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Identification of the suppressive factors for human immunodeficiency virus type-1 replication using the siRNA mini-library directed against host cellular genes

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Abstract

We performed the screening to find the novel host factors affecting human immunodeficiency virus type-1 (HIV-1) replication using the siRNA mini-library consisted with 257 siRNAs directed against cellular genes. J111 cells, a human acute monocytic leukemia cell line, were transfected with individual siRNA, followed by either infected or transfected with the HIV-1 molecular clone with luciferase reporter gene in 96-well plate format. The results showed that six siRNAs significantly enhanced the HIV-1 replication in J111 cells, indicating that the target cellular genes of those siRNAs may negatively regulate HIV-1 replication in normal cell culture condition. We also discuss the possible mechanisms by which those cellular proteins regulate viral replication.

Keywords: Human immunodeficiency virus type-1; RNA interference; Small interfering RNA; siRNA mini-library; Host factor

Human immunodeficiency virus type-1 (HIV-1) is a causative agent of acquired immune deficiency syndrome (AIDS). The HIV-1 replication is governed by complex regulatory mechanism, and many host factors are involved either positively or negatively in HIV-1 replication [1,2]. Some of such host factors were found to be the determinants of the cell tropism and/or host range of HIV-1 [3–10]. Although many host factors had been already identified, the regulatory mechanism of HIV-1 life cycle is still not fully understood.

RNA interference (RNAi) has found as a highly effective and widely used methodology for the suppression of specific gene expression in eukaryotic cells. The small interfering RNA (siRNA), comprised of a duplex of two 21-mer

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RNAs with 19 complimentary nucleotides and 3′ terminal 2 non-complementary nucleotides, can induce the RNAi-mediated specific suppression of target genes in eukaryotic cells [11].

In this study, we have studied the effects on the replication of the infectious molecular clone of HIV-1 in the human cells by transfection with the siRNA mini-library consisted with 257 siRNAs directed against cellular genes. Our results showed that six siRNAs significantly enhanced the HIV-1 replication, indicating that the target cellular genes of those siRNAs negatively regulate HIV-1 replication. We also discuss the possible mechanisms by which those cellular factors regulate HIV-1 replication.

Materials and methods

siRNA. The 257 siRNAs directed against cellular genes that we selected as functional molecules to be involved in the intracellular signal transduction pathways, intracellular transportation processes, and cytoskeletal system were prepared as siRNA mini-library (the list of target genes is available as the Supplementary file). In addition, siRNAs against adaptor-related protein complex 2 (AP-2) α-subunit (AP2α) [siRNA ID: 5397], ADP-ribosylation factor 6 (ARF6) [siRNA ID: 10338], Axin1 [siRNA ID: 121446], Ezrin [siRNA ID: 13018], dual specificity phosphatase 1 (DUSP1) [siRNA ID: 104724], heat shock transcription factor 1 (HSF-1) [siRNA ID: 115674], Janus kinase 1 (JAK1) [siRNA ID: 219], partitioning defective 6 (par-6) homolog-α (PARD6α) [siRNA ID: 135172], RAN binding protein 2 (RanBP2) [siRNA ID: 142957], and Rho-associated, coiled-coil containing protein kinase 2 (ROCK2) [siRNA ID: 595] were purchased from Ambion, and used as second set of siRNA. In addition, siRNAs against Ezrin [siRNA ID: 13110], HSF-1 [siRNA ID: 3234], JAK1 [siRNA ID: 218], RanBP2 [siRNA ID: 142956], and ROCK2 [siRNA ID: 596] were used as a third set of siRNA. As negative controls, siRNA against Apaf1 and control (non-silencing) siRNA were purchased from Qiagen.

siRNA transfection. The cells were transfected with siRNA using the RNAiFect transfection reagent (Qiagen), essentially as described [12]. For the transfection of siRNA mini-library, the cells ($3–4 \times 10^3$ cells/100 μl) were seeded in 257 wells of 96-well plates 24 h prior to siRNA-transfection. The 257 siRNAs were mixed with the RNAiFect transfection reagent individually using 257 vessels for forming the RNA/reagent complex. Then, the cells were transfected with individual siRNA (final 100 nM), according to the manufacturers' protocol.

Cells. 293T, HeLa and a human acute monocytic leukemia cell line, J111 cells, were maintained in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal calf serum, as described previously [12].

Preparation of vesicular stomatitis virus G protein (VSVG)-pseudotyped reporter virus. VSVG-pseudotyped HIV-1 reporter molecular clone (pNL-Luc/VSVG) was prepared by transfecting 293T cells with a pNL4-3 [13]-based, Env-deficient proviral construct bearing a firefly luciferase gene, pNL-Luc-E$^-$R$^+$ [14,15], and VSVG-expression vector, pHIT/G [16], using FuGENE 6 transfection reagent (Roche), as described previously [12]. The viral titer was determined by measuring the concentration of HIV-1 Gag p24 antigen in the cell culture supernatant by enzyme-linked immunosorbent assay (ELISA) (ZeptoMetrix Corp., Buffalo, NY).

HIV-1 infection. The cells were infected with pNL-Luc/VSVG (30 ng of p24). Twenty-four hours after infection, the firefly luciferase activity in the infected cells was measured using the Steady-Glo luciferase assay system (Promega) and the microplate luminometer, Centro LB960 (Berthold).

Transfection of HIV-1 proviral construct. The cells were transfected with pNL4-3-based proviral construct bearing a firefly luciferase gene, pNL-Luc-envCT (denoted as pNL-envCT in reference [17]), using Lipofectamine 2000 (Invitrogen). Forty-eight hours after transfection, the concentration of p24 antigen in the cell culture supernatant and the firefly luciferase activity in the transfected cells were measured, as described above. In some experiments, the cells were co-transfected with pNL-Luc-envCT and pTK-RL (Promega). Forty-eight hours after transfection, both firefly and *Renilla* luciferase activities were measured using the Dual-Glo luciferase assay system (Promega). *Renilla* luciferase activity was used to monitor the transfection efficiency.

Immunoblotting. Cells were lysed in sample buffer [62.5 mM Tris–HCl, pH 6.8, 2% sodium dodecylsulfate (SDS), 10% glycerol, 5% 2-mercaptoethanol, and 0.003% bromophenol blue]. Then, samples were separated by SDS–polyacrylamide gel electrophoresis (SDS–PAGE), and transferred to a polyvinylidene fluoride (PVDF) membrane. After blocking with 3% non-fat milk in PBS, the blots were immuno-stained with one of the following antibodies: anti-AP2α (Adaptin α) monoclonal antibody (#610502; BD Biosciences, San Jose, CA), anti-ARF6 monoclonal antibody (sc-7971; Santa Cruz Biotechnology, Inc., Santa Cruz, CA), anti-Axin1 polyclonal antibody (#34-5900; Zymed Laboratories, South San Francisco, CA), anti-JAK1 monoclonal antibody (#610231; BD Biosciences), anti-DUSP1 (MKP-1) polyclonal antibody (sc-1199; Santa Cruz Biotechnology, Inc.) and anti-PARD6 polyclonal antibody (sc-14405; Santa Cruz Biotechnology, Inc.). After incubation of the samples successfully with peroxidase-labeled secondary antibodies, the immuno-complex was visualized using ECL Plus Western blotting detection reagents (Amersham Pharmacia Biotech).

WST-1 test. The cell toxicity test was carried out using WST-1 cell proliferation assay system (Takara Bio, Shiga, Japan), according to the manufacturers' protocol.

Results and discussion

Study on early phase of the HIV-1 life cycle. We examined the efficiency of single-round infection of VSVG-pseudotyped luciferase reporter virus, pNL-Luc/VSVG, in the cells transfected with each of 257 siRNAs directed against cellular genes. By measuring the firefly luciferase activity in pNL-Luc/VSVG-infected cells, the efficiency of early phases of HIV-1 replication cycle, including reverse transcription, integration, RNA transcription and protein translation, can be monitored [14,15]. After the first screening of 257 siRNAs, we found that the siRNAs against AP2 α (RefSeq Accession No. NM_014203.2), ARF6 (NM_001663.2), PARD6α (NM_016948.1), and JAK1 (NM_002227.1) efficiently enhanced the level of luciferase activity in pNL-Luc/VSVG-infected cells (Fig. 1A). In contrast, no siRNA was found to reduce the luciferase activity by less than 25% compared with that in control cells (data not shown). The target gene sequences of siRNAs against AP2α, JAK1, PARD6α, and ARF6 included in siRNA mini-library are 5′-GTGGTACCGTGTGCTACA GATCG-3′, 5′-CACTACCGGATGAGGTTCTATTT-3′, 5′-GGGGCATCTGGGCGTTTGACAGG-3′, and 5′-GCCGCTCTGGCGGCATTACTACA-3′, respectively. To confirm the gene-specific effects of siRNAs against AP2α, ARF6, PARD6α, and JAK1, we next carried out the second screening experiments using the second set of siRNAs directed against the different gene region in each target gene. J111 cells were transfected with one of the second set of siRNAs against AP2α, ARF6, PARD6α, and JAK1, and then infected with pNL-Luc/VSVG. The results showed that those siRNA also enhanced the luciferase activities in infected cells (Fig. 1B). Those results indicate

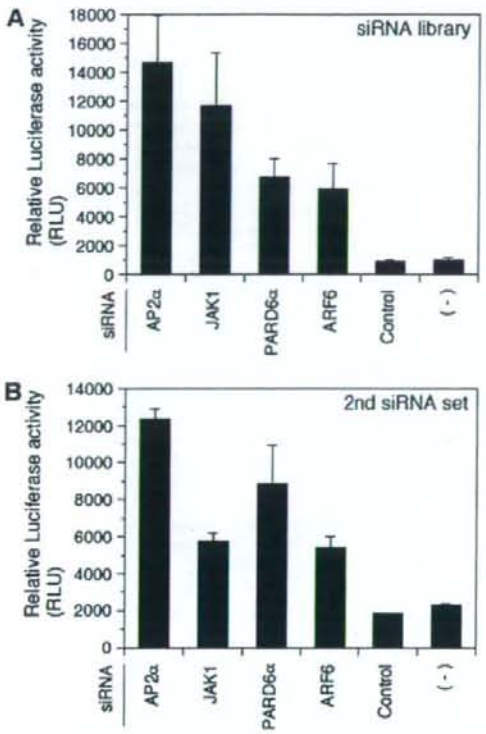

Fig. 1. siRNAs against AP2α, JAK1, PARD6α, and ARF6 enhanced the HIV-1 replication at the early phase(s) of the viral life cycle. J111 cells were transfected with the siRNAs included in siRNA mini-library (A) or the second set of siRNAs (B) directed against the indicated target gene. Forty-eight hours after transfection, the cells were infected with pNL-Luc/VSVG (30 ng of p24 antigen). Twenty-four hours after infection, cells were harvested, and the firefly luciferase activity was measured, as described in Materials and methods. Data are represented by means and standard deviations (error bars) of four independent experiments.

that the siRNAs against AP2α, ARF6, PARD6α, and JAK1 reproducibly enhanced the HIV-1 replication at the early phase(s) of viral life cycle, implying that those cellular proteins could negatively regulate HIV-1 replication in normal cell culture condition. In addition to J111 cells, we also examined the level of HIV-1 replication in HeLa cells transfected with those siRNAs. The results showed that siRNAs against AP2α, ARF6, PARD6α, and JAK1 enhanced the HIV-1 replication in HeLa cells (data not shown). These results suggest that the host factors we examined here may negatively regulate the HIV-1 replication not only in J111 cells but also in other human cell lines.

AP2α is a major component of the AP-2 that is known to regulate the receptor-mediated endocytosis of the plasma membrane proteins [18,19]. ARF6 is a member of the ARF gene family that is known to stimulate the ADP-ribosyltransferase activity of cholera toxin [20] and also to play a role in vesicular trafficking [21,22] and as activator of phospholipase D [23]. ARF6 regulates clathrin-dependent and -independent endocytosis [24–27], and interacts with AP-2 [27].

Several reports suggest that AP-2 [28–32] and ARF6 [33] play roles in HIV-1 replication, although these gene products were shown to function in the late phase of viral life cycle. Thus, the negative roles of AP2α and ARF6 for the early phase of the HIV-1 life cycle have not been studied. Maréchal et al. [34] had studied the efficiency of cellular uptake of HIV-1 and their results indicated that very low population (0.15% ± 0.04%) of the viral input was internalized into cells. In addition, roughly 10% of internalized virus (less than 0.01% of viral input) was found in the cytosolic fraction and participated in the infection process. In contrast, 90% of internalized virus found in the vesicular fraction seemed to be leaded to the dead end with respect to viral replication [34]. These results may suggest that the cellular internalization process of HIV-1 is largely restricted by cellular machinery involved in the sorting of membrane proteins. Therefore, our results suggest that AP2α and ARF6 may play roles for negatively regulating the HIV-1 replication in the sorting pathway of viral component in infected cells.

PARD6 family proteins play a role as the adaptor molecule in the regulation of the cellular polarization and the formation of tight junctions at epithelial cell–cell contacts [35,36]. We still have no information in regard to the negative regulation of HIV-1 replication by PARD6α, and plan to study the mechanism underlying this phenomenon. Finally, JAK1 is a protein–tyrosine kinase involved in the interferon-α/β and -γ signal transduction pathways [37,38]. Thus, our results may suggest that HIV-1 replication is inhibited by interferon antiviral response through pathway(s) involving JAK1.

Study on late phase of the HIV-1 life cycle. Next, we studied on the effect of siRNAs for the late phase of the HIV-1 life cycle. J111 cells transfected with one of 257 siRNAs were transfected further with the luciferase reporter virus, pNL-Luc-envCT. We can monitor the level of viral gene expression by measuring the firefly luciferase activity in pNL-Luc-envCT-transfected cells. In addition, the efficiency of the late phases of the HIV-1 life cycle, including individual steps such as gene expression, post-translational modification of viral proteins, virion assembly and budding steps, can be also monitored by measuring the level of p24 antigen in the culture supernatant. We found that siRNAs against Axin1 (RefSeq Accession No. NM_003502.2), JAK1, HSF-1 (NM_005526.1), ROCK2 (NM_004850.2), DUSP1 (NM_004417.2), RanBP2 (NM_006267.3), and Ezrin (NM_003379.3) enhanced the level of firefly luciferase activity in the transfected cells (Fig. 2A). In addition, those siRNAs enhanced the level of p24 antigens in the culture supernatant (Fig. 3A). Especially, siRNAs against Axin1, DUSP1, and HSF1 strongly enhanced the levels of p24 antigen (Fig. 3A). In contrast, no siRNA was found to significantly suppress the late phase of HIV-1 replication

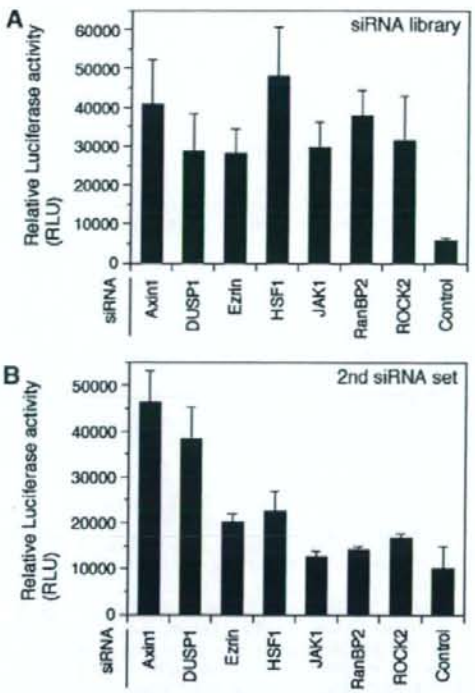

Fig. 2. siRNAs against Axin1 and DUSP1 reproducibly enhanced the HIV-1 gene expression. J111 cells were transfected with the siRNAs included in siRNA mini-library (A) or the second set of siRNAs (B) directed against the indicated target gene. Forty-eight hours after siRNA-transfection, the cells were transfected with pNL-Luc-envCT and pRL-TK. Forty-eight hours after the transfection of HIV-1 proviral construct, the cells were harvested, and the both of firefly and *Renilla* luciferase activities were measured, as described in Materials and methods. The firefly luciferase activity was normalized to the *Renilla* luciferase activity. Data are represented by means and standard deviations (error bars) of four independent experiments.

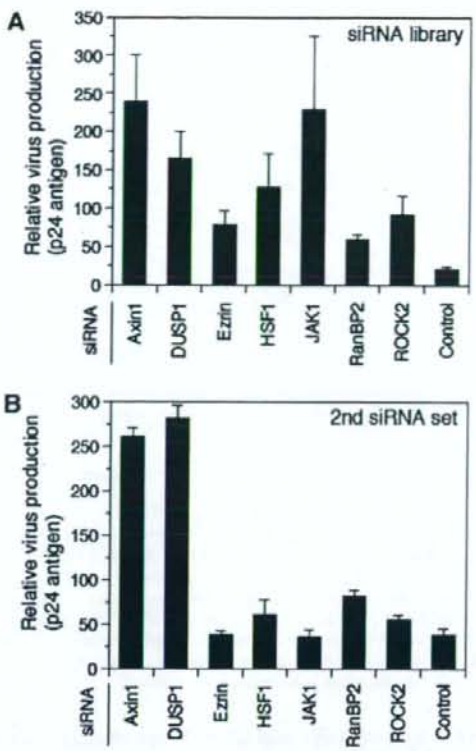

Fig. 3. siRNAs against Axin1 and DUSP1 reproducibly enhanced the HIV-1 replication at the late phase(s) of viral life cycle. J111 cells were transfected with siRNA, and then transfected with pNL-Luc-envCT and pRL-TK, as described in the legend of Fig. 2. Forty-eight hours after the transfection of HIV-1 proviral construct, the level of p24 antigens in the cell culture supernatants was measured, as described in Materials and methods. Data are represented by means and standard deviations (error bars) of four independent experiments.

in our assay system (data not shown). We next carried out the experiments using the second set of siRNAs directed against the different gene region of each target gene to confirm the gene-specific effects of siRNAs. The results showed that second set of the siRNAs against Axin1 and DUSP1, but not JAK1, HSF-1, ROCK2, RanBP2, and Ezrin, significantly enhanced the levels of luciferase activity (Fig. 2B) and p24 antigen (Fig. 3B). We also performed the experiments using the third set of siRNAs against JAK1, HSF-1, ROCK2, RanBP2, and Ezrin, but the results showed that those siRNAs did not enhanced the late phase of HIV-1 replication (data not shown). These results suggested that siRNAs against JAK1, HSF-1, ROCK2, RanBP2, and Ezrin non-specifically enhanced the HIV-1 replication. We carried out the sequential transfection of the cells with siRNA and HIV-1 molecular clone for studying the late phase of the HIV-1 life cycle. Although, we found no particular cell death in siRNA-transfected cells by WST-1 test (data not shown), the multiple transfection process may cause the physiological stress to the cells, and we consider that this is a part of reasons why some siRNAs showed such a high level of non-specific effects. Taken together with these results, we may conclude that the siRNAs against Axin1 and DUSP1 could enhance the HIV-1 replication at the late phase of viral life cycle, as a gene-specific manner. The target sequences of siRNAs against Axin1 and DUSP1 included in siRNA mini-library are 5′-TGGATACCTGCCGACCTTAAATG-3′ and 5′-TACCTTATGAGGACTAATCGAGT-3′, respectively.

DUSP1 is known as the mitogen-activated protein kinase (MAPK) phosphatase (MKP)-1, and is a negative regulator of MAPK signal transduction pathways [39] as well as of innate immune responses [40,41]. On the other hand, Axin1 is known as the regulator of WNT signaling pathway [42,43]. Our data suggest that Axin1 and DUSP1 negatively regulate the late phase of HIV-1 replication. The

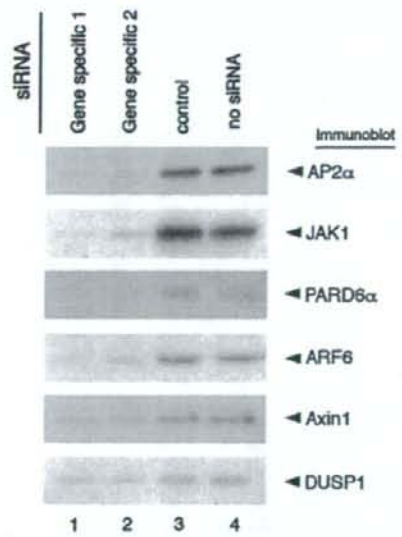

Fig. 4. Two sets of siRNAs against AP2α, ARF6, PARD6α, JAK1, Axin1, and DUSP1 suppressed the expression levels of target gene products. J111 cells were transfected with two sets of siRNA against indicated target gene (lanes 1 and 2), control siRNA (lane 3) or MOCK-transfected (lane 4). Then, the cells were subjected to immunoblot analysis. Indicated target gene products were immuno-stained using appropriate antibodies, as described in Materials and methods.

siRNA against Axin1 and DUSP1 reproducibly increased both of the luciferase activity in the cells transfected with HIV-1 proviral construct (Fig. 2) and the level of p24 antigen in the cell culture supernatant (Fig. 3), suggesting that these host factors may affect the transcription and/or translation step(s) in HIV-1 life cycle.

Study on protein levels of six host factors in siRNA-transfected cells. Finally, we confirmed the RNAi effects of siRNAs against six host factors using immunoblot analysis. The results showed that two sets of siRNAs against AP2α, ARF6, PARD6α, JAK1, Axin1, and DUSP1 reproducibly reduced the levels of target proteins (Fig. 4).

We revealed in this study that six host factors play roles as the negative regulators for HIV-1 replication. The precise mechanisms by which the host factors regulate viral replication are still unknown at moment. We are now underway to determine the target step(s) of those host factors in the HIV-1 life cycle.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2007.05.173.

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Rapid propagation of low-fitness drug-resistant mutants of human immunodeficiency virus type 1 by a streptococcal metabolite sparsomycin

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Here we report that sparsomycin, a streptococcal metabolite, enhances the replication of HIV-1 in multiple human T cell lines at a concentration of 400 nM. In addition to wild-type HIV-1, sparsomycin also accelerated the replication of low-fitness, drug-resistant mutants carrying either D30N or L90M within HIV-1 protease, which are frequently found mutations in HIV-1-infected patients on highly active antiretroviral therapy (HAART). Of particular interest was that replication enhancement appeared profound when HIV-1 such as the L90M-carrying mutant displayed relatively slower replication kinetics. The presence of sparsomycin did not immediately select the fast-replicating HIV-1 mutants in culture. In addition, sparsomycin did not alter the 50% inhibitory concentration (IC_{50}) of antiretroviral drugs directed against HIV-1 including nucleoside reverse transcriptase inhibitors (lamivudine and stavudine), non-nucleoside reverse transcriptase inhibitor (nevirapine) and protease inhibitors (nelfinavir, amprenavir and indinavir). The IC_{50}s of both zidovudine and lopinavir against multidrug resistant HIV-1 in the presence of sparsomycin were similar to those in the absence of sparsomycin. The frameshift reporter assay and Western blot analysis revealed that the replication-boosting effect was partly due to the sparsomycin's ability to increase the –1 frameshift efficiency required to produce the *Gag–Pol* transcript. In conclusion, the use of sparsomycin should be able to facilitate the drug resistance profiling of the clinical isolates and the study on the low-fitness viruses.

Keywords: drug resistant mutants, enhancement of replication, HIV-1, low-fitness mutants, sparsomycin

Introduction

Highly active antiretroviral therapy (HAART) has been successful in controlling the progression of AIDS caused by HIV-1. However, HAART has accelerated the emergence and spread of multidrug-resistant HIV-1. Once drug-resistant HIV-1 occurs in a HIV-1-infected patient, the success rate of HAART drops substantially. Resistance testing has been shown to be valuable to optimize HAART against HIV-1 infection (Hirsch *et al.*, 2000; Rodriguez-Rosado *et al.*, 1999). Profiling drug resistance might be necessary even before the initiation of HAART because of the spread of drug-resistant HIV-1 (Boden *et al.*, 1999; Gehringer *et al.*, 2000; Yerly *et al.*, 1999).

Genotypic and phenotypic resistance testing are the two major ways to determine the drug resistance of clinical HIV-1 isolates. For genotyping, the HIV-1 genome isolated from the infected individuals is sequenced. This HIV-1 genome is than cross-referenced with a database and we are able to predict the drug resistance profile of HIV-1. However, it is impossible to predict the phenotype when we encounter a combination of mutations that has never been documented. This may raise a concern when a new drug is released in the market. Another problem in the genotyping is the presence of genotype-phenotype discordance (Parkin *et al.*, 2003; Sarmati *et al.*, 2002).

Alternatively, for the phenotypic resistance testing, the drug resistance profiles are measured by many biological/virological assay systems (Hertogs *et al.*, 1998; Iga *et al.*, 2002; Jarmy *et al.*, 2001; Kellam & Larder, 1994; Menzo *et al.*, 2000; Walter *et al.*, 1999). Phenotypic resistance testing is powerful because the diagnosis is based on experimental observations. Among the systems, ones that depend on the multi-round HIV-1 replication seemed to provide the best drug resistance data reflecting the *in vivo* condition. However, many drug-resistant mutants have lower replication capabilities than wild-type (wt) HIV-1, which makes the phenotypic resistance testing difficult and time-consuming. In order to overcome these problems, it would be useful to develop a technique to make HIV-1

replicate faster without altering the effectiveness of antiretroviral compounds.

During our search for an inhibitor of HIV-1 replication, we found sparsomycin, a metabolite from *Streptomyces sparsogenes*, which reproducibly enhanced the replication of HIV-1. Therefore, we tested whether sparsomycin merits phenotypic drug resistance profiling studies on low-fitness HIV-1 isolates.

Materials and methods

Cells and viruses

Human embryonic kidney (HEK) 293T cells were maintained in Dulbecco's modified Eagle's medium (Sigma-Aldrich, Tokyo, Japan) supplemented with 10% fetal bovine serum (FBS; Hyclone, Logan, UT, USA), penicillin and streptomycin (Invitrogen, Carlsbad, CA, USA). H9, Jurkat, SupT1 and HPB-Ma cells were maintained in RPMI1640 (Sigma-Aldrich) supplemented with 10% FBS, penicillin and streptomycin. All the cell lines were incubated at 37°C in a humidified 5% CO_2 atmosphere. As previously described, HIV-1 (HXB2) was produced by transfecting proviral DNA into 293T cells and collecting the culture medium 3 days post-transfection (Komano *et al.*, 2004). The replication-incompetent HIV-1 (HXB2 Δ*vpr*, Δ*rev*, Δ*env*, Δ*nef*) was produced by transfecting the proviral DNA carrying renilla luciferase with the *nef* open reading frame into 293T cells, along with the expression plasmid for *env*, *tat*, *rev* and *nef* (pIIIex) as described previously in Komano *et al.* (2004). As previously described, the D30N, L90M, and D25N protease mutants of HIV-1 were generated by the site-directed mutagenesis (Sugiura *et al.*, 2002). The multidrug-resistant HIV-1 DR3577 was a clinical isolate from a patient on HAART in which reverse transciptase carried the following mutations M41L, D67N, K70R, V75M, K101Q, T215F and K219Q, and protease carried the following mutations L10I, K20R, M36I, M46I, L63P, A71V, V82T, N88S and L90M. For the generation of replication-incompetent murine leukaemia virus (MLV) vector expressing firefly luciferase, pCMMP luciferase was transfecting into 293T cells along with *gag/pol* and VSV-G expressing plasmids as described previously (Komano *et al.*, 2004).

Chemical compound

Sparsomycin was either purchased from Sigma-Aldrich (cat. S1667) or obtained from Dr Nakajima (Toyama Prefectural University, Toyama, Japan). Sparsomycin was dissolved in 2mM dimethyl sulphoxide and stored at –20°C until use.

Monitoring HIV-1 replication

For HIV-1 infection, 1×10^6 cells were incubated with the culture supernatant containing approximately 10 ng of p24. Alternatively, wt HIV-1, or D30N and L90M mutants were introduced into cells either by electroporation or DEAE-dextran-mediated protocol as previously described (Matsuda *et al.*, 1993; Miyauchi *et al.*, 2005). The culture supernatants were collected everytime the infected cells were split until they ceased to proliferate. The amount of p24 antigen of HIV-1 in the culture supernatants was quantified by using Retro TEK p24 antigen ELISA kit according to the manufacturer's protocol (Zepto Metrix, Buffalo, NY, USA). The signal was detected by Vmax ELISA reader (Molecular Devices, Palo Alto, CA, USA).

Determining 50% inhibitory concentrations (IC_{50})

IC_{50} was calculated by using a reporter cell line, MARBLE, developed by Sugiura *et al.* (personal communication). In brief, a clone of HPB-Ma carrying the long terminal repeat (LTR)-driven firefly luciferase cassette integrated in its genome was infected with HIV-1 and incubated in the presence of varying concentrations of antiretroviral compounds for a week. The cells were then lysed to measure the firefly luciferase activity, which represented the propagation of HIV-1 in culture. The firefly luciferase activity was normalized by constitutively-expressed renilla luciferase activity. The dual luciferase assay was performed according to the manufacturer's protocol (Promega, Madison, WI, USA). Chemiluminescence was detected by Lmax (Molecular Devices).

Reporter assay

The –1 frameshift reporters, pLuc (–1) and pLuc (0), were kindly provided by Dr Brakier-Gingras (Dulude *et al.*, 2002). The renilla luciferase expression vector phRL/CMV was purchased from Promega. pLTR Luc encoded GFP-luciferase under the regulation of HIV-1's LTR promoter (Komano *et al.*, 2004). pLTRΔnefLuc encoded renilla luciferase by substituting *nef* in the proviral context of HXB2 (Komano *et al.*, 2004). Plasmids were transfected into 293T cells by Lipofectamine 2000 plus reagent in accordance with the manufacturers' protocol (Invitrogen). For the detection of luciferase activities, the dual glo luciferase assay was performed at 2–3 days post-transfection or post-infection according to the manufacturers' protocol (Promega). The signal was detected by Vmax ELISA reader (Molecular Devices).

Western blot analysis

COS-7 cells were transfected with Lipofectamine 2000 (Invitrogen) or Fugen6 (Roche, Basel, Switzerland) according to the manufacturer's protocol with proviral DNA encoding the D25N protease mutant. At 48 h post-transfection, cells were washed with PBS and lysed in a buffer containing 4% SDS, 100 mM Tris-HCl (pH 6.8), 12% 2-ME, 20% glycerol and bromophenol blue.

Samples were boiled for 10 min. Protein lysates approximately equivalent to 5×10^4 cells were separated in 5–20% SDS-PAGE (Perfect NT Gel, DRC, Tokyo, Japan), transferred to a polyvinylidene fluoride (PVDF) membrane (Immobilon-P^{SQ}, Millipore, Billerica, MA, USA), and blocked with 5% dried non-fat milk (Yuki-Jirushi, Tokyo, Japan) in PBS. For the primary antibody, we used rabbit anti-*Gag* polyclonal antibody or mouse anti-*Gag* monoclonal antibody. For the secondary antibody, either a biotinylated anti-rabbit antibody or a biotinylated anti-mouse goat antibody (GE Healthcare Bio-Science, Piscataway, NJ, USA) was used. For the tertiary probe, a horseradish peroxidase-conjugated streptoavidin (GE Healthcare Bio-Science) was used. Signals were developed by incubating blots with a chemilumenescent horseradish peroxidase substrate (GE Healthcare Bio-Science) and detected by using Lumi-Imager F1 (Roche).

Results

The structure of sparsomycin, a metabolite from *Streptomyces sparsogenes*, is unique in that it comprises two unusual entities, a monooxodithioacetal moiety and a uracil acrylic acid moiety (Figure 1A). H9 cells were infected with HIV-1 and then maintained in the presence of varying concentrations of sparsomycin. Dimethyl sulphoxide was added in the absence of sparsomycin throughout this study. At 7 days post-infection, a massive syncytial formation was found in the presence of sparsomycin (Figure 1B). The higher the concentration of sparsomycin, the faster p24 accumulated in the culture supernatants (Figure 1C). Similar observations were made in Jurkat, SupT1 (Figures 1D and E), and HPB-Ma cells although the speed of p24 accumulation appeared different among the cell lines. On the other hand, sparsomycin did not show any detectable effect on the cell growth under concentrations of 500 nM.

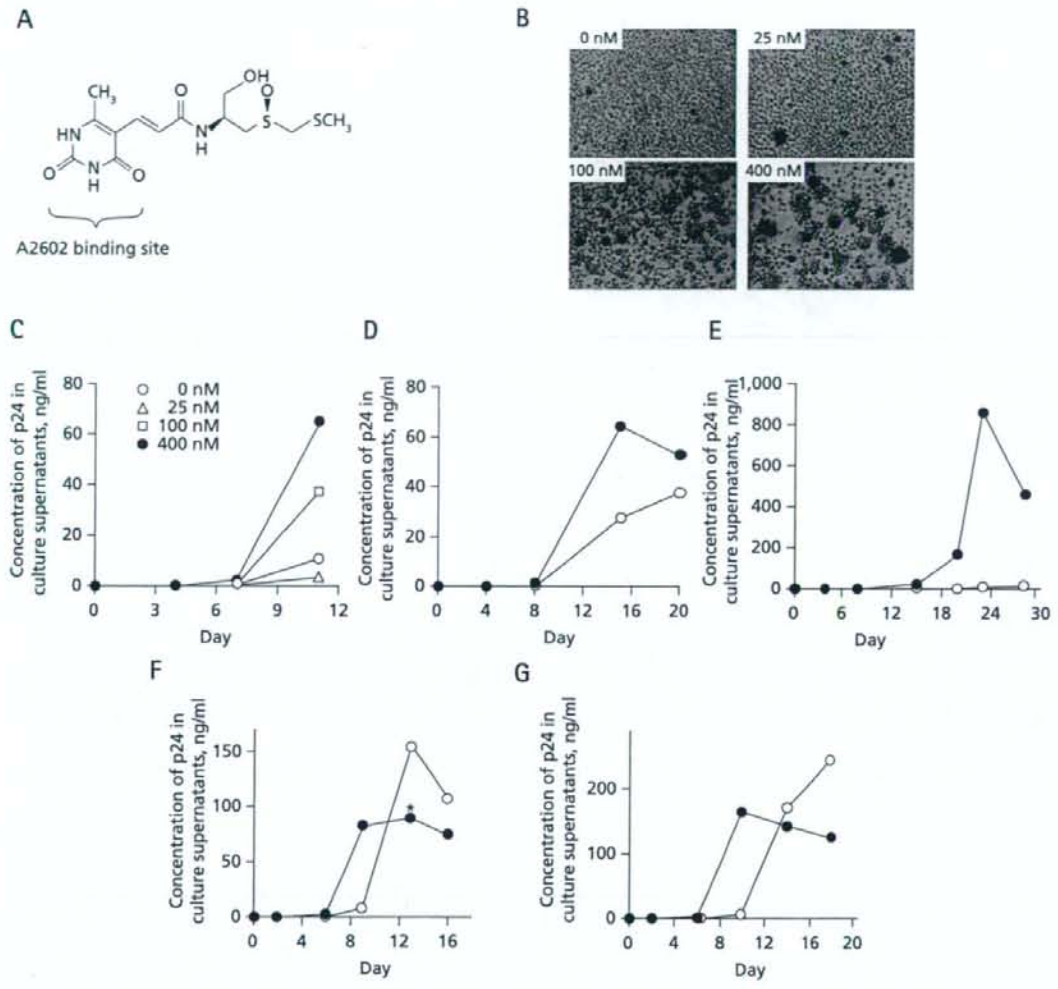
These results could be due to sparsomycin's ability to either boost HIV-1 replication or select a mutant that replicated substantially faster than the wt HIV-1. To differentiate these possibilities, we recovered the virus-containing culture supernatants from the H9 cell culture at the peak of HIV-1 replication in the presence of 400 nM sparsomycin (asterisk in Figure 1F). Then fresh H9 cells were infected with the recovered virus, the cells were split into two samples and 400 nM of sparsomycin was added to each sample. If sparsomycin selected fast-growing mutants, the replication profiles of HIV-1 should resemble the original sample with sparsomycin (solid circle, Figure 1F) regardless of sparsomycin's presence. However, the replication profile in the presence of sparsomycin shifted leftward (Figure 1G), suggesting that it was unlikely that sparsomycin selected the fast-replicating viral mutants. Therefore, it is likely that sparsomycin boosted HIV-1 replication. Replication-enhancing effects were also seen by using the chemically-synthesized derivatives of sparsomycin (unpublished data; Nakajima *et al.*, 2003). The replication-boosting effect levelled-out at 500 nM, an approximately 20-fold lower concentration than the 50% toxic dose (TD_{50}) of sparsomycin (Ash *et al.*, 1984).

To demonstrate the usefulness of sparsomycin in HIV-1 research, we have examined whether sparsomycin can also boost the replication of drug-resistant low-fitness isolates. The D30N and L90M are common drug-resistant mutations found within HIV-1 protease in HIV-1-infected patients on HAART (Devereux *et al.*, 2001; Kantor *et al.*, 2002; Pellegrin *et al.*, 2002; Sugiura *et al.*, 2002). We introduced proviral DNA carrying the D30N or L90M mutation into H9, Jurkat, and SupT1 cells. HIV-1 replication was than monitored in the presence of 400 nM of sparsomycin. The replication of both viral mutants was substantially enhanced in the presence of sparsomycin in H9 cells (Figures 2A and B). The replication of the L90M-carrying mutant was also enhanced in Jurkat and SupT1 cells (Figures 2C and D). Of note, the replication enhancement appeared profound when HIV-1 displayed relatively slower replication kinetics (for example, the replication of D30N-carrying mutant versus the wt HIV-1 in H9 cells or the replication of HIV-1 in SupT1 versus H9 cells).

Considering the use of sparsomycin in the phenotypic resistance testing, it is critical to know whether sparsomycin affects HIV-1's sensitivity to the antiretroviral drugs. The respective IC_{50} of representative antiretroviral drugs in the absence and the presence of 400 nM sparsomycin were as follows: reverse transcriptase inhibitors; lamivudine, 13.7 and 10.4 nM, and stavudine, 6.3 and 17.0 nM; an non-nucleoside reverse transcriptase inhibitor, nevirapine, 78.2 and 146.4 nM; and protease inhibitors, nelfinavir, 2.8 and 1.0 nM, indinavir, 4.2 and 3.0 nM, and amprenavir, 3.4 and 3.3 nM. Then, we examined whether the presence of sparsomycin affected the IC_{50} of both zidovudine (AZT) and lopinavir (LPV) against a multidrug-resistant HIV-1 isolate, DR3577. The magnitude of both AZT and LPV-resistance of DR3577 was in the order of 2 log (data not shown). The IC_{50}s of AZT in the presence and absence of 400 nM sparsomycin were 14.0 and 36.7 nM, respectively, and for LPV they were 103.1 and 78.9 nM, respectively. These data suggested that the presence of sparsomycin did not significantly influence the IC_{50} of antiretroviral drugs on the replication of both wt and drug-resistant HIV-1.

Finally, we investigated the possible mechanisms that sparsomycin enhanced the replication of HIV-1 and its mutants although the estimated magnitude of enhancement per single replication cycle was small. To do this, we used non-T cells to increase the sensitivity of assays. First, we examined if the early phase of HIV-1's life cycle was

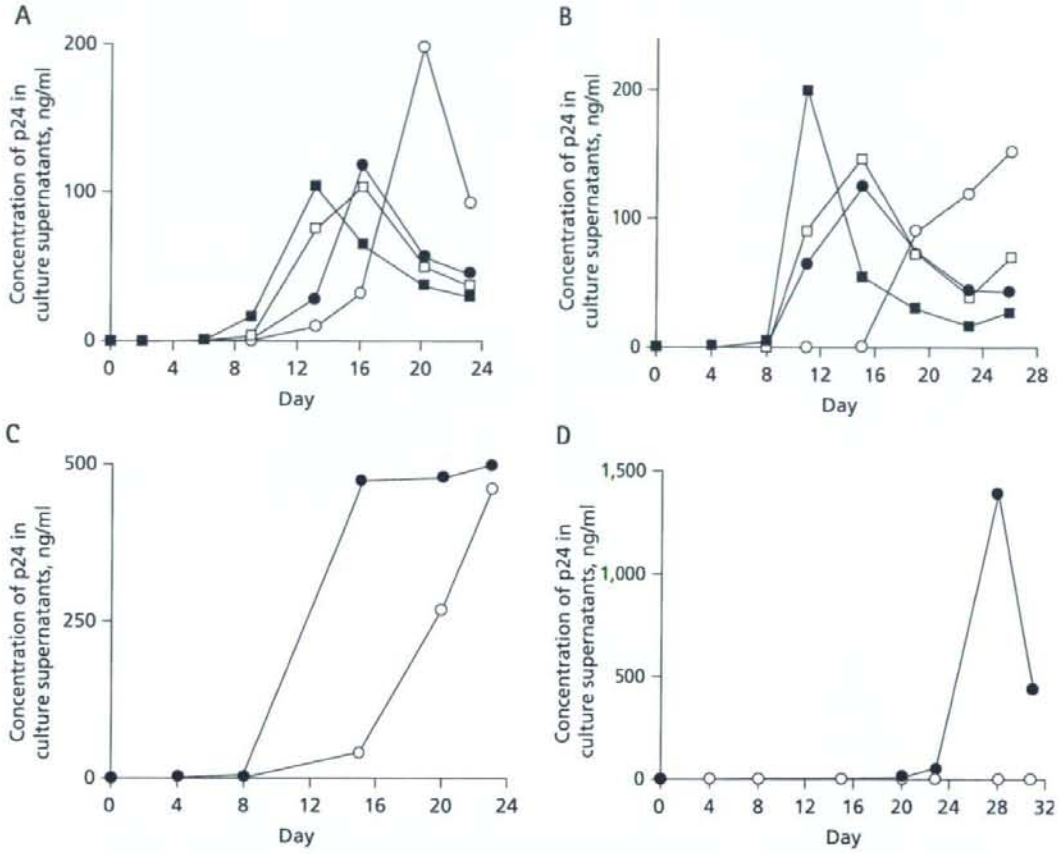
Figure 1. The enhancement of HIV-1 replication by sparsomycin



(A) Structure of sparsomycin. The uracil acrylic acid moiety confers the binding capacity to the conserved nucleobase A2602 of the large ribosomal subunit. **(B)** H9 cells infected with HIV-1 were photographed at a week after infection (magnification, ×200). **(C)** The replication profiles of HIV-1 in H9 cells in the presence of varying concentrations of sparsomycin. **(D-G).** The replication profiles of HIV-1 in Jurkat **(D)**, SupT1 **(E)**, and H9 cells **(F and G)** in the presence of sparsomycin (400 nM, solid circle) or in the absence (open circle; **F and G**). Virus-containing culture supernatant was collected at 13 days post-infection (asterisk, **F**) to infect fresh H9 cells and the replication profiles of HIV-1 were analysed in the presence of sparsomycin (400 nM, solid circle) or in the absence (open circle, **G**).

positively affected by sparsomycin. In the presence of increasing concentrations of sparsomycin, 293 CD4$^+$ T-cells and NP2 CD4 CXCR4 cells were infected with either a replication-deficient HIV-1 vector enveloped with its own *Env* or a VSV-G-psueodotyped MLV vector. Two days post-infection, cells were lysed to measure the luciferase activities representing the efficiency of viral infection. Our results indicate that luciferase activities were not significantly increased at the replication-enhancing dose for both HIV-1 and MLV vectors (Figure 3A). Thus suggesting that the early phase of the retroviral life cycle was not detectably affected by sparsomycin.

Figure 2. Sparsomycin's ability to enhance replication of low-fitness drug resistant HIV-1 mutants



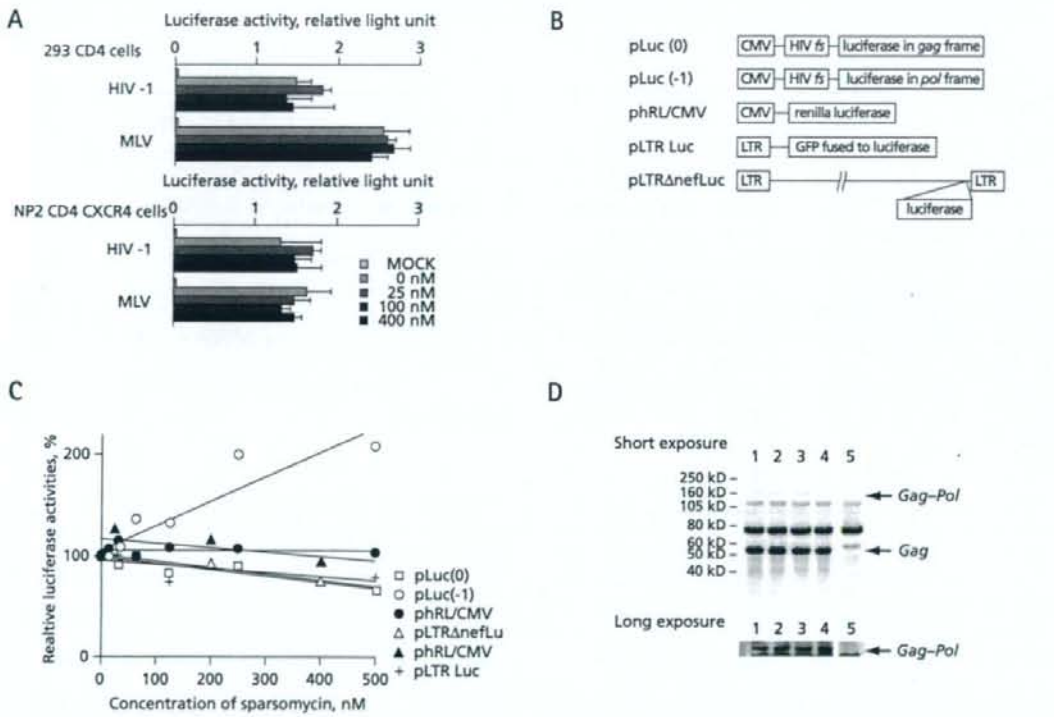
(A and B) The replication kinetics of the D30N-carrying (circle) and L90M-carrying (square) mutants in the presence of sparsomycin (400 nM, solid) or in the absence (open) were investigated twice independently in H9 cells. **(C and D)** The replication kinetics of the L90M-carrying mutant were examined in Jurkat cells **(C)** and SupT1 cells **(D)** in the presence of sparsomycin (400 nM, solid circle) or in the absence (open circle).

Next, we examined the possible active role of sparsomycin in the late phase of HIV-1's life cycle. Sparsomycin has been reported to be a potential enhancer of the −1 frameshift (Dinman *et al.*, 1997). Therefore, we tested whether sparsomycin could positively affect the efficiency of the translational −1 slip at HIV-1's frameshift signal using the reporter assay system established by Dulude *et al.* (2002). The −1 frameshift reporter was created by placing the firefly luciferase in the *pol* frame, pLuc(−1), whereas the control plasmid pLuc(0) has the luciferase in the *gag* frame after the frameshift signal (Figure 3B). In addition, HIV-1's LTR-driven luciferase reporter constructs were tested (pLTR Luc and pLTRΔnefLuc; Figure 3B). We transfected these reporter plasmids into 293T cells along with the renilla luciferase-expressing plasmid phRL/CMV (Promega) to measure the non-specific or toxic effects, if any, of sparsomycin. Cells were incubated in the presence of varying concentrations of sparsomycin for 3 days. Then the dual luciferase assay was performed. The pLuc(−1) behaved differently from the other groups in that the luciferase activities from the pLuc(−1) increased in a dose-dependent fashion. The magnitude of increasement was 2.3-fold at the replication-enhancing dose (Figure 3C). The positive correlation between the relative luciferase activity and the concentration of sparsomycin was statistically significant (r=0.926, P<0.001, n=8, Student's t-test). In contrast, the luciferase activities from the other reporters, even the renilla luciferase plasmid

co-transfected with the pLuc(-1) vector, remained unchanged (Figure 3C). These data suggested that sparsomycin positively affected the efficiency of HIV-1's −1 frameshift. It also suggested that sparsomycin did not enhance transcription from the viral promoter or the translation of proteins driven by the LTR promoter to enhance HIV-1 replication.

If the efficiency of −1 frameshift was increased, we would expect that the *Gag–Pol* to *Gag* ratio to increase. To test this, we transfected COS-7 cells with the HIV-1's proviral DNA carrying the D25N mutation in protease that produced catalytically inactive protease to increase the sensitivity of detecting *Gag–Pol* (Xie *et al.*, 1999). When sparsomycin was added, the intensities of *Gag–Pol* gradually increased in relation to the reporter assay. The *Gag–Pol* to *Gag* ratio reached 1.3-fold at 400 nM sparsomycin when normalized with results produced in the absence of sparsomycin (Figure 3D). The average and standard deviation of the *Gag–Pol* to *Gag* ratio from four independent experiments were 1.29 ± 0.14 at the replication enhancing concentration of sparsomycin (1.29-, 1.48-, 1.16-, and 1.24-fold increase). Similar results were obtained by using

Figure 3. The possible mechanism of HIV-1 replication enhancement by sparsomycin



(A) The single round infection efficiencies of HIV-1 and murine lukaemia virus (MLV) vectors measured by the virally-encoded luciferase activities in 293 CD4⁺ T-cells and NP2 CD4 CXCR4 cells in the presence of varying concentrations of sparsomycin. **(B)** The schematic drawing of constructs used in the reporter assay. The HIV-1's frameshift signal (*fs*) was placed between the CMV promoter and the luciferase. The luciferase was placed in either the *gag* frame (pLuc(0)) or the *pol* frame (pLuc(-1)). The renilla luciferase expression vector phRL/CMV was used in parallel. The pLTR Luc encodes the GFP-luciferase driven by HIV-1's LTR promoter. The pLTRΔnefLuc has the renilla luciferase substituting *nef* in the proviral context of HXB2. **(C)** The luciferase activities from the above reporter constructs without sparsomycin were set as 100% and the relative luciferase activities in the presence of sparsomycin were shown. The renilla luciferase activities from phRL/CMV were shown for the pLuc(-1) (solid circle) and pLTRΔnefLuc (solid triangle) transfections in particular. The pLuc(-1) behaved differently from the other groups and the positive correlation between the relative luciferase activity and the concentration of sparsomycin was statistically significant (r=0.926, P<0.001, n=8, Student's t-test). The SD was within 10% from the avgerage. Shown are the representative data from two independent experiments. **(D)** Western blot analysis to measure the *Gag–Pol* and *Gag* ratio. Cell extracts were separated in the SDS-polyacrylamide gel and immunoblotted by using the rabbit polyclonal antibodies raised against p24. (lane 1, 0 nM; lane 2, 20 nM; lane 3, 200 nM; lane 4, 400 nM; lane 5, MOCK). The lower panel shows the *Gag–Pol* signal obtained from the long exposure of the same blot.

two different antibodies recognizing *Gag*. We were unable to detect a significant increase in the *Env:Gag* ratio (unpublished data), suggesting that the sparsomycin's effect on *Gag–Pol:Gag* ratio was specific. These data suggested that the translational efficiencies of viral proteins were not equally enhanced by sparsomycin. Altogether, it was strongly suggested that the sparsomycin's replication-boosting effect on HIV-1 was partly due to the enhancement of the –1 frameshift efficiency.

Discussion

In the present study, we have demonstrated that sparsomycin is an enhancer of HIV-1 replication in many human T cell lines at concentrations between 400–500nM. Our preliminary observation suggested that HIV-1 replication was also enhanced in primary peripheral blood monocyte culture (data not shown). Sparsomycin should be able to accelerate the study on the low-fitness HIV-1 such as drug-resistant mutants. As sparsomycin did not alter the IC_{50} of multiple antiretroviral drugs on both wt and drug-resistant HIV-1, its usage should be able to facilitate the phenotypic resistance testing of clinical isolates and as a result, benefit HIV-1-infected patients. Our observation raised an immediate concern as to whether sparsomycin-producing Streptomyces species caused an opportunistic infection in humans, which influenced AIDS progression. However, we did not find any reports suggesting so.

Sparsomycin and puromycin are the only antibiotics that can inhibit protein synthesis in bacterial, archaeal and eukaryotic cells (Ottenheijm *et al.*, 1986; Porse *et al.*, 1999). Sparsomycin has the ability to enhance the –1 frameshift in mammalian cells as well as *S. cervisiae* (Dinman *et al.*, 1997). The proposed molecular mechanism behind this ability was either through a higher affinity of the donor stem for the ribosome and slowing down the rate of the peptidyl transfer reaction, or a change in the steric alignment between donor and acceptor tRNA stems resulting in decreased peptidyl-transfer rates. Conversely, puromycin is not known to enhance the –1 frameshift in mammalian cells. At sub-toxic concentrations, puromycin was unable to enhance the HIV-1 replication (unpublished data). These data, along with the data provided in this paper, implied that the sparsomycin's unique ability to enhance the –1 frameshift might play a role in boosting the HIV-1 replication.

The maintenance of the –1 frameshift efficiency at the optimal range is critical for HIV-1 to replicate (Jacks *et al.*, 1988). Therefore, limiting *Gag–Pol* production should lead to an inhibition of viral replication because *pol* encodes enzymes essential for viral replication (Levin *et al.*, 1993). In contrast, it was also reported that increasing the *Gag–Pol* to *Gag* ratio by twofold resulted in a reduction of viral replication (Hung *et al.*, 1998; Shehu-Xhilaga *et al.*, 2001). Thus, a modest alteration of the –1 frameshift efficiency should markedly affect the replication capacity of HIV-1. Our data indicated that sparsomycin increased the efficiency of –1 frameshift by 1.3-fold, which produces a better replication capacity for HIV-1. As a result, we hypothesize that HIV-1 has a 'suboptimal' –1 frameshift efficiency. In theory, the 1.3-fold difference per one replication cycle becomes approximately 10-fold after 10 rounds of viral replication cycle because the effect accumulates exponentially. The difference should become larger when HIV-1 replicates with the slower kinetics and the replication profile is monitored over a longer time course. In fact, our experimental data were in good agreement with the above estimation. In nature, HIV-1 does not accumulate mutations within the frameshift signal to achieve the higher frameshift efficiencies. This implies that there are multiple and complex regulatory mechanisms that keep the efficiency of the –1 frameshift at suboptimum. Under these conditions, the best efficiency of HIV-1 survival in the host might be achieved. Altogether, one of the possible mechanisms that sparsomycin boosted the HIV-1 replication could be the enhancement of the –1 frameshift efficiency.

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