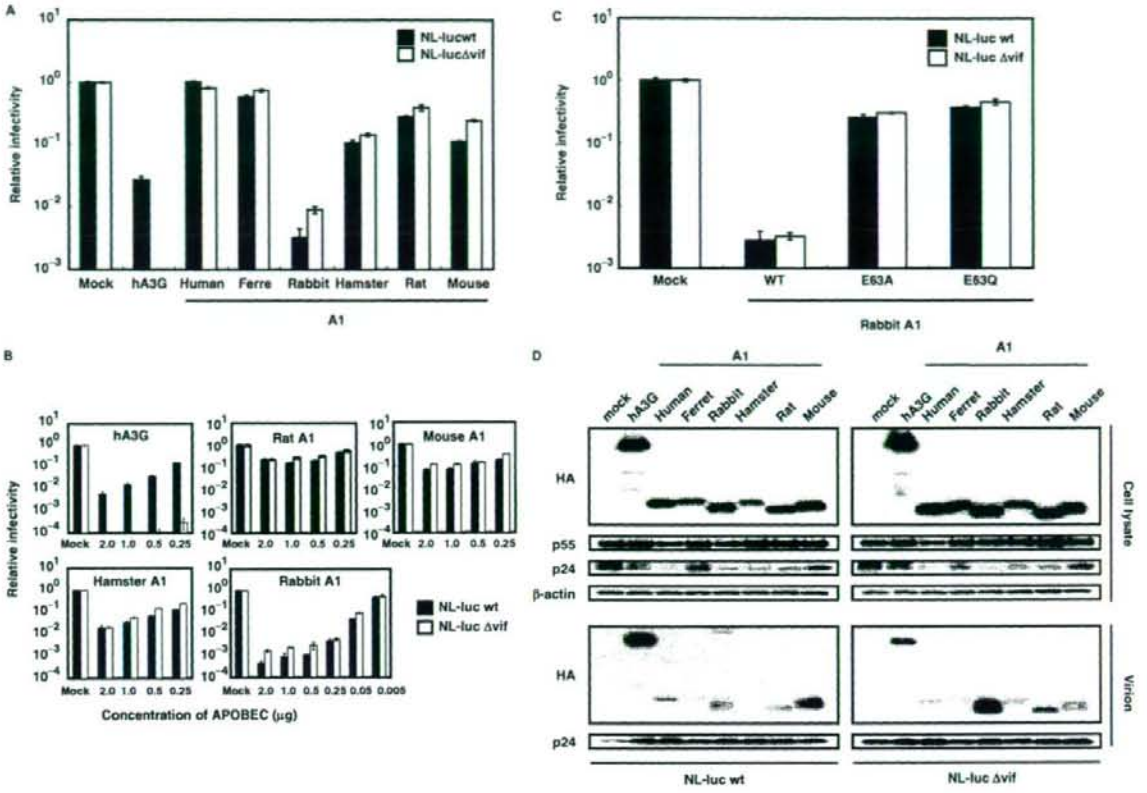

Figure 2. Inhibition of HIV-1 infection by A1s. (**A**) VSV-G pseudotyped wild-type and *Δvif* HIV-1 luciferase viruses were produced in 293T cells transfected with 1.5 μg of luciferase reporter viruses, 1.0 μg of pVSV-G and 0.5 μg of HA-tagged APOBEC expression vector or empty vector. Virus-containing supernatants were normalized for equal p24 content and used for the infection of the fresh 293T cells. Virus-induced intracellular luciferase activity was measured. 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 APOBEC protein. The average of three experiments with standard deviation is indicated. (**B**) Wild-type and *Δvif* HIV-1 luciferase reporter viruses pseudotyped were produced in 293T cells transfected with decreasing amounts of HA-tagged APOBEC expression vector. The amount of expression vector plasmid transfected is shown in micro gram on the *X*-axis. (**C**) The catalysis domain of A1 is required for the efficient anti-HIV-1 potency of the rabbit A1. The HA-tagged rabbit A1 (WT), A1 mutated in the active site motif (E63A and E63Q) were used. (**D**) A1 proteins from small animal species are encapsidated into HIV-1 virions. The producer cells were collected and lyzed, while the released virus in the supernatants were collected by ultracentrifugation. The cells and virion lysates were then subjected to Western analysis using an antibody specific for the HA tag and HIV-1 Gag CA. The immunoblot probed with anti-β-actin antibody of the proteins present in the cell lysates is shown.

found to be packaged into HIV-1 virions, but variations in the efficiency of the incorporation of each A1 protein were seen. Rabbit A1 was incorporated efficiently, which could explain for its potent antiviral activity. The incorporations into HIV-1 virions of A1s from small animal species were not affected by expression of the Vif protein (Figure 2D). We therefore conclude that A1s like hA3G are specifically packaged into HIV-1 virions. However, unlike hA3G, this incorporation is not inhibited with coexpression of HIV-1 Vif. The mutant rabbit A1 E63A E63Q proteins were correctly expressed within the producer cells and also incorporated into the HIV-1 virions efficiently, as judged by western blot analysis (data not shown).

The HIV-1 Gag NC domain is required for A1 packaging

We next determined whether parts of Gag were dispensable for A1 packaging into virions, by use of previously described Gag mutants (22). Two Gag deletion mutants, δ10-110 mutant lacking residues 10 through 110, δ10-277 mutant lacking residues 10 through 277 or the Zwt-p6 mutant, in which NC is replaced by a leucine zipper from GCN4 were tested (Figure 3A). A1s from human, rabbit and rat as well as hA3G were efficiently incorporated into virus like particles (VLPs) formed by both Gag deletion δ10-110 and δ10-277 mutants (Figure 3B). Consistent with previous observations (22), hA3G was not incorporated into VLP formed by Zwt-p6 mutant. A1 from human and rabbit did not exhibit any detectable incorporation, while, A1 from rat caused a relatively small amount of incorporation into VLP formed by Zwt-p6 mutant (Figure 3B). Thus, almost all matrix and the amino-terminal two-thirds of capsid appear to be largely dispensable for A1 packaging, similar to those observed in hA3G packaging.

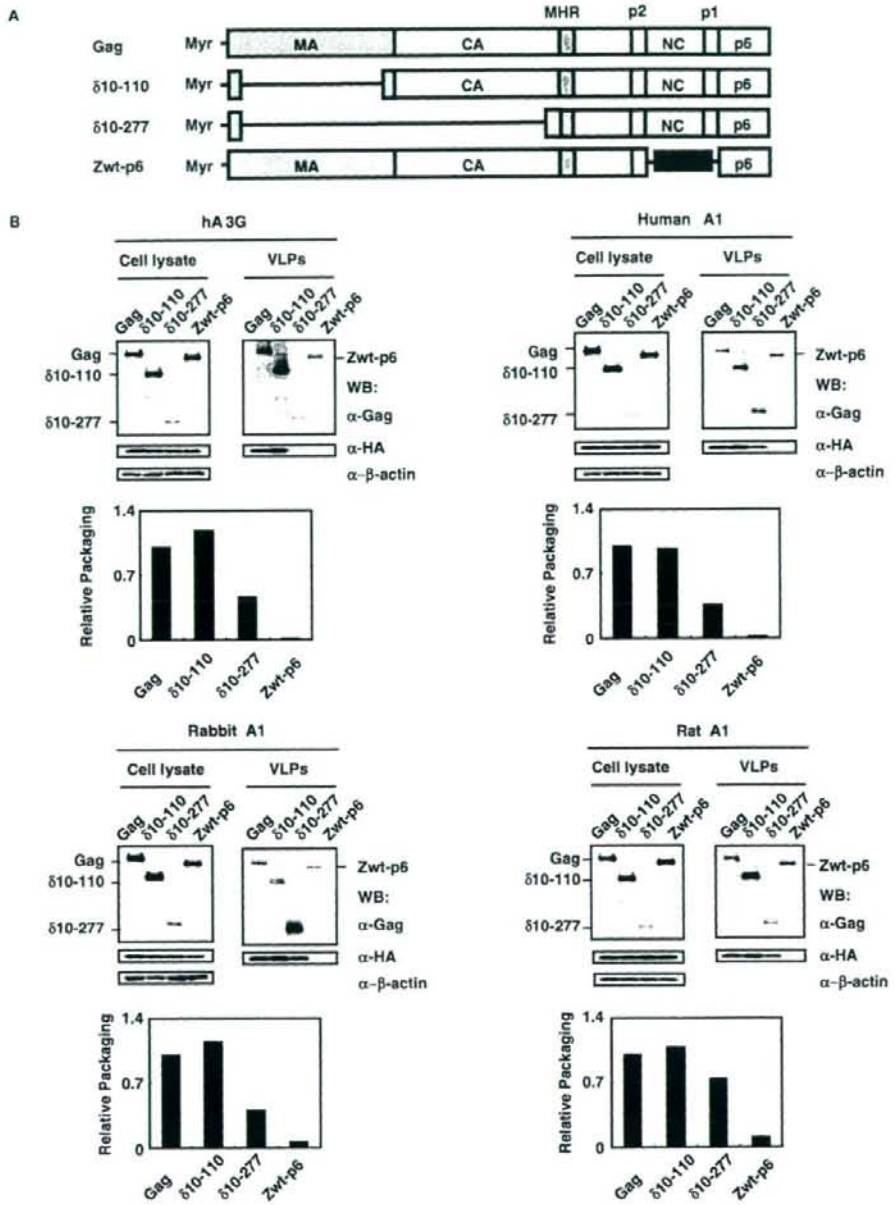

Figure 3. A1 proteins are encapsidated into HIV-1 virions via through the interaction with NC, but not the majority of Gag. (**A**) Schematic representation of the intact HIV-1 Gag precursor and its derivatives used in this study. The Gag are myristoylated (Myr-) on the N-tereminus of MA. The positions of the major homology region (MHR) and the various Gag cleavage sites are shown. (**B**) Western blot analysis of cell lysates and extracellular VLPs generated following transfection of 293T cells with plasmids expressing HIV-1 Gag or its derivatives shown in (A) and the HA-epitope tagged forms of the indicated APOBEC proteins. The producer cells were collected and lyzed, while the supernatants were harvested and released virus were collected by ultracentrifugation. The cells and virion lysates were then subjected to Western analysis using antibodies specific for the HIV-1 Gag CA and HA tag. The immunoblot probed with anti-β-actin antibody of the proteins present in the cell lysates is shown. Results were normalized to those obtained from the transfection of cells with the full-length Gag and APOBEC constructs, and were graphically shown as relative packaging.

Editing of HIV-1 proviral DNA and genomic RNA by A1 proteins

A3-mediated antiretroviral activity has been shown to be associated with cytidine deamination of nascently transcribed viral cDNAs following infection of target cells. Such dC-to-dU deamination in minus-strand DNA is detected as replacement of dG-to-dA in integrated proviral genomes. To address whether A1 functions in a similar manner, accumulation of dG-to-dA changes in the proviral DNA during reversetranscription was examined. Viral DNA was prepared from cells infected with wild-type or *Δvif* HIV-1 viruses produced in the presence of A1s from small animal species, and the 3′-end of the *pol* gene was analyzed for evidence of hypermutation. Consistent with previous reports, significant dG-to-dA hypermutation was observed for hA3G serving as a control for these studies (Table 1) and no dG-to-dA mutation was observed when the virus was prepared in the absence of an APOBEC protein (data not shown). In contrast, the predominant mutations induced by rabbit A1 that exhibited the most prominent antiviral activity were dC-to-dT changes (22 and 14 events in 23 664 and 24 480 bases in wild-type and *Δvif* viruses, respectively), which could have arisen through deamination of unpaired plus-stranded cDNA or virion RNA. To address the latter possibility, cell-free virions were purified and the genomic RNA was converted to cDNA for *pol* gene analyses. As can be seen in Table 2, there was a clear accumulation of dC-to-dT (U in the template RNA) changes in the RNAs of HIV-1 produced in the presence of rabbit A1 (39 and 51 events in 9792 and 8976 bases in wild-type and *Δvif* viruses, respectively), a frequency that is 4- to 5-fold higher than those observed in DNA sequencing. Thus, HIV-1 genome RNA as well as reversetranscribed proviral DNA could be a substrate for A1-mediated deamination.

The preferential sites of hypermutations induced by rabbit A1 were also examined (Figure 4). Consistent with previous observations (23–26), hA3G prefers to edit dC (marked by an asterisk) in the viral target DNA sequence C/TCC*. In constrast, rabbit A1, similar to mouse A3 (27) and human A3F (13,28,29), show preference for TTC* (Figure 4A). These results suggest that rabbit A1 inhibits HIV-1 infection by a deamination-dependent mechanism through targeting of proviral DNA sequences that are different from hA3G. Sequence context of the cytidine in viral RNA sequences mutated by rabbit A1 showed a clear

Table 1. Sequence analysis of reverse transcribed second-strand proviral DNA in the presence of mammalian A1s

			A1													
	hA3G		Human		Rabbit		Rabbit E63A		Rabbit E63Q		Hamster		Mouse		Rat	
	wt	*Δvif*	wt	*Δvif*	wt	*Δvif*	wt	*Δvif*	wt	*Δvif*	wt	*Δvif*	wt	*Δvif*	wt	*Δvif*
Clones sequenced	34	36	24	24	58	60	24	23	24	23	34	36	24	24	24	24
Total base pair sequenced	13 872	14 688	9792	9792	23 664	24 480	9792	9384	9792	9384	13 872	14 688	9792	9792	9792	9792
Clones with G to A	15	22	2	1	13	24	0	0	0	2	1	1	0	6	0	0
Number of G to A mutations	71	318	5	7	69	118	0	0	0	2	1	1	0	24	0	0
Number of G to A mutations per 1 kb	5.12	21.65	0.51	0.71	2.92	4.82	0	0	0	0.21	0.07	0.07	0	2.45	0	0
Clones with C to T	0	0	0	0	20	10	1	0	1	0	3	3	0	5	6	6
Number of C to T mutations	0	0	0	0	22	14	2	0	1	0	3	4	0	5	7	7
Number of C to T mutations per 1 kb	0	0	0	0	0.93	0.57	0.20	0	0.10	0	0.22	0.27	0	0.51	0.71	0.71
Number of Other mutations	0	1	0	0	1	3	0	0	0	0	0	0	0	0	0	0

A 408 bp fragment of HIV-1 *pol* region was amplified from reverse transcripts infected with wt or *Δvif* NL-Luc viruses produced in the presence of hA3G or mammalian A1s. The number in the clones of each group are shown (wt, wild-type). All mutations are designated using the conventional plus-strand nomenclature.

Table 2. Sequence analysis of HIV-1 genomic RNA in the presence of mammalian A1s

					A1							
	Mock		hA3G		Rabbit		Rabbit E63A		Rabbit E63Q		Rat	
	wt	*Δvif*	wt	*Δvif*	wt	*Δvif*	wt	*Δvif*	wt	*Δvif*	wt	*Δvif*
Clones sequenced	23	23	21	24	24	22	21	23	24	24	22	24
Total base pair sequenced	9384	9384	8568	9792	9792	8976	8568	9384	9792	9792	8976	9792
Clones with C to T	0	0	0	0	15	17	0	0	0	0	6	13
Number of C to T mutations	0	0	0	0	39	51	0	0	0	0	13	17
Number of C to T mutations per 1 kb	0	0	0	0	3.98	5.68	0	0	0	0	1.45	1.74
Number of Other mutations	0	2	1	0	1	0	0	1	1	0	0	0

This experiment was performed as described in the legend for Table 1, except that the genomic RNA of HIV-1 was amplified (wt, wild-type).

A hA3G

	−2	−1	C	1	2
A	18.3%	1.4%	0.0%	28.2%	33.8%
C	**40.8%**	**87.3%**	**100.0%**	33.8%	18.3%
G	0.0%	0.0%	0.0%	2.8%	11.3%
T	**40.8%**	11.3%	0.0%	**35.2%**	**36.6%**
	C/T	**C**	**C**	**T**	**T**

	−2	−1	C	1	2
A	20.4%	0.9%	0.0%	23.9%	**43.1%**
C	23.6%	**80.5%**	**100.0%**	29.2%	20.4%
G	0.3%	0.0%	0.0%	0.6%	6.0%
T	**55.7%**	17.9%	0.0%	**46.2%**	30.5%
	T	**C**	**C**	**T**	**A**

Rabbit A1

	−2	−1	C	1	2
A	34.8%	0.0%	0.0%	24.6%	27.5%
C	13.0%	2.9%	**100.0%**	20.3%	2.9%
G	11.6%	0.0%	0.0%	2.9%	5.8%
T	**40.6%**	**97.1%**	0.0%	**52.2%**	**63.8%**
	T	**T**	**C**	**T**	**T**

NL-luc wt

	−2	−1	C	1	2
A	28.0%	0.0%	0.0%	22.0%	38.1%
C	19.5%	14.4%	**100.0%**	37.3%	12.7%
G	0.0%	0.0%	0.0%	1.7%	6.8%
T	**52.5%**	**85.6%**	0.0%	**39.0%**	**42.4%**
	T	**T**	**C**	**T**	**T**

NL-luc Δvif

B Rabbit A1

	−2	−1	C	1	2
A	30.8%	41.0%	0.0%	**51.3%**	12.8%
C	**35.9%**	0.0%	**100.0%**	10.3%	20.5%
G	15.4%	0.0%	0.0%	5.1%	28.2%
T	17.9%	**59.0%**	0.0%	33.3%	**38.5%**
	C	**A/T**	**C**	**A/T**	**T**

NL-lucwt RNA

	−2	−1	C	1	2
A	31.4%	35.3%	0.0%	**54.9%**	19.6%
C	**47.1%**	0.0%	**100.0%**	0.0%	11.8%
G	9.8%	0.0%	0.0%	0.0%	**35.3%**
T	11.8%	**64.7%**	0.0%	45.1%	33.3%
	C	**A/T**	**C**	**A/T**	**G**

NL-luc ΔvifRNA

Figure 4. Comparison of the preferred sequence context for cytidine deamination by rabbit A1 in first strand cDNA and the genomic RNA of wild-type or*Δvif* viruses. (**A**) TTC*T in the HIV-1 minus-strand cDNA was the preferred tetranucleotide target of rabbit A1. Shown are percentages of each nucleotide found at the −2, −1, +1 and +2 positions relative to the dC residue targeted for deamination (position zero). The consensus DNA sequence is shown at the bottom of each minitable by bold. (**B**) WC*W sequence in the HIV-1 genome was the preferred RNA target of rabbit A1. Comparison of the preferred sequence observed in the viral genomic RNA produced in the presence of rabbit A1. All of the mutations in HIV-1 genomic RNA aligned with respect to the cytidine (**C**) targeted for deamination (position zero). The frequency with which each of the four bases found at positions adjacent to the deaminated C is indicated. The consensus RNA sequence is shown at the bottom of each minitable by bold.

WCW (W is A or T) trinucleotide preference on both the wild-type and *Δvif* HIV-1 genomic RNA (Figure 4B). The cytosine residue within WCW sequences was reported to be highly conserved as the apoB mRNA-editing site sequence by A1 protein among divergent of mammalian species (30). Rabbit A1 deaminated the cytosines in the genomic RNA but with different frequencies; some cytosines were changed at high frequencies, whereas others were not changed in any of the clones (Figure S4). Taken together, analysis of these mutations confirms that the inhibitory effects observed in rabbit A1s were based on, at least in part, cytosine-deaminating activity on viral genomic RNA, and the analysis of mutational hot spots indicated that the molecular mechanisms for editing of HIV-1 genome RNA and apoB mRNA overlap.

A1s from small animal species exhibit broad antilentiviral activity

To assess the breath of A1-mediated antiviral activity, the activities of these proteins on SIV infectivity were examined. A single-round assay was used to measure the infectivity of wild-type and *Δvif* SIVmac, and SIVagm luciferase reporter viruses (5), in the presence of A1s. VSV-G pseudotyped viral supernatants were collected, normalized for SIV CA p27 and used to infect 293T cells. As expected, hA3G caused a profound decrease in infectivity of both wild-type and *Δvif* SIV viruses (Figure 5A and C). A1 from rodents were found to be moderately active, but rabbit A1 significantly (>100-fold) reduced the infectivity of wild-type and *Δvif* SIV viruses. In contrast to A1 from small animal species, A1 from human intestine showed no antiviral activity. Expression of catalytic site mutant forms of rabbit A1 (E63A, E63Q) has little effect on the infectivity of SIV (Figure 5B and D), suggesting that the deaminase activity is also important for SIV repressive activities.

To measure the activities of A1 proteins on FIV infectivity, plasmids expressing APOBECs were cotransfected with an FIV (pFIV-H1/U6-copGFP) genome along with the packaging pFIV-34N plasmid and pVSV-G.

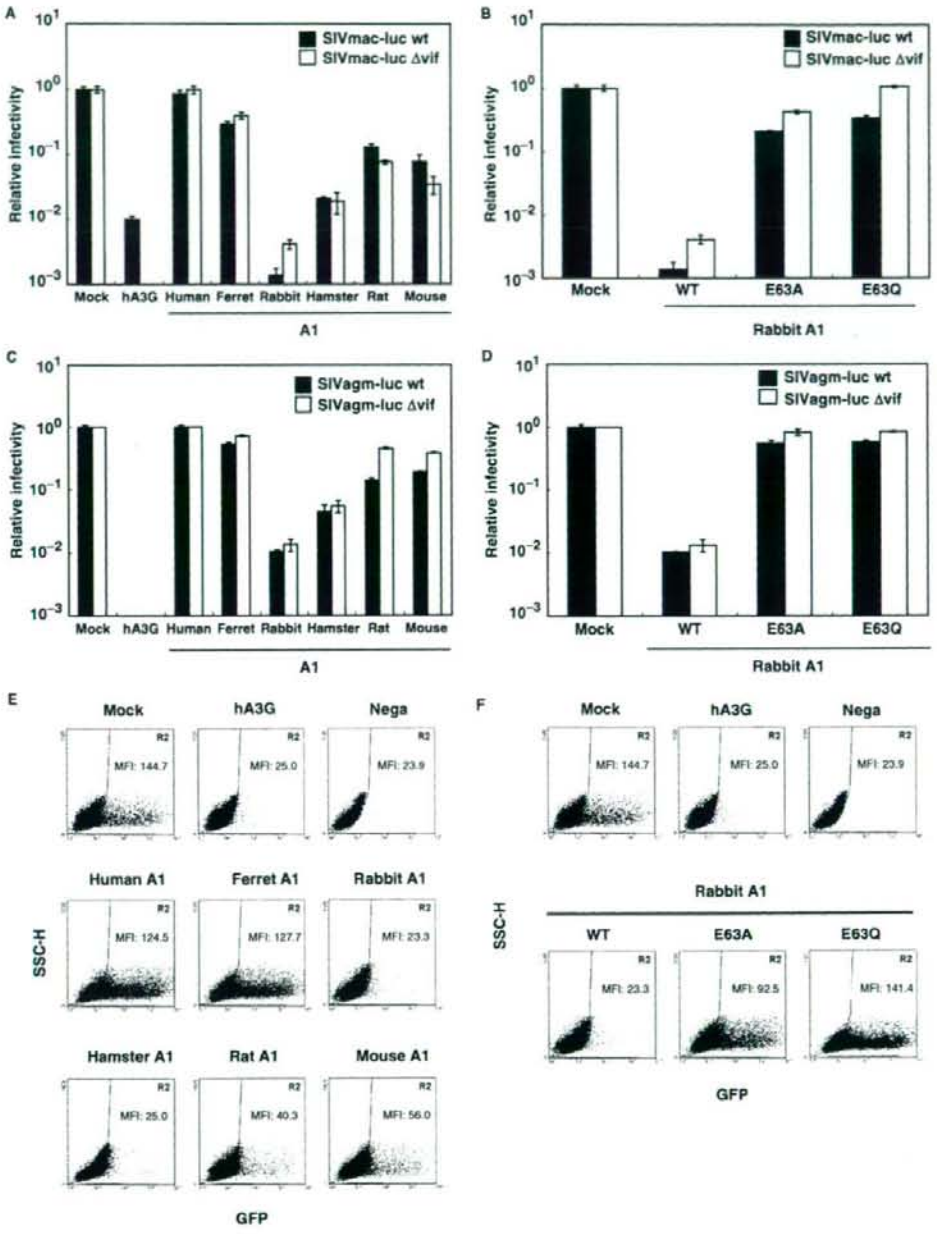

Figure 5. Inhibition of SIV and FIV infection by A1s. Wild-type and *Δvif* SIVmac (**A** and **B**), wild-type and *Δvif* SIVagm (**C** and **D**) luciferase reporter viruses pseudotyped were prepared. 293T cells were transfected with 1.5 μg of luciferase reporter viruses, 1.0 μg of pVSV-G and 0.5 μg of HA-tagged APOBECs (A and C) or rabbit A1 with catalytic site mutations (B and D). Virus-containing supernatants were normalized for equal p27 content and used for the infection. At 48 h postinoculation, virus-induced luciferase activity was measured and presented as described. VSV-G pseudotyped FIV GFP reporter viruses were produced in 293T cells transfected with 1.5 μg of pFIV-H1/copGFP and pFIV-34N, 1.0μg of pVSV-G and 0.5 μg of HA-tagged APOBECs (**E**) or rabbit A1 with catalytic active site mutations (**F**). At 48 h postinoculation with FIV-GFP virions normalized for equal RT activity, cells exhibiting GFP fluorescence of target 293T cells were analyzed on flow cytometry. The level of GFP MFI detected within the GFP-positive windows are indicated. Comparable results were obtained in three additional experiments.

Viral supernatants of pseudotyped virions were collected, normalized for RT activity and used to infect 293T cells. The GFP expression was analyzed by flow cytometry at 48 h postinfection. Large proportion of of 293T cells were positive for GFP expression, with a MFI of 144.7, while in the cells transduced with hA3G containing FIV virions, only a few green cells were present, with 5.7-fold lower MFI of 25.0 (Figure 5E). Interestingly, similar to primate lentiviruses, the infectivity of FIV was significantly reduced in the presence of rabbit A1, with a 6.2-fold lower MFI of 23.3. We also detected reduced GFP signals in the cells transduced with rodent A1-containing virions, while human and ferret A1s caused minimal reduction in the number of GFP positive cells (Figure 5E). As seen in Figure 5F, rabbit A1 with catalytic site mutation E63A caused a slight decrease in FIV infectivity, but the E63Q mutation had no effect. Thus, the results using HIV-1, SIVmac, SIVagm and FIV reporter viruses combined suggest that the A1s from small animal species have a relatively broad lentivirus restriction potential that is mainly mediated through deaminase-dependent mechanism.

A1s affect MLV virion infectivity

Evidence is mounting that some APOBEC proteins can target a variety of retroviral substrates, such as various oncovirus and spumavirus. The A3 orthologs from artiodactyls; cattle, pigs and sheep, as well as hAB and hA3G have been reported to exert antiviral activity on MLV (6,13,23,24). These findings suggest that A3s in artiodactyls could function as barriers of cross-subspecies transmission of MLV from mice. Interestingly, this simple oncovirus is resistant to the mouse A3, explaining the absence of a Vif-like activity in MLV (27,31), but the underlying mechanism is currently unknown (32). Therefore, we examined whether the A1s expressed in small animal species affect MLV infection. MLV-based reporter viruses were produced by transient transfection of pFB-Luc, encoding the luciferase gene, into the MLV packaging cell line GP293 with pVSV-G, in the presence of APOBEC proteins. As shown in Figure 6A, hA3G was able to restrict the infectivity of MLV, consistent with previous reports (13,23,24). Interestingly, MLV infectivity was inhibited ~7-fold by rabbit A1, while A1s from other mammalian species had none or only moderate effect. Similar results were obtained with another murine retroviral vector, pFB-hrGFP (data not shown). Deaminase-defective rabbit A1s retained only partial antiviral activity (Figure 6B) despite comparable levels of MLV virion incorporation (Figure 6C). These data suggest that A1 from small animal species functions as potential barriers of cross-species transmission of this gammmaretroviruses from mice.

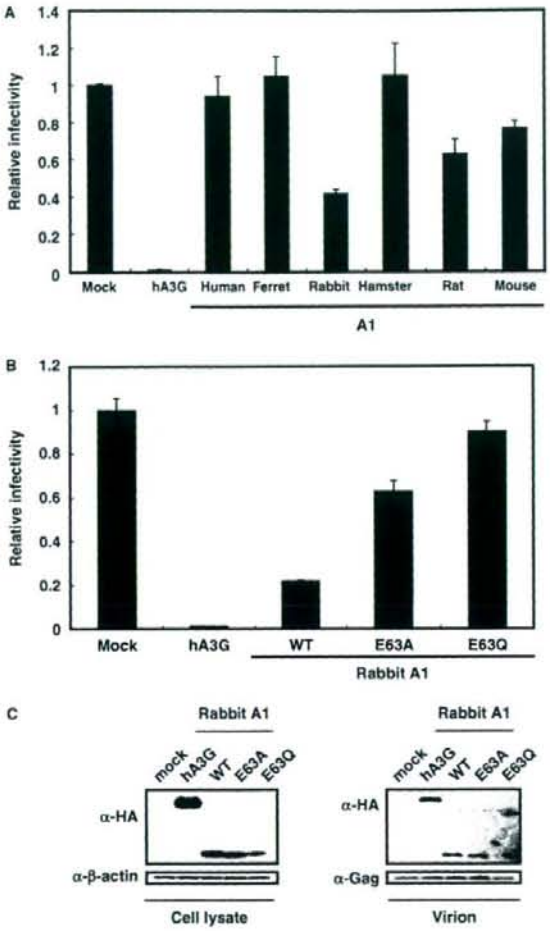

Figure 6. Inhibition of MLV infection by A1s. (**A** and **B**) MLV packaging cell line GP293 were transfected with 1.5 μg of luciferase pFB-Luc reporter plasmids, 1.0 μg of pVSV-G and 0.5 μg of HA-tagged APOBECs or rabbit A1 with catalytic site mutations. Virus-containing supernatants were normalized for equal MLV p30 CA content and used for the infection of the MDTF cells. Virus-induced intracellular luciferase activity was measured and presented as described. (**C**) Rabbit A1 proteins are encapsidated into MLV virions. After transfection, released virion was collected by ultracentrifugation, while the producer cells were collected and lyzed. The cells and virion lysates were then subjected to Western analysis using antibodies specific for the HA tag and MLV Gag CA. An immunoblot probed with anti-β-actin antibody of the proteins present in the cell lysates is also shown. While only the immunoblot of p30 CA performed with the disrupted virions is presented, closely similar results were also obtained using the cell lysates (data not shown).

DISCUSSION

In this study, we showed that single domain cytidine deaminase A1 from rodents (mouse, rat and hamster) and largomorphs (rabbit) are capable of inhibiting the infectivity of various lentiviruses in tissue culture models. A rank order in anti-HIV potency was seen, with rabbit A1 showing the greatest activity. The finding of more efficient virion incorporation of rabbit as compared to other small animal species A1 proteins may be a contributing factor. Catalytic site mutant analysis suggested a deaminase-dependent restriction mechanism, with genomic RNA as well as reversetranscribed proviral DNA serving as substrates for A1-mediated deamination.

A clear accumulation of C-T changes in the genomic RNAs of HIV-1 produced in the presence of rabbit A1 was observed, with G-A changes in the proviral DNA. Furthermore, expression of catalytic site mutant forms of rabbit A1 has little effect on the viral infectivity, supporting the importance of the deaminase activity for these repressive activities. Cytidine deaminase-defective A3 mutants have been shown to exhibit significant antiviral activity (33), implying that antiviral and deaminase activities can be uncoupled. Further evidence in support of editing-independent antiviral mechanism comes from studies on the enzymatically inactive, high-molecular mass complex of hA3G (34) as well as on the antiviral activity against hepatitis B virus (HBV) (35). Nevertheless, more recent studies using deaminase-defective A3 mutants show that efficient inhibition of HIV-1 or retroelements requires catalytically active A3 (36,37). In this regard, although deaminase-defective rabbit A1 mutants were shown to inhibit various lentivirus and MLV, these antiviral activities were significantly lower than those seen with wild-type A1. The suppressive activity of A1, therefore, is principally associated with its cytidine deaminase activity.

We demonstrated that the molecular mechanism for A1 editing of the HIV-1 genomic RNA and apoB mRNA overlapped. The C to U editing of apoB mRNA is shown to be a nuclear event (38), mediated by a complex composed of A1 homodimer and an A1 complementation factor (ACF) (39–41). Expression of ACF mRNA in cells such as Caco-2, a human colon cancer-derived cell line demonstrated to edit endogenous apoB mRNA has been documented (42). However, mRNA for ACF could not be detected by RT-PCR in 293T cells used for retorovirus production in this study (Supplementary Figure S5). Thus, the site(s) in virus-producing cells (e.g. nucleus, cytoplasm or both) where the deamination of HIV-1 genomic RNA takes place remains to be identified, and further experiments are needed to fully understand the role of ACF in the editing of retroviral genome observed in the present study.

In our study, A1s were expressed using both a chick β-actin (CAG) and a cytomegalovirus promoter-driven expression vector, and the concentration of A1s required to mediate antiviral activity was titrated carefully. We found that only 0.05 μg of rabbit A1 was required to achieve significant inhibition against HIV-1 (Figure 2B). Moreover, the antiviral activity of rabbit A1 appeared to be more potent than hA3G (Supplementary Figure S1), and this is unlikely to be explained by differences in virion incorporation of the two enzymes (Figure 2D). Taken together, the data suggest that antiretroviral activities of A1s observed in this study were not solely due to using overexpressed protein systems.

We found that human A1 exhibited no antiretroviral activity, consistent with reports of others (12,13,29,43). A1 from hominoids, therefore, appears to exclusively mediate the C to U editing of apoB mRNA, giving rise to two proteins with different sizes in the gastrointesitinal tissues that function in lipid transport and metabolism. As there have been no reports thus far of lentivirus infection in rodents, the antiviral activity of A1 proteins in rodents seen against HIV, SIV and FIV are also unlikely to have evolved originally to restrict infection of these lentiviruses. Nevertheless, the finding that rabbit A1 can restrict MLV raises the possibility that A1s from small animals species evolved to restrict cross-subspecies transmission of oncoviruses from mice. Furthermore, endogenous lentivirus of rabbits has recently been described (44), lending biological significance to the antilentiviral activity in lagomorphs reported here. Further studies of the antiviral activities of A1 proteins from other members of the placentalia super-order of Laurasiatheria; cetartiodactyla (cow, pig, sheep) and carnivore (cat) will be required to fully understand the complex evolutionary history of *APOBEC* genes as an intrinsic resistance mechanism against retroelements.

HIV-1 exhibits a highly restricted host cell tropism. The identification of chemokine receptors as entry cofactors with human CD4 raises the possibility that small animal species, in particular, rodents could be engineered to express these molecules, thereby rendering them able to support a productive HIV-1 infection. However, HIV-1 replication in rodents (45–47) and rabbits (48,49) expressing human versions of the HIV-1 receptors appeared to be limited and variable. Our findings with A1 suggest that this enzyme may be partly responsible for the inefficient replication of HIV-1 observed in rabbit as well as rodent cells. Blocking of antiviral A1 function by RNA interference in cells from small animal species should verify whether A1 acts as an intrinsic resistance factor. Further understanding of these species-nonspecific repressive activities to HIV-1 replication at the late phase, in conjugation with the early block owing to the different classes of activities, such as TRIM5α (50,51), may suggest approaches to the development of small animal models of HIV-1 infection.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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REFERENCES

1. Bieniasz,P.D. (2004) Intrinsic immunity: a front-line defense against viral attack. *Nat. Immunol.*, **5**, 1109–1115.
2. Holmes,R.K., Malim,M.H. and Bishop,K.N. (2007) APOBEC-mediated viral restriction: not simply editing? *Trends Biochem. Sci.*, **32**, 118–128.
3. Chiu,Y.L. and Green,W.C. (2008) The APOBEC3 cytidine deaminases: an innate defensive network opposing exogenous retroviruses and endogenous retroelements. *Annu. Rev. Immunol.*, **26**, 317–353.
4. Sheehy,A.M., Gaddis,N.C., Choi,J.D. and Malim,M.H. (2002) Isolation of a human gene that inhibits HIV-1 infection and is suppressed by the viral Vif protein. *Nature*, **418**, 646–650.
5. Mariani,R., Chen,D., Schrofelbauer,B., Navarro,F., Konig,R., Bollman,B., Munk,C., Nymark-McMahon,H. and Landau,N.R. (2003) Species-specific exclusion of APOBEC3G from HIV-1 virions by Vif. *Cell*, **114**, 21–31.
6. Jonsson,S.R., Hache,G., Stenglein,M.D., Fahrenkrug,S.C., Andresdottir,V. and Harris,R.S. (2006) Evolutionarily conserved and non-conserved retrovirus restriction activities of artiodactyl APOBEC3F proteins. *Nucleic Acids Res.*, **34**, 5683–5694.
7. Munk,C., Zielonka,J., Constabel,H., Kloke,B.P., Rengstl,B., Battenberg,M., Bonci,F., Pistello,M., Lochelt,M. and Cichutek,K. (2007) Multiple restrictions of human immunodeficiency virus in feline cells. *J. Virol.*, **81**, 7048–7060.
8. Munk,C., Beck,T., Zielonka,J., Hotz-Wagenblatt,A., Chareza,S., Battenberg,M., Thielebein,J., Cichutek,K., Bravo,I.G., O'Brien,S.J. *et al.* (2008) Functions, structure, and read-through alternative splicing of feline APOBEC3 genes. *Genome Biol.*, **9**, R48.
9. Chester,A., Scott,J., Anant,S. and Navaratnam,N. (2000) RNA editing: cytidine to uridine conversion in apolipoprotein B mRNA. *Biochem. Biophys. Acta.*, **1494**, 1–13.
10. Keegan,L.P., Gallo,A. and O'Connell,M.A. (2001) The many roles of an RNA editor. *Nat. Rev. Genet.*, **2**, 869–878.
11. Harris,R.S., Petersen-Mahrt,S.K. and Neuberger,M.S. (2002) RNA editing enzyme APOBEC1 and some of its homologs can act as DNA mutators. *Mol. Cell*, **10**, 1247–1253.
12. Bishop,K.N., Holmes,R.K., Sheehy,A.M. and Malim,M.H. (2004) APOBEC-mediated editing of viral RNA. *Science*, **305**, 645.
13. Bishop,K.N., Holmes,R.K., Sheehy,A.M., Davidson,N.O., Cho,S.-J. and M. H. Malim. (2004) Cytidine deamination of retroviral DNA by diverse APOBEC proteins. *Curr. Biol.*, **14**, 1392–1396.
14. Niwa,H., Yamamura,K. and Miyazaki,J. (1991) Efficient selection for high-expression transfectants with a novel eukaryotic vector. *Gene*, **108**, 193–199.
15. Ohsugi,T. and Koito,A. (2007) Human T cell leukemia virus type 1 is resistant to the antiviral effects of APOBEC3. *J. Virol. Methods*, **139**, 93–96.
16. Koito,A., Kameyama,Y., Cheng-Mayer,C. and Matsushita,S. (2003) Susceptibility of mink (*Mustera vision*)-derived cells to replication by human immunodeficiency virus type 1. *J. Virol.*, **77**, 5109–5117.
17. Burns,J.C., Friedmann,T., Driever,W., Burrascano,M. and Yee,J.-K. (1993) Vesicular stomatitis virus G glycoprotein pseudotyped retroviral vectors: concentration to very high titer and efficient gene transfer into mammalian and nonmammalian cells. *Proc. Natl Acad. Sci. USA*, **90**, 8033–8037.
18. Koito,A., Hattori,T., Matsushita,S., Maeda,Y., Nozaki,C., Sagawa,K. and Takatsuki,K. (1988) . Conserved immunogenic region of a major core protein (p24) of human and simian immunodeficiency viruses. *AIDS Res. Hum. Retroviruses*, **4**, 409–417.
19. Yamanaka,S., Poksay,K.S., Balestra,M.E., Zeng,G.-Q. and Innerarity,T.L. (1994) Cloning and mutagenesis of the rabbit apoB mRNA editing protein. A zinc motif is essential for catalytic activity, and noncatalytic auxiliary factor(s) of the editing complex are widely distributed. *J. Biol. Chem.*, **269**, 21725–21734.
20. Harris,R.S. and Liddament,M.T. (2004) Retroviral restriction by APOBEC proteins. *Nat. Rev. Immunol.*, **4**, 868–877.
21. Betts,L., Xiang,S., Short,S.A., Wolfenden,R. and Carter,C.W.J. (1994) Cytidine deaminase. The 2.3 A crystal structure of an enzyme: transition-state analog complex. *J. Mol. Biol.*, **235**, 635–656.
22. Zennou,V., Perez-Caballero,D., Gottlinger,H. and Bieniasz,P.D. (2004) APOBEC3G incorporation into human immunodeficiency virus type 1 particles. *J. Virol.*, **78**, 12058–12061.
23. 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) DNA deamination mediates innate immunity to retroviral infection. *Cell*, **113**, 803–809.
24. Mangeat,B., Turelli,P., Caron,G., Friedli,M., Perrin,L. and Trono,D. (2003) Broad antiretroviral defence by human APOBEC3G through lethal editing of nascent reverse transcript. *Nature*, **424**, 99–103.
25. Zhang,H., Yang,B., Pomerantz,R.J., Zhang,C., Arunachalam,S.C. and Gao,L. (2003) The cytidine deaminase CEM15 induces hypermutation in newly synthesized HIV-1 DNA. *Nature*, **424**, 94–98.
26. Yu,Q., Konig,R., Pillai,S., Chiles,K., Kearney,M., Palmer,S., Richman,D., Coffin,J.M. and Landau,N.R. (2004) Single-strand specificity of APOBEC3G accounts for minus-strand deamination of the HIV genome. *Nat. Struct. Mol. Biol.*, **11**, 435–442.
27. Kobayashi,M., Takaori-Kondo,A., Shindo,K., Abudu,A., Fukunaga,K. and Uchiyama,T. (2004) APOBEC3G targets specific virus species. *J. Virol.*, **78**, 8238–8244.
28. Liddament,M.T., Brown,W.L., Schumacher,A.J. and Harris,R.S. (2004) APOBEC3F properties and hypermutation preferences indicate activity against HIV-1 in vivo. *Curr. Biol.*, **14**, 1385–1391.
29. Wiegand,H.L., Doehle,B.P., Bogerd,H.P. and Cullen,B.R. (2004) A second human antiretroviral factor, APOBEC3F, is suppressed by the HIV-1 and HIV-2 Vif proteins. *EMBO J.*, **23**, 2451–2458.
30. Fujino,T., Navaratnam,N., Jarmuz,A., Von Haeseler,A. and Scott,J. (1999) C – U editing of apolipoprotein B mRNA in marsupials: identification and characterization of APOBEC-1 from the American opossum *Monodelphus domestica*. *Nucleic Acids Res.*, **27**, 2662–2671.
31. Doehle,B.P., Schafer,A., Wiegand,H.L., Bogerd,H.P. and Cullen,B.R. (2005) Differential sensitivity of murine leukemia virus to APOBEC3-mediated inhibition is governed by virion exclusion. *J. Virol.*, **79**, 8201–8207.
32. Browne,E.P. and Littman,D.R. (2008) Species specific restriction of Apobec3, mediated hypermutation. *J. Virol.*, **82**, 1305–1313.
33. Newman,E.N., Holmes,R.K., Craig,H.M., Klein,K.C., Lingappa,J.R., Malim,M.H. and Sheehy,A.M. (2005) Antiviral function of APOBEC3G can be dissociated from cytidine deaminase activity. *Curr. Biol.*, **15**, 166–170.
34. Chiu,Y.-L., Soros,V.B., Kreisberg,J.F., Stopak,K., Yonemoto,W. and Greene,W.C. (2005) Cellular APOBEC3G restricts HIV-1 infection in restricting CD4+ T cells. *Nature*, **435**, 108–114.
35. Turelli,P., Mangeat,B., Jost,S.S., Vianin,S. and Trono,D. (2004) Inhibition of hepatitis B virus replication by APOBEC3G. *Science*, **303**, 1829.
36. Miyagi,E., Opi,S., Takeuchi,H., Khan,M., Goila-Gaur,R., Kao,S. and Strebel,K. (2007) Enzymatically active APOBEC3G is required for efficient inhibition of human immunodeficiency virus type 1. *J. Virol.*, **81**, 13346–13353.
37. Schumacher,A.J., Hache,G., MacDuff,D.A., Brown,W.L. and Harris,R.S. (2008) The DNA deaminase activity of human APOBEC3G is required for Ty1, MusD and human immunodeficiency virus type 1 restriction. *J. Virol.*, **82**, 2652–2660.
38. Lau,P.P., Xiong,W.J., Zhu,H.J., Chen,S.H. and Chan,L. (1991) Apolipoprotein B mRNA editing is an intranuclear event that occurs posttranscriptionally coincident with splicing and polyadenylation. *J. Biol. Chem.*, **266**, 20550–20554.
39. Lellek,H., Kirsten,R., Diehl,L., Apostel,F., Buck,F. and Greeve,J. (2000) Purification and molecular cloning of a novel essential component of the apolipoprotein B mRNA editing enzyme-complex. *J. Biol. Chem.*, **275**, 19848–19856.
40. Mehta,A., Kinter,M.T., Sherman,N.E. and Driscoll,D.M. (2000) Molecular cloning of apobec-1 complementation factor, a novel RNA-binding protein involved in the editing of apolipoprotein B mRNA. *Mol. Cell. Biol.*, **20**, 1846–1854.
41. Chester,A., Somasekaram,A., Tzimina,M., Jarmuz,A., Gisbourne,J., O'Keefe,R., Scott,J. and Navaratnam,N. (2003) The apolipoprotein B mRNA editing complex performs a multifunctional cycle and suppresses nonsense-mediated decay. *EMBO J.*, **22**, 3971–3982.

42. Jiao,S., Moberly,J.B. and Schonfeld,G. (1990) Editing of apolipoprotein B messenger RNA in differentiated Caco-2 cells. *J. Lipid Res.*, **31**, 695–700.
43. Zheng,Y.-H., Irwin,D., Kurosu,T., Tokunaga,K., Sata,T. and Peterlin,B.M. (2004) Human APOBEC3F is another host factor that blocks human immunodeficiency virus type 1 replication. *J. Virol.*, **78**, 6073–6076.
44. Katzourakis,A., Tristem,M., Pybus,O.G. and Gifford,R.J. (2007) Discovery and analysis of the first endogenous lentivirus. *Proc. Natl Acad. Sci. USA*, **104**, 6261–6265.
45. Browning,J., Horner,J.W., Pettoello-Mantovani,M., Raker,C., Yurasov,S., DePinho,R.A. and Goldstein,H. (1997) Mice transgenic for human CD4 and CCR5 are susceptible to HIV infection. *Proc. Natl Acad. Sci. USA*, **94**, 14637–14641.
46. Sawada,S., Gowrishankar,K., Kitamura,R., Suzuki,M., Suzuki,G., Tahara,S. and Koito,A. (1998) Disturbed $CD4^+$ T cell homeostasis and in vitro HIV-1 susceptibility in transgenic mice expressing T cell line-tropic HIV-1 receptors. *J. Exp. Med.*, **187**, 1439–1449.
47. Keppler,O.T., Welte,F.J., Ngo,T.A., Chin,P.A., Patton,K.S., Tsou,C.-L., Abbey,N.W., Sharkey,M.E., Grant,R.M., You,Y. *et al.* (2002) Progress toward a human CD4/CCR5 transgenic rat model for de novo infection by human immunodeficiency virus type 1. *J. Exp. Med.*, **195**, 719–736.
48. Dunn,C.S., Mehtali,M., Houdebine,L.M., Gut,J.-P., Kirn,A. and Aubertin,A.-M. (1995) Human immunodeficiency virus type 1 infection of human CD4-transgenic rabbits. *J. Gen. Virol.*, **76**, 1327–1336.
49. Speck,R.F., Penn,M.L., Wimmer,J., Esser,U., Hague,B.F., Kindt,T.J., Atchison,R.E. and Goldsmith,M.A. (1998) Rabbit cells expressing human CD4 and human CCR5 are highly permissive for human immunodeficiency virus type 1 infection. *J. Virol.*, **72**, 5728–5734.
50. Stremlau,M., Owens,C.M., Perron,M.J., Kiessling,M., Autissier,P. and Sodroski,J. (2004) The cytoplasmic body component TRIM5alpha restricts HIV-1 infection in old world monkeys. *Nature*, **427**, 848–853.
51. Schaller,T., Hue,S. and Towers,G.J. (2007) An active TRIM5 in rabbits indicates a common antiviral ancestor for mammalian TRIM5 proteins. *J. Virol.*, **81**, 11713–11721.