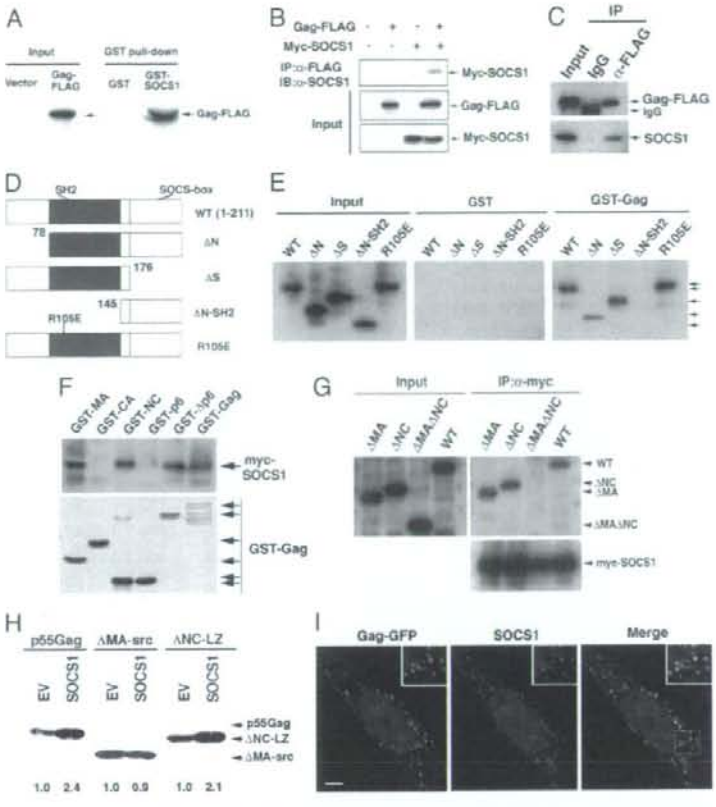


**Fig. 2.** SOCS1 interacts with HIV-1 Gag. (*A*) Extracts of 293T cells transfected with either empty vector or Gag-FLAG were subjected to pull-down analyses using glutathione–agarose beads with GST-SOCS1 in the presence of 10 ng/ml RNase followed by immunoblotting with anti-FLAG antibodies. (*B*) Extracts 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

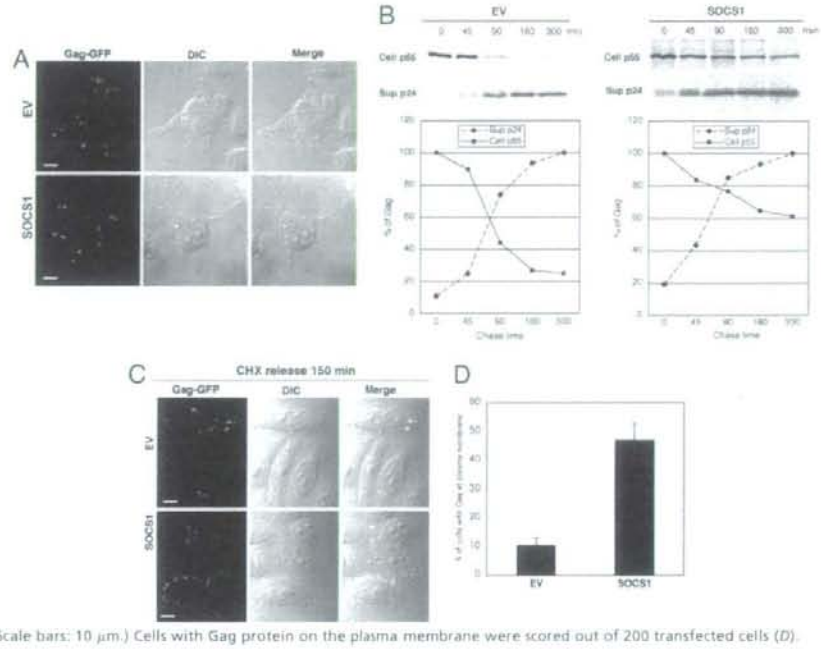


**Fig. 3.** SOCS1 enhances both the stability and trafficking of HIV-1 Gag. (*A*) HeLa cells cotransfected with pNL4-3 and either control vector (EV) or SOCS1 were immunostained with antibodies targeting anti-p24 (CA). Confocal microscopy with differential interference contrast (DIC) was then performed. (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 $Z_{II}$-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

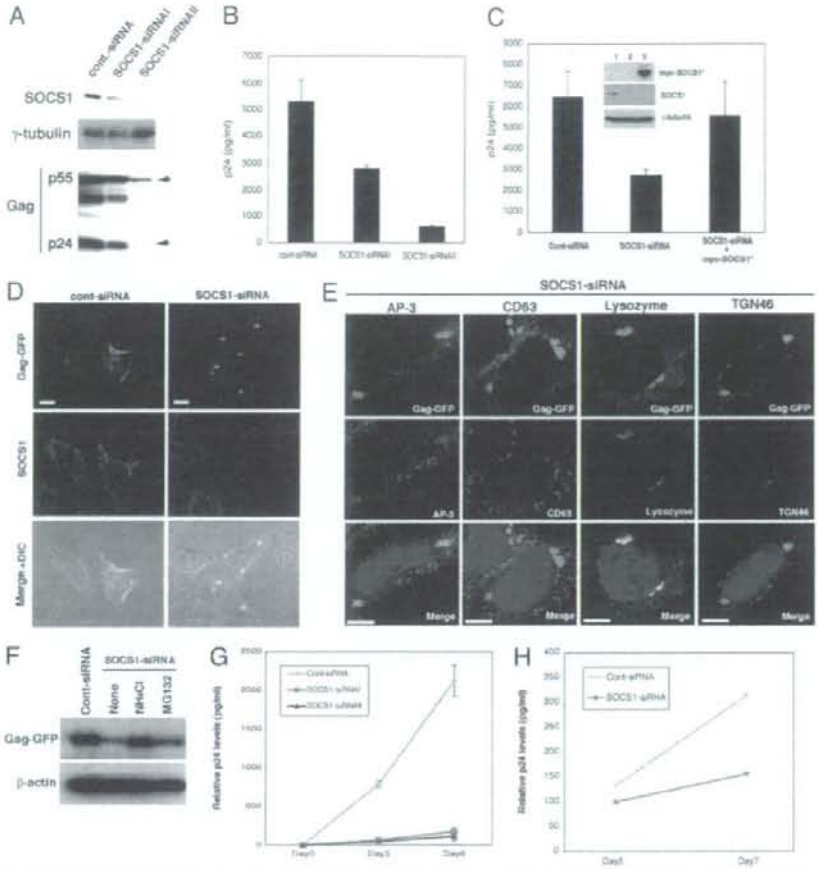


**Fig. 4.** The targeted inhibition of SOCS1 suppresses Gag trafficking and HIV-1 particle production and enhances Gag degradation in lysosomes. (*A* and *B*) 293T cells were transfected with either control siRNA or two different SOCS1-specific siRNAs (I or II) together with pNL4-3. At 48 h after transfection, 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 $\mu$M MG132 for another 16 h. Cell were then harvested and subjected to immunoblotting analysis with anti-GFP or anti-$\beta$-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).

**ACKNOWLEDGMENTS.** We thank Dr. H Gottlinger (University of Massachusetts) for providing plasmids. This work was supported in part by grants from the Ministry of Education, Culture, Sports, Science, and Technology of Japan and Human Health Science of Japan.

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JOURNAL OF VIROLOGY, Apr. 2009, p. 3059–3068 Vol. 83, No. 7
0022-538X/09/$08.00+0 doi:10.1128/JVI.02539-08


# Non-Cleavage Site Gag Mutations in Amprenavir-Resistant Human Immunodeficiency Virus Type 1 (HIV-1) Predispose HIV-1 to Rapid Acquisition of Amprenavir Resistance but Delay Development of Resistance to Other Protease Inhibitors▽

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Received 10 December 2008/Accepted 20 January 2009

**In an attempt to determine whether mutations in Gag in human immunodeficiency virus type 1 (HIV-1) variants selected with a protease inhibitor (PI) affect the development of resistance to the same or a different PI(s), we generated multiple infectious HIV-1 clones carrying mutated Gag and/or mutated protease proteins that were identified in amprenavir (APV)-selected HIV-1 variants and examined their virological characteristics. In an HIV-1 preparation selected with APV (33 passages, yielding $HIV_{APVp33}$), we identified six mutations in protease and six apparently critical mutations at cleavage and non-cleavage sites in Gag. An infectious recombinant clone carrying the six protease mutations but no Gag mutations failed to replicate, indicating that the Gag mutations were required for the replication of $HIV_{APVp33}$. An infectious recombinant clone that carried wild-type protease and a set of five Gag mutations ($rHIV_{WTpro}^{12/75/219/390/409gag}$) replicated comparably to wild-type HIV-1; however, when exposed to APV, $rHIV_{WTpro}^{12/75/219/390/409gag}$ rapidly acquired APV resistance. In contrast, the five Gag mutations significantly delayed the acquisition of HIV-1 resistance to ritonavir and nelfinavir (NFV). Recombinant HIV-1 clones containing NFV resistance-associated mutations, such as D30N and N88S, had increased susceptibilities to APV, suggesting that antiretroviral regimens including both APV and NFV may bring about favorable antiviral efficacy. The present data suggest that the preexistence of certain Gag mutations related to PI resistance can accelerate the emergence of resistance to the PI and delay the acquisition of HIV resistance to other PIs, and these findings should have clinical relevance in the therapy of HIV-1 infection with PI-including regimens.**

Combination antiretroviral therapy using reverse transcriptase inhibitors and protease inhibitors (PIs) produces substantial suppression of viral replication in human immunodeficiency virus type 1 (HIV-1)-infected patients (3, 27, 28, 42). However, the emergence of drug-resistant HIV-1 variants in such patients has limited the efficacy of combination chemotherapy. HIV-1 variants resistant to all of the currently available antiretroviral therapeutics have emerged both in vitro and in vivo (6, 16, 27, 30). Of note, a number of PI resistance-associated amino acid substitutions in the active site of protease have been identified, and such substitutions have considerable impact on the catalytic activity of protease. This impact is reflected by impaired processing of Gag precursors in mutated-protease-carrying virions and by decreased catalytic efficiency of the protease toward peptides with natural cleavage sites (7, 29, 31, 43).

However, the highly PI-resistant viruses frequently have amino acid substitutions at the p7-p1 and p1-p6 cleavage sites in Gag. These mutations have been identified in PI-resistant HIV-1 variants selected in vitro (2, 5, 8, 29) and in HIV-1 isolated from patients with AIDS for whom chemotherapy including PIs was failing (26, 40, 47, 48). These mutations are known to compensate for the enzymatic impairment of protease, per se, resulting from the acquisition of PI resistance-conferring mutations within the protease-encoding region. Moreover, certain mutations at non-cleavage sites in Gag have been shown previously to be essential for the replication of HIV-1 variants in the presence of PIs (14, 15). Although a few amino acid substitutions at cleavage and non-cleavage sites in Gag have been shown to be associated with resistance to PIs, the roles and impact of amino acid substitutions in Gag for the HIV-1 acquisition of PI resistance remain to be elucidated.

In the present study, we identified novel Gag non-cleavage site mutations in addition to multiple mutations in the protease gene during in vitro selection of HIV-1 variants highly resistant to amprenavir (APV). We show that the non-cleavage site mutations were important for not only the replication of the mutated-protease-carrying HIV-1 but also the accelerated acquisition of HIV-1 resistance to APV and an unrelated PI, nelfinavir (NFV). We also show that recombinant HIV-1 clones containing NFV resistance-associated mutations, such

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▽ Published ahead of print on 28 January 2009.

as D30N and N88S, had increased susceptibility to APV, suggesting that antiretroviral regimens including both APV and NFV may bring about favorable antiviral efficacy.

## MATERIALS AND METHODS

**Cells and antiviral agents.** MT-2 and MT-4 cells were grown in RPMI 1640-based culture medium, and 293T cells were propagated in Dulbecco's modified Eagle's medium. These media were supplemented with 10% fetal calf serum (HyClone, Logan, UT), 50 U/ml penicillin, and 50 μg/ml streptomycin. APV was kindly provided by GlaxoSmithKline, Research Triangle Park, NC. Saquinavir (SQV) and ritonavir (RTV) were provided by Roche Products Ltd. (Welwyn Garden City, United Kingdom) and Abbott Laboratories (Abbott Park, IL), respectively. NFV and indinavir (IDV) were kindly provided by Japan Energy Inc., Tokyo.

**Generation of PI-resistant HIV-1 in vitro.** For the generation of PI-resistant HIV-1, various PI-resistant HIV-1 strains were propagated in the presence of increasing concentrations of a drug in a cell-free fashion as described previously (44, 45). In brief, on the first passage, MT-2 or MT-4 cells ($5 \times 10^5$) were exposed to 500 50% tissue culture infective doses ($TCID_{50}$) of each infectious molecular HIV-1 clone and cultured in the presence of various PIs at initial concentrations of 0.01 to 0.06 μM. On the last day of each passage (approximately day 7), 1 ml of the cell-free supernatant was harvested and transferred to a culture of fresh uninfected cells in the presence of increased concentrations of the drug for the following round of culture. In this round of culture, three drug concentrations (increased by one-, two-, and threefold compared to the previous concentration) were employed. When the replication of HIV-1 in the culture was confirmed by substantial Gag protein production (greater than 200 ng/ml), the highest drug concentration among the three concentrations was used to continue the selection (for the next round of culture). This protocol was repetitively used until the drug concentration reached the targeted concentration. Proviral DNA from the lysates of infected cells at various passages was subjected to nucleotide sequencing.

**Determination of nucleotide sequences.** Molecular cloning and the determination of nucleotide sequences of HIV-1 passaged in the presence of each PI were performed as described previously (44, 45). In brief, high-molecular-weight DNA was extracted from HIV-1-infected MT-2 and MT-4 cells by using the InstaGene matrix (Bio-Rad Laboratories, Hercules, CA) and was subjected to molecular cloning, followed by sequence determination. The primers used for the first-round PCR amplification of the entire Gag- and protease-encoding regions of the HIV-1 genome were LTR F1 (5'-GAT GCT ACA TAT AAG CAG CTG C-3') and PR12 (5'-CTC GTG ACA AAT TTC TAC TAA TGC-3'). The first-round PCR mixture consisted of 5 μl of proviral DNA solution, 2.0 U of premix *Taq* (Ex *Taq* version; Takara Bio Inc., Otsu, Japan), and 12.5 pmol of each of the first-round PCR primers in a total volume of 50 μl. The PCR conditions used were an initial 2-min step at 94°C, followed by 30 cycles of 30 s at 94°C, 30 s at 58°C, and 3 min at 72°C, with a final 8 min of extension at 72°C. The first-round PCR products (1 μl) were used directly in the second round of PCR with primers LTR F2 (5'-GAG ACT CTG GTA ACT AGA GAT C-3') and Ksma2.1 (5'-CCA TCC CGG GCT TTA ATT TTA CTG GTA C-3') under the same PCR conditions described above. The second-round PCR products were purified with spin columns (MicroSpin S-400 HR; Amersham Biosciences Corp., Piscataway, NJ), cloned directly, and subjected to sequencing with a model 377 automated DNA sequencer (Applied Biosystems, Foster City, CA).

**Generation of recombinant HIV-1 clones.** The PCR products obtained as described above were digested with two of the three enzymes BssHII, ApaI, and SmaI, and the obtained fragments were introduced into $pHIV\text{-}1_{NLSma}$, designed to have a SmaI site by changing two nucleotides (2590 and 2593) of $pHIV\text{-}1_{NL4\text{-}3}$ (15, 19). To generate HIV-1 clones carrying the mutations, site-directed mutagenesis using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) was performed, and the mutation-containing genomic fragments were introduced into $pHIV\text{-}1_{NLSma}$. Determination of the nucleotide sequences of plasmids confirmed that each clone had the desired mutations but no unintended mutations. 293T cells were transfected with each recombinant plasmid by using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA), and the thus-obtained infectious virions were harvested 48 h after transfection and stored at −80°C until use.

**Drug sensitivity assays.** Assays for HIV-1 p24 Gag protein production were performed with MT-4 cells as described previously (1, 20, 24). In brief, MT-4 cells ($10^5$/ml) were exposed to 100 $TCID_{50}$ of infectious molecular HIV-1 clones in the presence or absence of various concentrations of drugs and were incubated at 37°C. On day 7 of culture, the supernatant was harvested and the amounts of p24 Gag protein were determined by using a fully automated chemiluminescent enzyme immunoassay system (Lumipulse F; Fujirebio Inc., Tokyo). The drug concentrations that suppressed the production of p24 Gag protein by 50% (50% inhibitory concentrations [$IC_{50}$]) were determined by comparing the levels of p24 production with that in a drug-free control cell culture. All assays were performed in triplicate.

**Replication kinetic assay.** MT-2 or MT-4 cells ($10^5$) were exposed to each infectious HIV-1 clone (5 ng of p24 Gag protein/ml) for 3 h, washed twice with phosphate-buffered saline, and cultured in 10 ml of complete medium as described previously (1, 14). Culture supernatants (50 μl) were harvested every other day, and the p24 Gag amounts were determined as described above.

**CHRA.** Two titrated infectious clones to be compared for their replicative capabilities or fitness in the competitive HIV-1 replication assay (CHRA) were combined and added to freshly prepared MT-4 cells ($2 \times 10^5$) in the presence or absence of various concentrations of PIs as described previously (21, 36). Briefly, a fixed amount (200 $TCID_{50}$) of one infectious clone was combined with three different amounts (100, 200, and 300 $TCID_{50}$) of the other infectious clone, and the mixture was added to the culture of MT-4 cells. On the following day, one-third of infected MT-4 cells were harvested and washed twice with phosphate-buffered saline, and cellular DNA was extracted and subjected to nested PCR and sequencing as described above. The HIV-1 coculture that best approximated a 50:50 mixture on day 1 was further propagated, and the remaining cultures were discarded. Every 7 days, the cell-free supernatant of the virus coculture was transmitted to fresh uninfected MT-4 cells. The cells harvested at the end of each passage were subjected to direct DNA sequencing, and viral population changes were determined. The persistence of the original amino acid substitutions was confirmed for all infectious clones used in this assay.

**Statistical analysis of selection profiles of infectious HIV-1 clones.** The selection profiles of various infectious HIV-1 clones were compared as follows. The logarithms of the concentrations were modeled as normally distributed variables with possible left censoring. The mean was assumed to be a quadratic function of the passage number. The difference between two curves was assessed by combining the estimated covariance-weighted differences of the linear and quadratic coefficients and comparing the result to computer simulations for the same quantity generated under the specific null hypothesis for that difference. SAS 9.1.3 (SAS Institute, Cary, NC) was used for all the computations. All *P* values are two tailed, and for figures with more than two curves, the values were corrected by the Hochberg method for multiple pairwise comparisons.

## RESULTS

**Amino acid sequences of Gag and protease of HIV-1 passaged in the presence of APV.** A wild-type HIV-1 strain ($HIV_{WT}$) was propagated in MT-2 cells in the presence of increasing concentrations of APV, and the proviral DNA sequences in those MT-2 cells were determined at passages 3, 12, and 33 (Fig. 1). By passage 3, when HIV-1 was propagating in the presence of 0.04 μM APV (yielding $HIV_{APVp3}$), no amino acid substitutions in protease were identified but 5 of 10 clones had acquired the substitution of arginine for leucine at position 75 (L75R) in Gag. By passage 12 (at 0.18 μM APV), two APV-related resistance mutations (L10F and M46L) in protease had emerged and one mutation (H219Q) in Gag had been added. By passage 33 (at 10 μM; yielding $HIV_{APVp33}$), six APV-related amino acid substitutions, one primary mutation (I84V) and five secondary mutations (L10F, V32I, M46I, I54M, and A71V), in protease had emerged (Fig. 1A). In addition, a p1-p6 cleavage site mutation in Gag (L449F) was identified in all 10 HIV-1 clones of $HIV_{APVp33}$ examined, and five non-cleavage site mutations (E12K, L75R, H219Q, V390D, and R409K) were seen in Gag of $HIV_{APVp33}$ (Fig. 1B). Cleavage site mutations have been known to emerge when amino acid substitutions in protease are accumulated and HIV-1 develops resistance to PIs both in vitro and in vivo (5, 8). Intriguingly, the present data suggest that certain amino acid substitutions in non-cleavage sites of Gag (i.e., L75R and

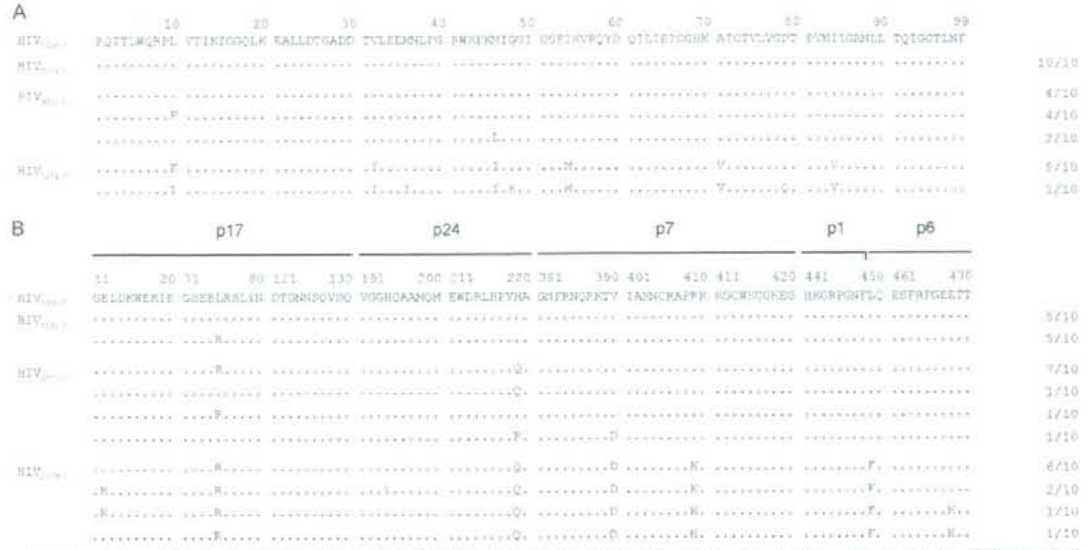


FIG. 1. Amino acid sequences deduced from the nucleotide sequences of protease (A)- and Gag (B)-encoding regions of proviral DNA isolated at the indicated passages (p3, p12, and p33) from HIV-$1_{NL4-3}$ variants selected in the presence of APV. The amino acid sequences of the protease and Gag proteins of wild-type HIV-$1_{NL4-3}$ are shown at the top as a reference. Identity to the sequence at individual amino acid positions is indicated by dots. The numbers of clones with the given amino acid substitutions among a total of 10 clones are listed.

H219Q) may emerge earlier and in greater numbers than amino acid substitutions in protease, at least in the case of HIV-1 selection with APV. The amino acid substitutions that emerged in the virus and the pattern and order of such substitutions were largely in agreement with the data in the previous report by Gatanaga et al. (15). The present results suggested that the non-cleavage site mutations observed may play a key role in the development of HIV-1 resistance against PIs and that especially the two Gag mutations H219Q and R409K may be required for the development of PI resistance.

**Mutations in Gag are required for the replication of $HIV_{APVp33}$.** In order to examine the effects of the mutations identified in Gag as described above on the replication profile of HIV-1, we generated infectious recombinant HIV-1 clones containing the six mutations (L10F, V32I, M46I, I54V, A71V, and I84V) in protease seen in $HIV_{APVp33}$. A recombinant HIV-1 clone containing the protease of $HIV_{APVp33}$ plus a wild-type Gag ($rHIV_{APVp33pro}^{WTgag}$) or the L449F cleavage site mutation-containing Gag ($rHIV_{APVp33pro}^{449gag}$) failed to replicate in MT-2 cells over the 7-day period of culture (Fig. 2A), indicating that these Gag species do not support the growth of $HIV_{APVp33}$. Therefore, we next generated a recombinant HIV-1 clone containing the protease of $HIV_{APVp33}$ and the Gag protein with the five non-cleavage site mutations (E12K, L75R, H219Q, V390D, and R409K; $rHIV_{APVp33pro}^{12/75/219/390/409gag}$), which replicated moderately under the same conditions (Fig. 2A). The addition of the cleavage site mutation L449F, generating $rHIV_{APVp33pro}^{12/75/219/390/409/449gag}$, further improved the replication of the virus. In MT-4 cells, in which HIV-1 generally replicates more quickly and efficiently than in MT-2 cells, $rHIV_{APVp33pro}^{WTgag}$ and $rHIV_{APVp33pro}^{449gag}$ replicated moderately; however, both $rHIV_{APVp33pro}^{12/75/219/390/409gag}$ and $rHIV_{APVp33pro}^{12/75/219/390/409/449gag}$ replicated comparably to $HIV_{WT}$ (Fig. 2B), due presumably to the greater replication of HIV-1 in MT-4 cells, making the difference relatively indistinct. These data clearly indicate that both non-cleavage site and cleavage site mutations in Gag contribute to the robust fitness of $HIV_{APVp33}$. We also attempted to examine the effects of combined Gag mutations on the replication of HIV-1 containing wild-type protease and generated three recombinant HIV clones, $rHIV_{WTpro}^{75/219gag}$, $rHIV_{WTpro}^{219/409gag}$, and $rHIV_{WTpro}^{12/75/219/390/409gag}$. The replication rates of these three recombinant clones turned out to be comparable to that of $HIV_{WT}$ when examined in MT-2 and MT-4 cells (Fig. 2C and D), unlike the finding by Doyon and his colleagues that the cleavage site mutation L449F compromised the replication of HIV-1 containing wild-type protease (8).

**Gag mutations predispose HIV-1 to rapidly acquire APV resistance.** The appearance of two non-cleavage site mutations (L75R and H219Q) in Gag prior to the emergence of mutations in protease (Fig. 1) prompted us to examine whether these two Gag mutations predisposed the virus to the acquisition of APV resistance-associated mutations in protease. We thus attempted to select APV-resistant HIV-1 by propagating $HIV_{NL4-3}$ ($HIV_{WT}$) and $rHIV_{WTpro}^{75/219gag}$ in the presence of increasing concentrations of APV (Fig. 3). When we compared the selection curves of these two viruses, there was no significant difference ($P$, 0.53 and 0.65 for propagation in MT-2 and MT-4 cells, respectively). We then examined the effects of two mutated Gag species containing two and five mutations (H219Q and R409K and E12K, L75R, H219Q, V390D, and

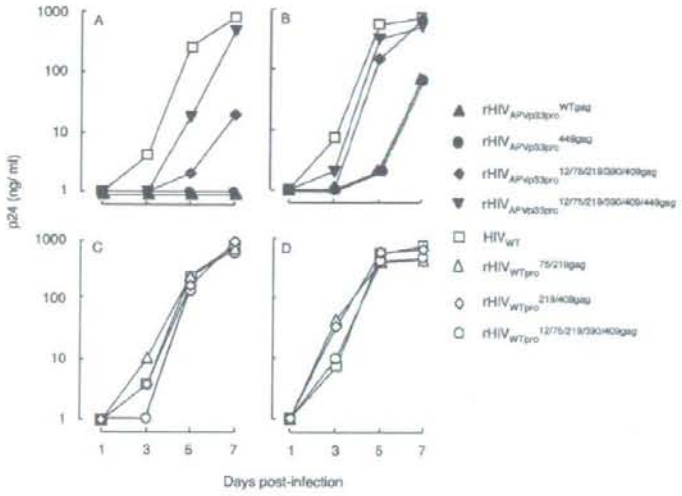


FIG. 2. Replication kinetics of Gag mutant clones with or without protease mutations. MT-2 cells (A and C) and MT-4 cells (B and D) were exposed to Gag mutant clones with (A and B) or without (C and D) protease mutations. Virus replication was monitored by the amounts of p24 Gag produced in the culture supernatants. The results shown are representative of results from three independent experiments. HIV$_{APVp33}$ variants had six mutations (L10F, V32I, M46I, I54M, A71V, and I84V) in the viral protease.

R409K [yielding mGag$^{12/75/219/390/409gag}$], respectively) on the selection curves. The selection profile of a newly generated recombinant HIV clone (rHIV$_{WTpro}^{219/409gag}$) was not different from that of HIV$_{WT}$ in MT-2 cells ($P = 0.22$); however,

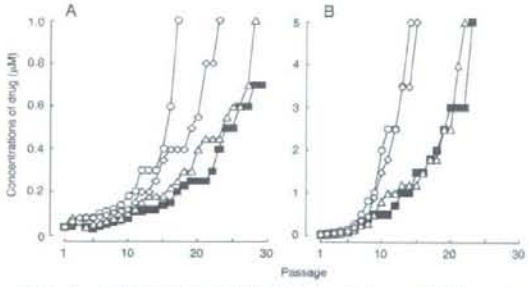


FIG. 3. In vitro selection of APV-resistant variants using HIV-1 carrying Gag mutations. HIV$_{WT}$ (■) and three infectious HIV clones, rHIV$_{WTpro}^{75/219gag}$ (△), rHIV$_{WTpro}^{219/409gag}$ (◇), and rHIV$_{WTpro}^{12/75/219/390/409gag}$ (○), were propagated in the presence of increasing concentrations of APV (starting at 0.03 μM) in MT-2 cells (A) or MT-4 cells (B). The selection was carried out in a cell-free manner for a total of 14 to 29 passages. The results of statistical evaluation of the selection profiles are as follows: panel A, HIV$_{WT}$ versus rHIV$_{WTpro}^{75/219gag}$, $P = 0.53$; HIV$_{WT}$ versus rHIV$_{WTpro}^{12/75/219/390/409gag}$, $P = 0.0080$; HIV$_{WT}$ versus rHIV$_{WTpro}^{219/409gag}$, $P = 0.22$; rHIV$_{WTpro}^{75/219gag}$ versus rHIV$_{WTpro}^{12/75/219/390/409gag}$, $P = 0.0065$; rHIV$_{WTpro}^{75/219gag}$ versus rHIV$_{WTpro}^{219/409gag}$, $P = 0.15$; and rHIV$_{WTpro}^{12/75/219/390/409gag}$ versus rHIV$_{WTpro}^{219/409gag}$, $P = 0.0018$, and panel B, HIV$_{WT}$ versus rHIV$_{WTpro}^{75/219gag}$, $P = 0.65$; HIV$_{WT}$ versus rHIV$_{WTpro}^{12/75/219/390/409gag}$, $P < 0.0001$; HIV$_{WT}$ versus rHIV$_{WTpro}^{219/409gag}$, $P < 0.0001$; rHIV$_{WTpro}^{75/219gag}$ versus rHIV$_{WTpro}^{12/75/219/390/409gag}$, $P < 0.0001$; rHIV$_{WTpro}^{75/219gag}$ versus rHIV$_{WTpro}^{219/409gag}$, $P < 0.0001$; and rHIV$_{WTpro}^{12/75/219/390/409gag}$ versus rHIV$_{WTpro}^{219/409gag}$, $P = 0.088$.

rHIV$_{WTpro}^{219/409gag}$ acquired resistance to APV much earlier than HIV$_{WT}$ when propagated in MT-4 cells ($P < 0.0001$). The recombinant clone with five non-cleavage site mutations (rHIV$_{WTpro}^{12/75/219/390/409gag}$) started to propagate in both cell lines in the presence of APV significantly earlier than HIV$_{WT}$, with $P$ values of 0.0080 and <0.0001 for MT-2 and MT-4 cells, respectively (Fig. 3).

We then asked whether additional amino acid substitutions occurred and accelerated the acquisition of APV resistance by the virus when the Gag mutations were present. To investigate this issue, we determined the nucleotide sequence of the protease-encoding gene of each virus. Only one protease mutation (L10F) was seen by passage 20 when HIV$_{WT}$ and rHIV$_{WTpro}^{75/219gag}$ were propagated in MT-2 cells in the presence of APV (Fig. 4A and B). In contrast, two mutations (M46L and I84V) had been acquired by rHIV$_{WTpro}^{219/409gag}$ by passage 20. Of note, when rHIV$_{WTpro}^{12/75/219/390/409gag}$ was propagated in MT-2 cells in the presence of APV, a mutation (L10F) had occurred by an early passage (passage 5) and four mutations (L10F, V32I, M46I, and I84V) had emerged by passage 17 (Fig. 4D). When examined in MT-4 cells, HIV$_{WT}$ and rHIV$_{WTpro}^{75/219gag}$ had acquired two mutations (L10F and I84V and M46L and I84V, respectively) by passage 10, although rHIV$_{WTpro}^{219/409gag}$ and rHIV$_{WTpro}^{12/75/219/390/409gag}$ had acquired three and four mutations (L10F, M46I, and I84V and L10F, V32I, M46I, and I84V, respectively) by the same passage (Fig. 4E to H). These data, taken together, indicate that the two sets of Gag mutations (H219Q and R409K and E12K, L75R, H219Q, V390D, and R409K) clearly predisposed the virus to rapidly acquire APV resistance-associated mutations in protease and begin to propagate in the presence of APV.

**Gag mutations in HIV$_{APVp33}$ delay viral acquisition of resistance to other PIs.** We next asked whether the presence of

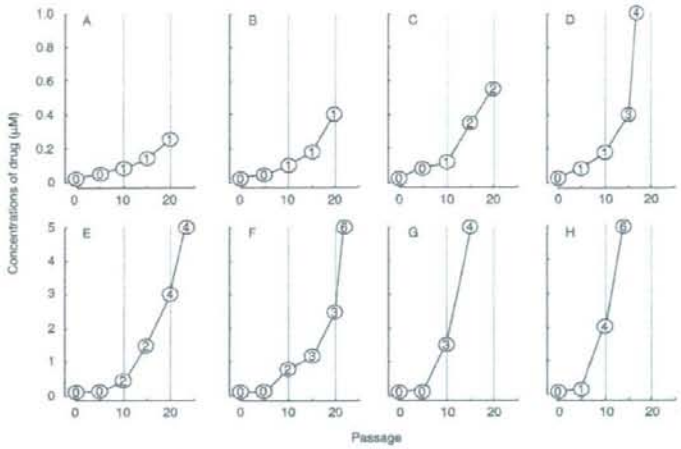


FIG. 4. Number of amino acid substitutions corresponding to the protease-encoding region of each infectious HIV-1 clone selected in the presence of APV. Nucleotide sequences of proviral DNA of $HIV_{WT}$ (A and E) and three infectious HIV-1 clones, $rHIV_{WTpro}^{75/219gag}$ (B and F), $rHIV_{WTpro}^{219/409gag}$ (C and G), and $rHIV_{WTpro}^{12/75/219/390/409gag}$ (D and H), were determined using lysates of HIV-1-infected MT-2 cells (A to D) and MT-4 cells (E to H) at the termination of each passage and compared to the nucleotide sequence of $HIV\text{-}1_{NL4\text{-}3}$. The number within each symbol represents the number of mutations identified in the protease when each infectious HIV-1 clone was selected in the presence of APV.

the five Gag mutations (E12K, L75R, H219Q, V390D, and R409K) accelerated the viral acquisition of resistance to other currently available PIs (SQV, IDV, RTV, and NFV) (Fig. 5). To this end, we propagated two HIV-1 strains ($HIV_{WT}$ and $rHIV_{WTpro}^{12/75/219/390/409gag}$) in MT-4 cells in the presence of increasing concentrations of each PI and compared the replication profiles. The initial drug concentrations used were 0.01 μM for SQV, 0.03 μM for IDV and NFV, and 0.06 μM for RTV, and each virus was selected by a concentration of up to 5 μM. The selection was carried out in a cell-free manner for a total of 13 to 32 passages as described previously (44, 45). There was no significant difference in the selection profiles of the two strains when they were passaged in the presence of SQV ($P = 0.8$) or IDV ($P = 0.22$) (Fig. 5A and B). However, $rHIV_{WTpro}^{12/75/219/390/409gag}$ started to replicate significantly later in the presence of RTV ($P = 0.0001$ (Fig. 5C). The selection profiles of $HIV_{WT}$ and $rHIV_{WTpro}^{12/75/219/390/409gag}$ in the presence of NFV were examined in two independent experiments. Both curves in the first and second sets depicted in Fig. 5D showed statistically significant difference, with $P$ values of <0.0001 and 0.0016, respectively. These data strongly suggest that the Gag mutations seen in $HIV_{APVp33}$ predispose HIV-1 to the rapid acquisition of APV resistance; however, such Gag mutations delay the viral acquisition of resistance to other PIs.

**Gag mutations seen in $HIV_{APVp33}$ do not affect viral susceptibilities to PIs.** Since the Gag mutations seen in $HIV_{APVp33}$ were found to contribute to the rapid acquisition of viral resistance to APV but they delayed the emergence of viral resistance to other PIs, we examined whether such Gag mutations affected the susceptibilities of HIV-1 to various PIs in the HIV-1 drug susceptibility assay. As shown in Table 1, none of three sets of Gag mutations, as examined in the context of $rHIV_{WTpro}^{75/219gag}$, $rHIV_{WTpro}^{219/409gag}$, and

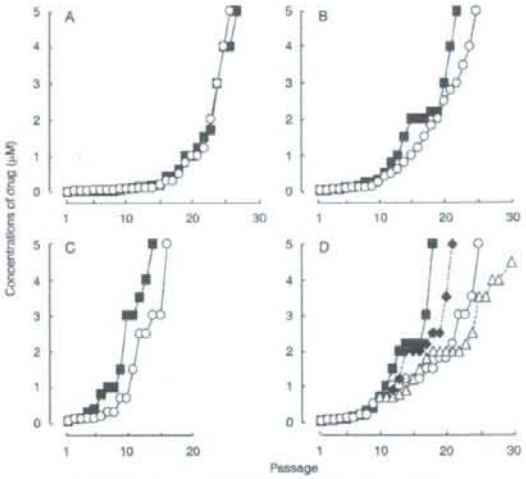


FIG. 5. In vitro selection of PI-resistant variants using HIV-1 carrying Gag mutations. $HIV_{WT}$ (■ and ◆) and $rHIV_{WTpro}^{12/75/219/390/409gag}$ (○ and △) were propagated in MT-4 cells in the presence of increasing concentrations of SQV (A), IDV (B), RTV (C), or NFV (D). The initial drug concentrations used were 0.01 μM for SQV, 0.03 μM for IDV and NFV, and 0.06 μM for RTV, and each virus was selected by up to a 5 μM concentration of each PI. The selection was carried out in a cell-free manner for a total of 13 to 32 passages. NFV selection was performed twice. Data from the first selection are shown with a solid line; the second selection was started using the HIV-1 from passage 10 of the first selection (with NFV at 0.7 μM), and the data are shown with a dashed line. The results of statistical evaluation of the selection profiles are as follows: panel A, $P = 0.80$; panel B, $P = 0.22$; panel C, $P = 0.0001$; and panel D, first selection, $P < 0.0001$, and second selection, $P = 0.0016$.

TABLE 1. Sensitivities of infectious HIV-1 clones with Gag mutations to various PIs

| Infectious HIV-1 clone | $IC_{50}$[a] (μM) of: | | | | |
|---|---|---|---|---|---|
| | APV | SQV | IDV | RTV | NFV |
| $HIV_{WT}$ | 0.031 ± 0.0008 | 0.021 ± 0.002 | 0.032 ± 0.002 | 0.032 ± 0.0005 | 0.028 ± 0.002 |
| $rHIV_{WTpro}^{75/219gag}$ | 0.031 ± 0.003 | 0.017 ± 0.003 | 0.032 ± 0.003 | 0.031 ± 0.0007 | 0.029 ± 0.003 |
| $rHIV_{WTpro}^{219/409gag}$ | 0.029 ± 0.003 | 0.020 ± 0.01 | 0.032 ± 0.001 | 0.031 ± 0.004 | 0.028 ± 0.002 |
| $rHIV_{WTpro}^{12/75/219/390/409gag}$ | 0.032 ± 0.0001 | 0.023 ± 0.005 | 0.032 ± 0.003 | 0.032 ± 0.0001 | 0.028 ± 0.002 |

[a] Data shown are mean values (with 1 standard deviation) derived from the results of three independent experiments conducted in triplicate. The $IC_{50}$s were determined by employing MT-4 cells exposed to each infectious HIV-1 clone (50 $TCID_{50}$) in the presence of each PI, with the inhibition of p24 Gag protein production as an end point.

$rHIV_{WTpro}^{12/75/219/390/409gag}$, affected the susceptibility of HIV-1 to any of five PIs (APV, SQV, IDV, RTV, and NFV). Indeed, the $IC_{50}$s for $HIV_{WT}$ were highly comparable to those for any of the three recombinant clones carrying combined Gag mutations.

**Replication rate difference is not the cause of the contrasting resistance acquisition patterns.** Our observations of the contrasting resistance acquisition patterns, in which $rHIV_{WTpro}^{12/75/219/390/409gag}$ acquired resistance to APV more rapidly than $HIV_{WT}$ when selected with APV (Fig. 3) and $rHIV_{WTpro}^{12/75/219/390/409gag}$ significantly delayed the acquisition of resistance to other PIs compared to $HIV_{WT}$ (Fig. 5), prompted us to ask whether the replication rates of $rHIV_{WTpro}^{12/75/219/390/409gag}$ and $HIV_{WT}$ were differentially affected by the presence of PIs. We therefore compared the replication rates of $rHIV_{WTpro}^{12/75/219/390/409gag}$ and $HIV_{WT}$ in the presence or absence of APV, SQV, IDV, RTV, or NFV by using the CHRA (21). As shown in Fig. 6, $rHIV_{WTpro}^{12/75/219/390/409gag}$ outgrew $HIV_{WT}$ regardless of the absence or presence of PIs. Comparing the divergence patterns of the curves for $rHIV_{WTpro}^{12/75/219/390/409gag}$ and $HIV_{WT}$ in the absence and presence of APV (Fig. 6A and B) revealed that those for growth in the presence of APV diverged more quickly than those for growth in the absence of APV (Fig. 6B). However, similar divergence patterns were seen with SQV, IDV, RTV, and NFV (Fig. 6C, D, E, and F), suggesting that the replication advantage of $rHIV_{WTpro}^{12/75/219/390/409gag}$ seen in the CHRA was not the cause for the observed contrasting resistance acquisition patterns.

**NFV resistance-conferring protease mutations increase HIV-1 susceptibility to APV.** There have been reports that an

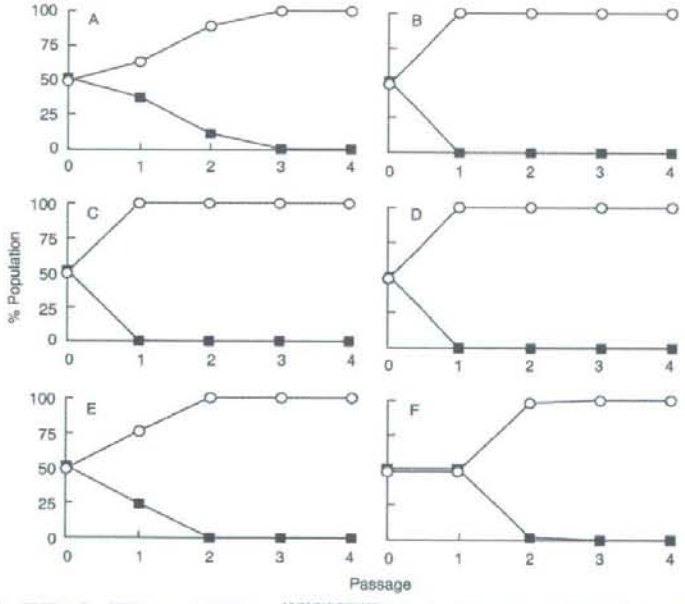


FIG. 6. Results from the CHRA for $HIV_{WT}$ and $rHIV_{WTpro}^{12/75/219/390/409gag}$ in the absence or presence of each drug. Replication profiles of $HIV_{WT}$ (■) and $rHIV_{WTpro}^{12/75/219/390/409gag}$ (○) in the absence (A) or presence of 0.03 μM APV (B), 0.02 μM SQV (C), 0.03 μM IDV (D), 0.03 μM RTV (E), or 0.03 μM NFV (F) were examined by the CHRA. The cell-free supernatant was transferred to fresh MT-4 cells every 7 days. High-molecular-weight DNA extracted from infected cells at the end of each passage was subjected to nucleotide sequencing, and the proportions of Arg and Lys at position 409 in Gag were determined.

TABLE 2. Phenotypic sensitivities of recombinant HIV-1 clones passaged with NFV[a]

| Infectious HIV-1 clone | $IC_{50}$ (μM) ± SD (change, *n*-fold) of: | |
|---|---|---|
| | APV | NFV |
| $HIV_{WT}$ | 0.031 ± 0.0008 (1) | 0.028 ± 0.002 (1) |
| $rHIV_{N88S_{pro}}^{WTgag}$ | 0.0015 ± 0.0007 (0.05) | 0.028 ± 0.001 (1) |
| $rHIV_{D30N_{pro}}^{WTgag}$ | 0.0031 ± 0.0001 (0.1) | 0.045 ± 0.001 (1.6) |
| $rHIV_{10/30/45/71_{pro}}^{WTgag}$ | 0.014 ± 0.0021 (0.45) | 0.26 ± 0.03 (9) |
| $rHIV_{10/30/45/71_{pro}}^{12/75/219/390/409_{gag}}$ | 0.020 ± 0.002 (0.64) | 0.32 ± 0.03 (11) |
| $rHIV_{30/46/77_{pro}}^{WTgag}$ | 0.0069 ± 0.0024 (0.22) | 0.25 ± 0.04 (9) |
| $rHIV_{30/46/77_{pro}}^{12/75/219/390/409_{gag}}$ | 0.0046 ± 0.0019 (0.15) | 0.21 ± 0.06 (8) |

[a] Recombinant HIV clones $rHIV_{10/30/45/71_{pro}}^{WTgag}$ and $rHIV_{10/30/45/71_{pro}}^{12/75/219/390/409_{gag}}$ were generated to have a set of four protease mutations (L10F, D30N, K45I, and A71V) and wild-type Gag or Gag with five mutations, while other clones, $rHIV_{30/46/77_{pro}}^{WTgag}$ and $rHIV_{30/46/77_{pro}}^{12/75/219/390/409_{gag}}$, were generated with three protease mutations (D30N, M46I, and V77I) and wild-type Gag or Gag with five mutations. Both sets of protease mutations were seen when HIV-1 was propagated in the presence of NFV. The $IC_{50}$s were determined by employing MT-4 cells exposed to each recombinant HIV-1 clone (50 $TCID_{50}$) in the presence of each PI, with the inhibition of p24 Gag protein production as an end point. All values were determined in triplicate, and the data are shown as mean values ±1 standard deviation of results from two or three independent experiments. The numbers in parentheses are changes (*n*-fold) compared to the $IC_{50}$ of each PI for $HIV_{WT}$.

NFV-related resistance mutation, N88S, renders HIV-1 susceptible to APV (33, 49). Since the acquisition of viral resistance to PIs such as NFV was significantly delayed when HIV-1 had the Gag mutations seen in $HIV_{APVp33}$, we asked if another NFV-related resistance mutation (D30N) would render HIV-1 more susceptible to APV. We also asked whether the presence of multiple NFV resistance-associated mutations (D30N, M46I, and V77I) would make HIV-1 susceptible to APV. Moreover, we examined the effects of the Gag mutations seen in $HIV_{APVp33}$ on HIV-1 susceptibilities to APV and NFV.

As shown in Table 2, the N88S mutant clone $rHIV_{N88S_{pro}}^{WTgag}$ was more susceptible to APV than $HIV_{WT}$ by a factor of 20, in agreement with the reports by Ziermann et al. and Resch et al. (33, 49). We found that the D30N mutation in $rHIV_{D30N_{pro}}^{WTgag}$ also made HIV-1 more susceptible to APV, by a factor of 10. Interestingly, $rHIV_{10/30/45/71_{pro}}^{WTgag}$, with the four mutations L10F, D30N, K45I, and A71V, was more resistant to NFV than $HIV_{WT}$ by a factor of 9; however, the recombinant virus remained more susceptible to APV than $HIV_{WT}$ (Table 2). The introduction of the five Gag mutations (E12K, L75R, H219Q, V390D, and R409K) into $rHIV_{10/30/45/71_{pro}}^{WTgag}$, generating $rHIV_{10/30/45/71_{pro}}^{12/75/219/390/409_{gag}}$, did not change the susceptibility profile (Table 2). Another recombinant HIV-1 clone with three protease mutations (D30N, M46I, and V77I), $rHIV_{30/46/77_{pro}}^{WTgag}$, was also more resistant to NFV (by a factor of 9) and more susceptible to APV than $HIV_{WT}$. The introduction of the five Gag mutations, generating $rHIV_{30/46/77_{pro}}^{12/75/219/390/409_{gag}}$, did not affect the susceptibility of $rHIV_{30/46/77_{pro}}^{WTgag}$ to APV or NFV (Table 2).

Taken together, the data suggest that, as seen in the case of the lamivudine (3TC) resistance-associated mutation M184V that restores zidovudine (ZDV) sensitivity (37), NFV resistance-associated mutations paradoxically render HIV-1 more susceptible to APV.

## DISCUSSION

Certain amino acid substitutions in Gag are known to occur in common with resistance to PIs (11, 15, 32, 36); however, no salient features such as patterns and orders of the occurrence have been identified for a number of amino acid substitutions seen in Gag in PI-resistant HIV-1 variants. The roles and impact of such amino acid substitutions in Gag for the replication of HIV-1 have not been delineated, either. These limitations have been worsened since the functions and tertiary structures of entire HIV-1 Gag proteins remain to be determined, although some structures of certain parts of Gag proteins have been lately elucidated (13, 34, 41).

In the present study, we attempted to determine the effects of non-cleavage site mutations in Gag which emerged during the in vitro selection of HIV-1 in the presence of APV on the viral acquisition of resistance to APV and other currently existing PIs. When we selected HIV-1 in vitro in the presence of increasing concentrations of APV, six amino acid substitutions apparently critical for the development of APV resistance emerged. Such substitutions included five non-cleavage site mutations (E12K, L75R, H219Q, V390D, and R409K) and one cleavage site mutation, L449F (Fig. 1B).

HIV-1 variants containing PI resistance-conferring amino acid substitutions in protease plus wild-type Gag often have highly limited replicative abilities (7, 31). Indeed, in the present study, the recombinant HIV-1 clone containing the protease of $HIV_{APVp33}$ plus a wild-type Gag ($rHIV_{APVp33_{pro}}^{WTgag}$) or the L449F cleavage site mutation-containing Gag ($rHIV_{APVp33_{pro}}^{449gag}$) failed to replicate in MT-2 cells (Fig. 2A), indicating that neither of the two Gag species supported the growth of $HIV_{APVp33}$. However, a recombinant HIV-1 clone containing the protease of $HIV_{APVp33}$ and the five Gag non-cleavage site mutations, $rHIV_{APVp33_{pro}}^{12/75/219/390/409gag}$, replicated moderately under the same conditions (Fig. 2A), an observation in agreement with reports by others that some PI resistance-associated mutations compromise the catalytic activity of protease and/or alter polyprotein processing, often leading to slower viral replication (29, 36, 43). Since some of the five non-cleavage site mutations emerged before mutations in protease developed, we examined the effects of three sets of non-cleavage site amino acid mutations upon the emergence of APV resistance. Interestingly, HIV-1 with either of two sets of Gag mutations ($rHIV_{WTpro}^{219/409gag}$ and $rHIV_{WTpro}^{12/75/219/390/409gag}$) acquired APV resistance significantly faster than $HIV_{WT}$ (Fig. 3), while such mutations alone did not alter the susceptibilities of HIV to the PIs examined (Table 1), a finding providing the first report that Gag mutations expedite the emergence of PI-resistant HIV-1 variants. At this time, it is apparently unknown whether certain Gag mutations associated with viral resistance to PIs persist when highly active antiretroviral therapy (HAART) regimens including a PI(s) are interrupted or changed to regimens containing no PIs. However, the non-cleavage site mutations in Gag examined in this study did not reduce the viral fitness (Fig. 2 and 6), suggesting that Gag mutations may persist longer in circulation and/or in the HIV-1 reservoir in the body than mutations in protease when antiretroviral therapy including a PI(s) is interrupted. Such persisting Gag mutations may enable HIV-1 to rapidly acquire resistance

to that very PI when treatment with the PI is resumed. It is of note that on the other hand, two sets of Gag non-cleavage site mutations seen in $HIV_{APVp33}$ (H219Q and R409K and E12K, L75R, H219Q, V390D, and R409K) significantly delayed the emergence of resistance to other PIs such as RTV and NFV (Fig. 5). These data suggest that if a HAART regimen including APV is changed to an alternative regimen, the inclusion of a different PI in the alternative regimen is likely to delay the emergence of resistance to the different PI.

It is known that the L449F cleavage site mutation renders recombinant HIV-1 carrying a protease mutation (I50V) more resistant to APV (25). In the present study, a recombinant HIV-1 clone containing the protease of $HIV_{APVp33}$ plus the L449F cleavage site mutation-containing Gag ($rHIV_{APVp33pro}^{449gag}$) failed to replicate (Fig. 2A). These data strongly suggest that the L449F mutation alone prevents $HIV_{APVp33}$ from replicating, although $HIV_{APVp33}$ did not contain the I50V mutation. The observation in the present study that the addition of five non-cleavage site mutations to $rHIV_{APVp33pro}^{449gag}$, generating $rHIV_{APVp33pro}^{12/75/219/390/409/449gag}$, restored the replicative ability of the virus indicates that the presence of non-cleavage site Gag mutations plays an important role in the replication of APV-resistant HIV-1 variants.

Since $rHIV_{WTpro}^{12/75/219/390/409gag}$ acquired resistance to APV more rapidly than $HIV_{WT}$ (Fig. 3), while $rHIV_{WTpro}^{12/75/219/390/409gag}$ significantly delayed the acquisition of resistance to other PIs (Fig. 5), we examined whether the replication rates of $rHIV_{WTpro}^{12/75/219/390/409gag}$ and $HIV_{WT}$ were associated with the observed contrasting resistance acquisition patterns by using the CHRA (21). We found that $rHIV_{WTpro}^{12/75/219/390/409gag}$ outgrew $HIV_{WT}$ regardless of the presence or absence of PIs (Fig. 6), suggesting that the difference in the replication rates of $rHIV_{WTpro}^{12/75/219/390/409gag}$ and $HIV_{WT}$ was not the cause for the contrasting resistance acquisition patterns. As for the reason why $rHIV_{WTpro}^{12/75/219/390/409gag}$ outgrew $HIV_{WT}$, it is well explained by the presence of the H219Q mutation. His-219 is located within the cyclophilin A (CypA) binding loop of p24 Gag protein. It is thought that CypA plays an essential role in the HIV-1 replication cycle (4, 35) by destabilizing the capsid (p24 Gag protein) shell during viral entry and uncoating (12) and/or by performing an additional chaperone function, thus facilitating correct capsid condensation during viral maturation (17, 39). CypA is also known to support the replication of HIV-1 by binding to the Ref-1 restriction factor and/or TRIM5α, the human cellular inhibitors that impart resistance to retroviral infection (18, 38). It has also been demonstrated previously that the effect of CypA on HIV-1 replicative ability is bimodal: both high and low CypA contents limit HIV-1 replication (14). We have demonstrated previously that certain human cells, such as MT-2 and H9 cells, contain large amounts of CypA (14). We have determined more recently that MT-2 cells contain more CypA by about fivefold and that MT-4 cells contain about three times more than peripheral blood mononuclear cells (PBMCs) (unpublished data). In fact, HIV-1 produced in MT-4 cells contains large amounts of CypA, presumably resulting in compromised replication of the HIV-1. However, the H219Q mutation apparently reduces the incorporation of CypA into the virions through significantly distorting the CypA binding loop and restores the replicative ability of virions produced in MT-4 cells (14). Therefore, H219Q should contribute at least in part to the replication advantage of $rHIV_{WTpro}^{12/75/219/390/409gag}$. It is noteworthy that of 156 different HIV-1 strains whose sequences were compiled in the *HIV Sequence Compendium 2008* (22), 95 and 45 strains had histidine and glutamine, respectively, at position 219. Hence, position 219 is a polymorphic amino acid site, and it is thought that this polymorphic position is associated with the acquisition of resistance to certain PIs. Indeed, we have observed that $rHIV_{WTpro}^{219gag}$ overgrew $rHIV_{WTpro}^{WTgag}$ in the CHRA using fresh phytohemagglutinin-stimulated PBMCs (14). Since H219Q confers a replication advantage on HIV-1 in PBMCs, it is likely that HIV-1 with H219Q may acquire resistance more rapidly than HIV-1 without H219Q.

Two groups, Ziermann et al. and Resch et al., have reported that an NFV-related resistance mutation, N88S, renders HIV-1 susceptible to APV (33, 49), and indeed, Zachary et al. have reported an anecdotal finding that the infection of an individual with HIV-1 containing N88S was successfully managed with an ensuing APV-based regimen (46). Therefore, we examined the effect of another NFV resistance-associated mutation, D30N, in addition to that of the N88S mutation on HIV-1 susceptibility to APV. It was found that the mutations (D30N and N88S) clearly increased the susceptibility of HIV-1 to APV by 10- and 20-fold, respectively. These data are reminiscent of the observation that the 3TC resistance-associated mutation M184V in a background of mutations conferring resistance to ZDV restores ZDV sensitivity (37) and that ZDV-3TC combination therapy has proven to be more beneficial than ZDV monotherapy in patients harboring HIV-1 with the M184V mutation (9, 23), although the structural mechanism of the restoration of ZDV sensitivity by M184V is not clear. When a set of four protease mutations (L10F, D30N, K45I, and A71V), which had emerged by passage 10 when $HIV_{WT}$ was selected with NFV, were introduced into $HIV_{WT}$, generating $rHIV_{10/30/45/71pro}^{WTgag}$, the recombinant HIV-1 clone was more resistant to NFV than $HIV_{WT}$ by a factor of 9 while the clone was slightly more sensitive to APV (Table 2). When we introduced $mGag^{12/75/219/390/409gag}$ into HIV-1 containing a set of three NFV resistance-associated protease mutations (D30N, M46I, and V77I), generating $rHIV_{30/46/77pro}^{12/75/219/390/409gag}$, the recombinant clone was more resistant to NFV by a factor of 8 but more sensitive to APV by a factor of 6.7 (Table 2).

There has been a report that dual PI therapy with APV plus NFV is generally safe and well tolerated and that the combination of APV with NFV may have the most beneficial pharmacokinetic interactions, based on the results of a phase II clinical trial of dual PI therapies, APV in combination with IDV, NFV, or SQV, although this phase II trial was handicapped by the presence of substantial PI resistance at the baseline and the small number of patients in the study, precluding conclusions about the relative activities or toxicities of the dual PI combinations (10). The hypothesis that a HAART regimen combining APV with NFV may bring about more

favorable antiviral efficacy for HIV-1-infected individuals should merit further study.

## ACKNOWLEDGMENTS

This work was supported in part by a grant-in-aid for scientific research (priority areas) from the Ministry of Education, Culture, Sports, Science, and Technology of Japan (Monbu-Kagakusho); a grant to the Cooperative Research Project on Clinical and Epidemiological Studies of Emerging and Reemerging Infectious Diseases (Renkei Jigyo; no. 78, Kumamoto University) of Monbu-Kagakusho (H.M.); a grant for the promotion of AIDS research from the Ministry of Health, Welfare, and Labor of Japan (Kosei-Rohdosho; H15-AIDS-001); a grant-in-aid from the Institute of Health Sciences, Kumamoto Health Science University (M.A.); and in part by the intramural research program of the Center for Cancer Research, National Cancer Institute, National Institutes of Health.

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# Characterization of a CD4-independent clinical HIV-1 that can efficiently infect human hepatocytes through chemokine (C-X-C motif) receptor 4

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**Objective:** HIV-1 isolates are prominently CD4-dependent and, to date, only a few laboratory-adapted CD4-independent strains have been reported. Therefore, whether CD4-independent viruses may exist in HIV-1-infected patients has remained unclear. Here, we report the successful isolation of a CD4-independent clinical HIV-1 strain, designated SDA-1, from the viral quasispecies of a therapy-naive HIV-1 and *Pneumocystis jirovecii* pneumonia patient in the late-stage of AIDS with extremely low CD4 cell count (CD4 = 1/μl). We characterized this virus and further explored whether it could infect or induce pathological effects in human hepatocytes.

**Design and methods:** To determine coreceptor usage and CD4-independent infection, the HIV-1 envelope (Env)-pseudotypes and Env-chimeric viruses were used.

**Results:** SDA-1 was able to infect $CD4^-$ cell lines through either chemokine (C-X-C motif) receptor 4 or CCR5. It still maintained the ability to infect $CD4^+$ cells through multiple coreceptors of chemokine (C-X-C motif) receptor 4, chemokine (C-C motif) receptor 5, chemokine (C-C motif) receptor 3 and chemokine (C-C motif) receptor 8. Productive infection by SDA-1 was noted in both CD4-negative hepatoma cells and primary cultured human hepatocytes. Moreover, we demonstrated that SDA-1 could efficiently infect human hepatocytes on both static and mitotic phases through chemokine (C-X-C motif) receptor 4, without inducing apoptotic cell death.

**Conclusion:** The present study provides evidence that emergence of CD4-independent HIV-1 virus *in vivo* may occur in HIV-1-infected patients. In addition, these results shed light on the mechanisms involved in liver damage in HIV-1-infected individuals, which could have important implications concerning the range of mutability and the pathogenesis of AIDS. 

*AIDS* 2008, **22**:1749–1757

**Keywords: CD4-independence, HIV-1, human hepatocytes, human hepatoma cells**

## Introduction

The entry of HIV-1 into target cells requires interaction of the viral envelope (Env) with CD4 and a chemokine coreceptor [1,2]. Macrophage-tropic HIV-1 viruses primarily use chemokine (C-C motif) receptor 5 (CCR5) (R5) as a coreceptor, whereas T-cell-tropic viruses use chemokine (C-X-C motif) receptor 4

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Received: 17 January 2008; revised: 21 April 2008; accepted: 9 May 2008.

DOI:10.1097/QAD.0b013e328308937c

(CXCR4) (X4). Dual-tropic viruses (R5X4) use both coreceptors [3]. A few rare viruses can also use alternative coreceptors such as chemokine (C-C motif) receptor 1 (CCR1), chemokine (C-C motif) receptor 2b (CCR2b), chemokine (C-C motif) receptor 8 (CCR8), chemokine (C-X-C motif) receptor 6 (CXCR6), G protein-coupled receptor 1 (GPR1) or GPR15/Bob for entry into coreceptor-transfected $CD4^+$ cell lines [4]. Whatever the coreceptor specificity of an HIV-1 isolate, an interaction with CD4 is always the first step in a chain of events leading to fusion of the viral envelope with the cellular membrane. However, previous studies have shown that SIV [5] and HIV-2 [6] can also infect cells independently of CD4.

In contrast to SIV and HIV-2, HIV-1 CD4-independent viruses are rarely isolated. To date, only a few laboratory CD4-independent HIV-1 variants [7–10] have been reported. Therefore, whether such viruses may exist in HIV-1-infected patients has remained unclear. However, several studies [11–14] have shown that HIV-1-DNA and p24, a core HIV-1 antigen, were detected in CD4-negative cells or tissues such as brain, kidney and liver in HIV-infected individuals, suggesting the possibility that low levels of CD4-independent variants exist *in vivo*. Among such $CD4^-$ cells or tissues, liver is an important organ in determining the prognosis of HIV-1-infected patients. End-stage liver disease is becoming a frequent cause of death in HIV-1-infected hospitalized patients [15–17]. Although the cause of liver injury in HIV-1 patients might be multifactorial, such as hepatitis B virus (HCV) and hepatitis B virus (HBV) coinfection and the side effects of antiretroviral therapy, a number of reports have documented that histological liver abnormalities occurred solely as a result of HIV-1 infection [13,18,19]. Nonetheless, few attempts have been made to elucidate the mechanisms of the liver damage in HIV-1-infected individuals.

In this study, we successfully isolated a CD4-independent clinical HIV-1 strain, designated SDA-1, from the viral quasispecies of a therapy-naive HIV-1 and *Pneumocystis jirovecii* pneumonia (PJP) patient in the late stage of AIDS with extremely low CD4 cell numbers. We characterized the phenotype of this virus and further explored whether it could infect or induce pathological effects in human hepatocytes.

## Materials and methods

### Patient's information

A 53-year-old Japanese man infected with HIV-1 was admitted to Tohoku University Hospital owing to prolonged fever and severe dyspnea in 2000. His plasma viral load and CD4 cell count at the time of admission was 220 000 copies/ml and 1 cell/μl, respectively. He was diagnosed with PJP, and his clinical stage was classified as IV-C3 [20]. The onset and route of HIV-1 infection were unknown. No evidence of coinfection with HBV or HCV in this patient was found. The patient was treated with trimethoprim and sulfamethoxazole (TMP–SMX) and highly active antiretroviral therapy (HAART). His condition deteriorated rapidly and he died 33 days after admission. Consent for autopsy was denied by the patient's family.

Before HAART, plasma samples and peripheral blood mononuclear cells (PBMC) were collected from this patient and cryopreserved in liquid nitrogen until use. The institutional Ethics Committee approved this study and written informed consent was obtained from the patient.

### Virus isolation

HIV-1 isolation was achieved by using an in-vitro short-term phytohemagglutinin (PHA)–PBMC coculture method. Briefly, cryopreserved PBMC ($2 \times 10^6$) from the patient were cocultivated with PHA-stimulated PBMC ($5 \times 10^6$) from an HIV-1 seronegative healthy donor. The culture was maintained in RPMI-1640 (Invitrogen, California, USA) containing 10% fetal calf serum and 5 U/ml of recombinant interleukin-2 (IL-2) (Sigma, St. Louis, Missouri, USA). Proliferation of HIV-1 was examined by measuring p24 antigen in the cell culture supernatant using a p24 ELISA kit (RETRO-TEK, ZeptoMetrix Corp., New York, USA). The virus stocks were kept at −80 °C until use.

### Amplification of *env* and sequence analysis

The full-length HIV-1 *env* genes were amplified by limiting dilution nested PCR from proviral PBMC DNA or plasma RNA as previously described [21,22]. To avoid artificial recombination and resampling of the viral genomes, independent nested PCR reactions were carried out per specimen [23,24].

The first round PCR was conducted with a F5852–R8935 primer pair (F5852, 5′-TAGAGCCCTGGAAG CATCCAGGAAG, HIV-1 HXB2 nucleotide position 5852–5876; R8935, 5′-TTGCTACTTGTGATTGCT CCATGT, HXB2 nucleotide position 8912–8935). The second round PCR was performed with a F5957–R8903 primer pair (F5957, 5′-GATCGAATTCTAGGCATC TCCTATGGCAGGAAGAAG, HXB2 nucleotide position 5957–5982, containing an additional *Eco*RI site (underlined) to facilitate cloning; R8903, 5′-AGCT CTC GAGGTCTCGAGATACTGCTCCCACCC, HXB2 nucleotide position 8881–8903, containing an additional *Xho*I site (underlined)). The purified PCR products were subcloned into the *Eco*RI and *Xho*I sites of the pSM-HXB2 plasmid. All correctly oriented *env* clones were then screened for biological function [22] followed by sequencing and phylogenetic analysis as previously described [25,26].

### Cell lines and cell culture

All the cell lines, unless otherwise specifically mentioned, were cultured in Dulbecco's modified Eagle's medium (DMEM, Invitrogen) containing 10% fetal calf serum. Human glioma NP-2-CD4$^+$ cells transfected with a variety of chemokine receptors as indicated [27] were maintained in medium containing 500 μg/ml of G-418 (Promega, Wisconsin, USA) and 1 μg/ml of puromycin (Sigma). Human CD4-negative osteosarcoma (HOS) cells expressing either CXCR4 or CCR5 [28] were cultured in medium containing 1 μg/ml of puromycin. Human hepatoma cells Huh-7 and Hep-G2 [29] were obtained through the Cell Resource Center for Biomedical Research, Tohoku University, Japan. Human primary cultured hepatocytes (p-hepatocytes, BD Bioscience, California, USA) were maintained on BD Matrigel with Hepato-STIM hepatocyte culture medium (BD Bioscience).

### Reagents and antibodies

The CXCR4 antagonist AMD3100 [30], and the CCR5 antagonist TAK-779 [31] were provided by the NIH AIDS Research and Reference Reagent Programme and Takeda Chemical Industries, Ltd., Osaka, Japan, respectively. Recombinant human soluble CD4 (sCD4) was from ImmunoDiagnostics, Inc. (Woburn, Massachusetts, USA). Antialbumin-fluorescein isothiocyanate (FITC) antibody was from Cedarlane Laboratories Ltd. (Hornby, Ontario, Canada). Anticytokeratin-18–phycoerythrin and antialpha fetoprotein (AFP)–FITC antibodies were from Santa Cruz Biotechnology, Inc. (Santa Cruz, California, USA). Anti-HIV-1-p24 (clone KC57)-FITC antibody was from Beckman Coulter. All other antibodies were from BD Pharmingen (San Diego, California, USA).

### Pseudotyped virus infection assay

The HIV-1 Env-pseudotypes were generated as previously described [32]. Briefly, 293T cells ($5 \times 10^6$ cells/10 cm-dish) were transfected with 5 μg of luciferase-expressing pNL4-3-Luc-R$^-$E$^-$ [33] or green fluorescent protein (GFP)-expressing pNL4-3-GFP [34] plasmid in combination with 10 μg of one of the *env*-expressing plasmids, pSM-SDA-1, pSM-HXB2 (X4), pSM-ADA (R5), or pSM-89.6 (R5X4). The vesicular stomatitis virus-G pseudotypes were also prepared [35].

For infection assays of luciferase-pseudotypes (luc-p), 10 ng p24 of luc-p were added into each well of 24-well plates ($5 \times 10^4$ cells/well). After 12 h infection, the cells were washed and incubated for an additional 36 h at 37 °C. The cells were then lysed using a Luciferase Assay kit (Promega) and the luciferase activity was examined by a luminometer (Lumat 9507, Germany). To determine the effects of various reagents related to the viral receptors, target cells were preexposed for 1 h with the indicated concentration of the antagonists, or the antibodies. For GFP-pseudotypes (GFP-p) infection, target cells were infected with 10 ng p24 of GFP-p virus for 48 h and fixed by 5% paraformaldehyde. Infectivities were visualized under a Zeiss LSM510 confocal microscopy and DIC images with a 512 × 512 resolution were acquired.

### Chimeric viruses

All *env* recombinant chimeric viruses in this study were generated in the background of pNL43, an X4-tropic HIV-1 infectious clone [36]. Briefly, the fragment of pNL43 containing *Eco*RI (nt 5743–5748) and *Kpn*I (nt 6343–6348) was amplified by PCR with a F5671–R6472 primer pair (F5671, 5′-GGCTCCATAACTTAGGA CAAC, pNL43 nucleotide position 5671–5691; R6472, 5′-TACTTCTTGTGGGTTGGGGTC, pNL43 position 6452–6472), followed by insertion into the pSM–SDA-1 using *Eco*RI and *Kpn*I. The new *Eco*RI–*Xho*I fragment (3155 bp) covering the entire SDA-1 *env* gene was then replaced with the equivalent region of pNL43 to construct the Env-chimeric virus NL43_SDA-1. Similarly, Env-chimeras of ADA (NL43_ADA), 89.6 (NL43_89.6) or truncated *env* (NL43_env (-)) were created, respectively. All Env-chimeric viruses were prepared by transfecting 293T cells as described above. For infection assays, 100 ng p24 of the chimeric viruses or virus stock supernatants were added in each well of 24-well plates ($5 \times 10^4$ cells/well). After 2 h adsorption, the cells were washed and incubated for 48 h. Viral replication was monitored by p24 antigen production.

### Flow cytometry and apoptosis assay

We performed cell-surface staining for CD4, CXCR4 and CCR5 by flow cytometry. To determine the purification and differentiation of p-hepatocytes, we tested the specific markers using antialbumin–FITC, anti-AFP–FITC and anticytokeratin-18–phycoerythrin antibodies. Appropriate class matched antibodies were used in each experiment. To detect the proliferation and intracellular p24, p-hepatocytes were fixed and permeabilized using a Cytofix-Cytoperm kit (BD Bioscience). Subsequently, the cells were stained with anti-Ki-67–phycoerythrin and antip24–FITC antibodies. Apoptosis of the p-hepatocytes was determined using the Apoptosis Detection kit I (BD Pharmingen). Flow cytometry analysis was performed using FACSCalibur (Becton Dickinson, New Jersey, USA). All Data were acquired and analyzed using Cell Quest software (BD Bioscience).

### Nucleotide sequence accession number

The GenBank accession number for the sequence determined in this study is AY902478 (SDA-1).

## Results

### Evaluation of SDA-1 viral quasispecies

In an attempt to isolate CD4-independent clinical HIV-1 strain(s), we performed virus isolation from a

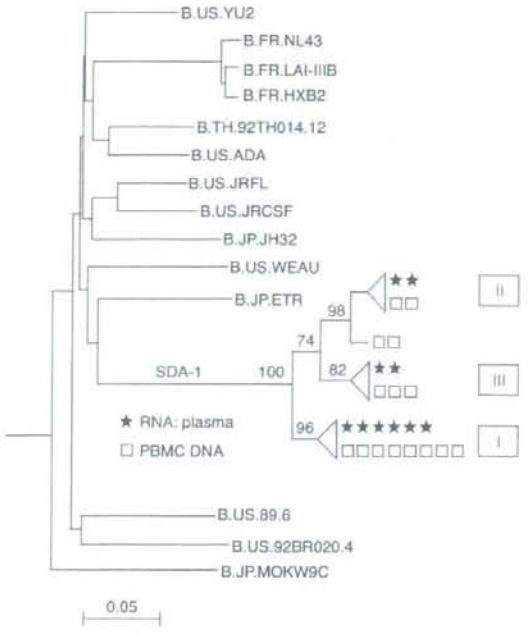


**Fig. 1. Evolution of SDA-1 *env* quasispecies in plasma and PBMC.** Phylogenetic analysis of newly characterized, SDA-1 gp120 *env* nucleotide sequences obtained from plasma ($n = 10$) and PBMC ($n = 15$) with representative sequences of HIV-1 subtype B. Numbers at branch nodes refer to the percentage of bootstrap values and symbols indicate individual clones.

therapy-naive HIV-1 and PJP patient with extremely low CD4 cell number, and successfully isolated the virus (peak of p24, 500 ng) from this patient and designated it SDA-1. To assess the quasispecies diversity present in *vivo*, we analyzed the SDA-1 *env* clones derived from plasma RNA and PBMC. As shown in Fig. 1, SDA-1 is grouped within the HIV-1 subtype B reference sequences. Within SDA-1's sequence cluster, three phylogenetic forms were identified. Supported by a significant bootstrap value (96%), form I was the predominant quasispecies, representing 70% of all sequences. Two minor quasispecies (forms II and III) had similar structures but differed in the position of the first breakpoint. The mean distances between major and minor quasispecies did not differ significantly from the sequence heterogeneity. Furthermore, the quasispecies diversities between plasma and PBMC were similar within each form, and were all below 5.0%.

## Multicoreceptor usage and CD4-independent entry of SDA-1

To determine the receptor usage of SDA-1, we randomly selected five clones from the predominant quasispecies and generated Env-pseudotypes and Env-chimeric viruses as representatives. As a control, the Envs from a variety of HIV-1 subtypes with X4 (HXB2), R5 (ADA), and R5X4 (89.6) tropism were used. Utilizing luciferase-pseudotypes (luc-p), we first examined the coreceptor usage of SDA-1. We found that in the presence of CD4, all representative SDA-1 Env-pseudotypes were able to use efficiently both CXCR4 and CCR5, with additional moderate usage of CCR3 and CCR8 (Fig. 2a).

We next investigated whether SDA-1 Envs are capable of inducing CD4-independent infection. We found that SDA-1 Envs mediated entry into both HOS–CXCR4 and HOS–CCR5. However, the infectivities of SDA-1 for HOS–CXCR4 were approximately 2.5-fold higher than that for HOS–CCR5 (Fig. 2b). In stark contrast, none of the other types of luc-p viruses entered either of those cells. Furthermore, we evaluated the ability of SDA-1 Envs in mediating cell–cell fusion, a dye-transfer cell–cell fusion assay [37] was used with HOS–CXCR4 and HOS–CCR5 cells. Only in the cells expressing SDA-1 Envs (effector cells) did cell–cell fusion with CD4-negative, CXCR4- or CCR5-positive HOS cells (target cells) occur (data not shown).

In addition to the results with HOS–CXCR4 and CCR5, preexposure of HOS cells to Leu-3A, a CD4 monoclonal antibody (mAb) that recognizes the gp120 binding site on CD4 [38], failed to block SDA-1 infection. In contrast, pretreatment with antagonists for CXCR4 or CCR5 effectively inhibited infection (Table 1). Furthermore, the infectivities of SDA-1 on HOS–CXCR4 and HOS–CCR5 were enhanced by preexposure of the virus to sCD4 indicating that the binding of SDA-1 Env to CD4 induces further conformational changes in gp120 to fully expose the chemokine receptor binding domain. Collectively, SDA-1 Envs mediated the CD4-independent infection via both CXCR4 and CCR5.

Having clarified that SDA-1 is a CD4-independent isolate, we next investigated what types of $CD4^-$ cells are able to support SDA-1's entry. We focused first on human liver-derived cell lines, as the mechanisms of the liver damage in HIV-1-infected individuals are still unclear.

Two hepatoma cell lines, Huh-7 and Hep-G2, were used as targets. We first examined the expression of the receptors on the cell surface by flow cytometry and found that both CXCR4 and CCR5 were expressed on Huh-7 and Hep-G2 cells. In contrast, CD4 was not detected on either, which was confirmed by RT–PCR (data not shown). We then evaluated whether SDA-1 can enter into hepatoma cells with luc-p viruses. We found that only SDA-1 luc-p viruses efficiently infected Huh-7; however, its infectivity was marginal in Hep-G2 (Fig. 2c). Previous studies have shown that few HIV-1 variants can infect $CD8^+$ cells using CD8 as receptor [10,39]. Therefore, we further explored receptors used by

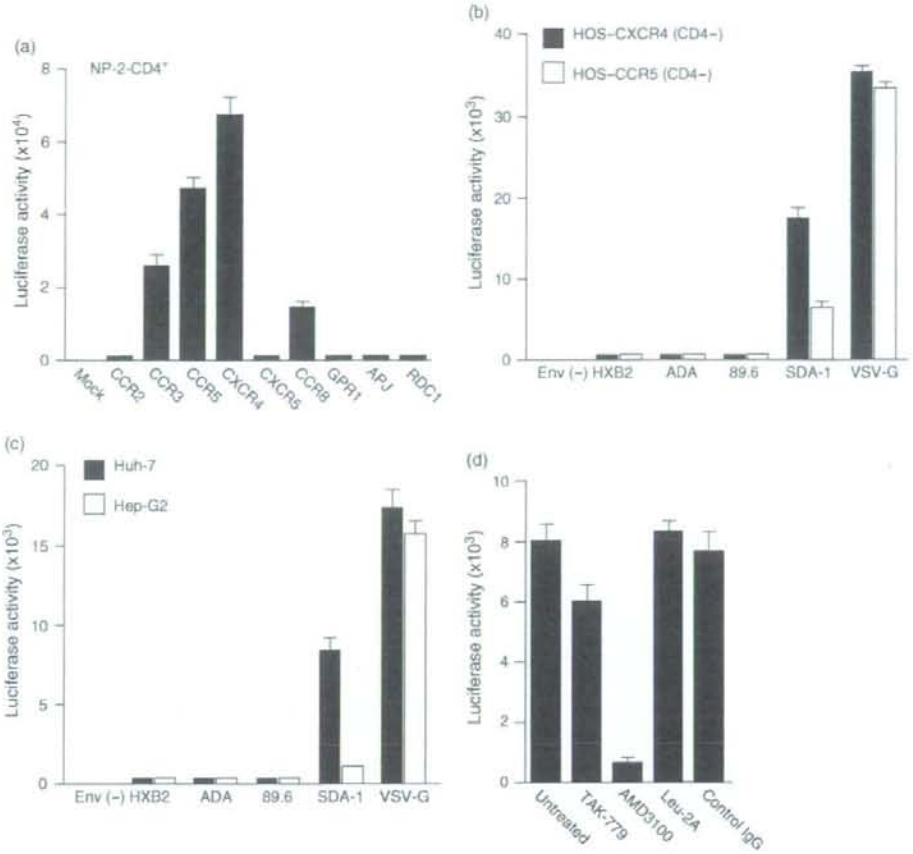


**Fig. 2. Multicoreceptor usage and CD4-independent entry of SDA-1.** (a) SDA-1 Envs mediate entry of $CD4^+$ cells using multiple coreceptors. NP-2-$CD4^+$ cells coexpressing one of the indicated chemokine receptors were exposed to SDA-1 luc-p viruses for 48 h and the luciferase activities were measured. (b) SDA-1 Envs mediate entry of $CD4^-$ cell lines through either CXCR4 or CCR5. The HOS cells ($CD4^-$) expressing either CXCR4 or CCR5 were exposed to the indicated HIV-1 luc-p viruses or VSV-G for 48 h, after which the infectivities were determined. (c) Entry of SDA-1 into $CD4^-$ human hepatoma cells. Huh-7 and Hep-G2 were exposed to the indicated HIV-1 luc-p viruses or VSV-G. Infectivities were determined at 48 h. (d) Effects of receptor-related antagonists or antibodies on the entry of SDA-1 entry into Huh-7 cells. Interaction of SDA-1 luc-p viruses with Huh-7 cells was tested in the absence or presence of AMD3100 (1.0 μM), TAK-779 (100 nM), anti-CD8 Leu-2A antibody (30 μg/ml) or class-matched control antibody (30 μg/ml). Results shown (a–d) are means of triplicate experiments. Bars, standard deviation. IgG, immunoglobulin G; VSV, vesicular stomatitis virus.

SDA-1 for entry into hepatoma cells. As shown in Fig. 2d, preexposure of Huh-7 to anti-CD8 Leu-2A mAb, as well as the CCR5 antagonist, TAK-779, failed to block SDA-1 infection of Huh-7, whereas anti-CXCR4 with AMD3100 effectively suppressed the infectivity. These results suggested that SDA-1 enters Huh-7 cells principally via CXCR4.

## Replication of SDA-1 in human hepatoma cells

Although SDA-1 luc-p viruses infected some cells independently of CD4 cells, it was necessary to determine whether SDA-1 can replicate in those $CD4^-$ cells, particularly in hepatoma cells. For this purpose, we constructed NL43-based Env-chimeric viruses described above. We then examined whether the chimeric viruses were able to replicate in $CD4^-$ cells. As shown in Fig. 3a, the SDA-1 Env-chimeric viruses replicated efficiently in HOS–CXCR4 and HOS–CCR5 cells to similar levels. In contrast, none of the other Env-chimeric viruses infected either of those cells lines. Furthermore, we examined whether SDA-1 Env-chimeric viruses could replicate in hepatoma cells. As shown in Fig. 3b, high levels of NL43-SDA-1 replication were observed in Huh-7 cells. However, marginal replication was detected

**Table 1. Inhibition of SDA-1 by blocking reagents in CD4⁻ cells.**

| Reagent | % Inhibition | |
|---|---|---|
| | HOS–CXCR4 | HOS–CCR5 |
| Medium | 0 | 0 |
| Control mAb (30 μg/ml) | 0 | 0 |
| Leu-3A (30 μg/ml) | 10 | 12 |
| Soluble CD4 (10 μg/ml) | 225[a] | 120[a] |
| AMD3100 (1.0 μM) | 99 | 0 |
| TAK-779 (100 nM) | 0 | 97 |

CCR5, chemokine (C-C motif) receptor 5; CXCR4, chemokine (C-X-C motif) receptor 4; HOS, Human CD4-negative osteosarcoma; mAb, monoclonal antibody.
[a]Enhancement of entry.

in Hep-G2 cells. Although both Huh-7 and Hep-G2 cells are derived from human hepatoma, many potential host factors [40] could influence HIV replication, which for the most part remain unknown. Similarly, only Huh-7 cells, but not Hep-G2 cells, were susceptible to HCV [41,42]. These reasons may be related to the difference between Huh-7 and Hep-G2 regarding the level of replication by SDA-1.

## SDA-1 replicates in both proliferating and static hepatocytes

To investigate further whether normal human hepatocytes could sustain entry and replication of SDA-1, p-hepatocytes were used for the following experiments. Among the three specific markers of human hepatocytes, both albumin and cytokeratin-18, but not alpha-fetoprotein were detected in the p-hepatocytes suggesting that the hepatocytes we used were well differentiated (data not shown). We also found that CXCR4 was expressed on the surface of p-hepatocytes. In contrast, neither CD4 nor CCR5 was detected on the p-hepatocyte surface or by real-time PCR (RT–PCR) (data not shown).

We next explored whether SDA-1 can enter p-hepatocytes by using GFP-p. As shown in Fig. 4a, only SDA-1 GFP-p viruses gave GFP-positive cells in p-hepatocytes, whereas other HIV-1 GFP-p viruses did not. The GFP-positive cells showed spindle-like shapes suggesting that the infection occurred in the p-hepatocytes but not in the contaminating lymphocytes. Furthermore, we studied whether SDA-1 can replicate in the p-hepatocytes. As shown in Fig. 4b, the p-hepatocytes were productively infected by the SDA-1 Env-chimeric viruses and SDA-1 virus stock itself but not by the other HIV-1 Env-chimeric viruses. Moreover, we found that AMD3100 inhibited the replication of SDA-1 in p-hepatocytes in a dose-dependent manner (Fig. 4c) indicating that the infection of p-hepatocytes by SDA-1 was mediated through CXCR4.

A previous study [19] reported that the HIV-1 gp120 *env* directly caused hepatocyte death by signaling through CXCR4 *in vitro*; however, most studies were performed using the hepatoma Huh-7 cells not hepatocytes, therefore, it may not really reflect the nature of liver damage. To explore the pathological effects of HIV-1 CD4-independent infection on hepatocytes, we exposed p-hepatocytes to the SDA-1 and analyzed cell viability. We found that the viability of the p-hepatocytes in cells cultured with or without SDA-1 Env-chimeric viruses was comparable (96%, $P$ was not significant) indicating that HIV-1 CD4-independent infection rarely induces hepatocyte death via an apoptotic process (data not shown). To further examine whether the infection or replication of SDA-1 is limited only to a certain number of p-hepatocytes or whether the infectivity or replication is influenced by the cell cycle, we studied the intracellular expression by flow cytometry of p24 and Ki-67 [43], a marker strictly associated with cell proliferation, in the HIV-1-infected p-hepatocytes. As shown in Fig. 4d, we found that 32.49% of p-hepatocytes were infected by SDA-1. However, there was no significant difference in

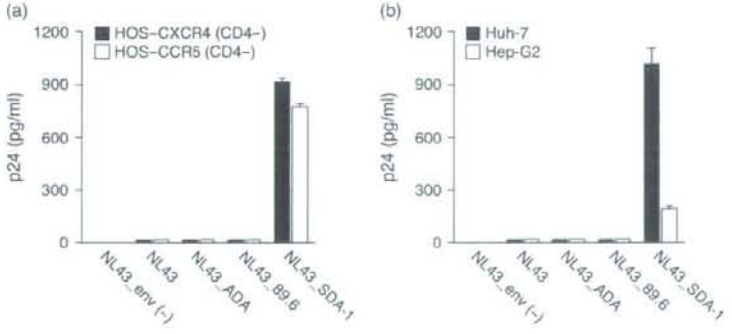


**Fig. 3. CD4-independent infection of SDA-1 Env-chimeric viruses.** The HOS cells (CD4⁻) expressing either CXCR4 or CCR5 (a) and two CD4⁻ human hepatoma cells (b) were incubated with the indicated HIV-1 Env-chimeric viruses. Virus replication was then monitored by p24 antigen production on day 3. Results shown (a, b) are means of triplicate experiments. Bars, standard deviation.