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Inhibiting lentiviral replication by HEXIM1, a cellular negative regulator of the CDK9/cyclin T complex

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Objective: Tat-dependent transcriptional elongation is crucial for the replication of HIV-1 and depends on positive transcription elongation factor b complex (P-TEFb), composed of cyclin dependent kinase 9 (CDK9) and cyclin T. Hexamethylene bisacetamide-induced protein 1 (HEXIM1) inhibits P-TEFb in cooperation with 7SK RNA, but direct evidence that this inhibition limits the replication of HIV-1 has been lacking. In the present study we examined whether the expression of FLAG-tagged HEXIM1 (HEXIM1-f) affected lentiviral replication in human T cell lines.

Methods: HEXIM1-f was introduced to five human T cell lines, relevant host for HIV-1, by murine leukemia virus vector and cells expressing HEXIM1-f were collected by fluorescence activated cell sorter. The lentiviral replication kinetics in HEXIM1-f-expressing cells was compared with that in green fluorescent protein (GFP)-expressing cells.

Results: HIV-1 and simian immunodeficiency virus replicated less efficiently in HEXIM1-f-expressing cells than in GFP-expressing cells of the five T cell lines tested. The viral revertants were not immediately selected in culture. In contrast, the replication of vaccinia virus, adenovirus, and herpes simplex virus type 1 was not limited. The quantitative PCR analyses revealed that the early phase of viral life cycle was not blocked by HEXIM1. On the other hand, *Tat*-dependent transcription in HEXIM1-f-expressing cells was substantially repressed as compared with that in GFP-expressing cells.

Conclusion: These data indicate that HEXIM1 is a host factor that negatively regulates lentiviral replication specifically. Elucidating the regulatory mechanism of HEXIM1 might lead to ways to control lentiviral replication.

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Keywords: CDK9, cyclin T, HEXIM1, lentivirus, *tat*

Introduction

Activation of transcription elongation requires the positive transcription elongation factor b complex (P-TEFb) composed of cyclin dependent kinase 9 (CDK9) and cyclin T1, T2, or K [1]. P-TEFb is essential for efficient transcriptional elongation from the promoter of human immunodeficiency virus type 1 (HIV-1), the long terminal repeat (LTR) (reviewed in [2,3]). The functional interaction between P-TEFb and the viral protein Tat has been well studied. Immediately after viral transcription starts at the LTR of the integrated proviral genome, the nascent viral transcript forms a three-dimensional structure called TAR. In the presence of P-TEFb, Tat binds to TAR. Through the Tat–TAR interaction, Tat activates P-TEFb and therefore assures the efficient

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completion of viral gene transcription and the propagation of HIV-1.

Recently, the regulatory mechanisms of P-TEFb function have been elucidated. In 2001, the interaction of P-TEFb with 7SK RNA was found to be necessary to inactivate the kinase activity of CDK9 within P-TEFb [4–6]. However, the binding of 7SK RNA alone is not sufficient to inactivate P-TEFb. More recently, Yik *et al.* demonstrated that the inactivation of P-TEFb requires hexamethylene bisacetamide-induced protein 1 (HEXIM1; synonyms CLP1, MAQ1, and HIS1) [7–9]. The inactivation of P-TEFb by the HEXIM1-7SK RNA complex appears to regulate the transcriptional elongation of cellular genes.

The HEXIM1-7SK RNA complex has been shown to physically compete with Tat for binding to P-TEFb [10]. In agreement with this finding, HEXIM1 was shown to inhibit Tat-dependent transcription from the HIV-1 LTR in transient transfection assays [8,11,12]. However, no data demonstrating that HEXIM1 is able to limit HIV-1 replication has been provided. Here we provide direct experimental evidence that the constitutive expression of HEXIM1 specifically limits lentiviral replication.

Methods

Plasmids

The FLAG-tagged HEXIM1 expression constructs were generated by reverse-transcription PCR using RNA isolated from CEM cells as templates. The primers used were 5′-CACCTCGAGCCACCATGGACTACAAAGACGATGACGACAAGGCCGAGCCATTCTTGTC-3′ and 5′-CAATTGCTAGTCTCCAAACTTGGAAAGCGGCGC-3′′ for amino terminus FLAG tagging, and 5′-CACCTCGAGCCACCATGGCCGAGCCATTCTTGTCAGAATATC-3′ and 5′-CAATTGCTAGTCGTCATCGTCTTTGTAGTCGTCTCCAAACTTGGAAAGCGGCGCTC-3′ for carboxy terminus FLAG tagging. The *Xho*I-*Mfe*I fragments of the PCR products were cloned into the *Xho*I-*Mfe*I sites of pCMMP IRES GFP, generating pCMMP f-HEXIM1 and pCMMP HEXIM1-f [13]. The cytomegalovirus (CMV) promoter-driven *gag-pol* expression vector psyn*gag-pol* has been previously described by Wagner *et al.* [14] and pLTR*gag-pol* was constructed by cloning the *Mlu*I-*Hin*dIII fragment encoding the LTR from pNL-luc [15] into the *Mlu*I-*Hin*dIII sites of psyn*gag-pol*. The tax expressing plasmid pCGtax and pHTLV LTR luciferase were kindly provided by Dr. Watanabe (Tokyo Medical Institute). The *tat*-expressing plasmid pSVtat was a generous gift from Dr. Freed (National Cancer Institute-Frederick, Frederick, Maryland, USA). The plasmid pLTR-luc has been described previously (Miyauchi *et al.*, *Antiviral Chemistry and Chemotherapy*, in press). The following plasmids have been described previously by Komano *et al.* [13]: pVSV-G, pMD*gag-pol*, pTM3Luci, phRL-CMV and pSIVmac239ΔnefLuc.

Cells and transfection

All the mammalian cells were maintained in RPMI 1640 (Sigma, St Louis, Missouri, USA) supplemented with 10% fetal bovine serum (Japan Bioserum, Tokyo, Japan), penicillin and streptomycin (Invitrogen, Tokyo, Japan). Cells were incubated at 37°C in a humidified 5% CO_2 atmosphere. Cells were transfected using Lipofectamine 2000 according to the manufacturer's protocol (Invitrogen).

Western blotting

Cells were lysed with sample buffer, sonicated, and boiled for 5 min. Samples were separated on 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels and transferred to polyvinylidene difluoride membranes (Millipore, Billerica, Massachusetts, USA) for western blotting according to standard techniques. Membranes were blocked with Tris-buffered saline containing 0.05% Tween-20 (TBS-T) containing 5% (w/v) non-fat skim milk (Yuki-Jirushi, Tokyo, Japan) for 1 h at room temperature and incubated with primary antibodies including the M2 anti-FLAG epitope monoclonal antibody (Sigma), an anti-actin monoclonal antibody (MAB1501R; Chemicon/Millipore, Billerica, Massachusetts, USA), an anti-cyclin T1 rabbit polyclonal antibody (H-245; Santa Cruz Biotechnology, Santa Cruz, California, USA), an anti-cyclin T2a/b goat polyclonal antibody (A-20; Santa Cruz), an anti-p24 monoclonal antibody (183-H12-5C; NIH AIDS Research and Reference Reagent Program), an anti-HIS1 chicken polyclonal antibody (N-150; GenWay, San Diego, California, USA), and an anti-Bip/GRP78 monoclonal antibody (clone 40; BD Biosciences/Transduction Laboratories, San Jose, California, USA) for 1 h at room temperature. Membranes were washed with TBS-T and incubated with appropriate second antibodies including biotinylated anti-goat (GE Healthcare Bio-Sciences, Piscataway, New Jersey, USA) or anti-chicken IgY (Promega, Madison, Wisconsin, USA), and EnVision+ (Dako, Glostrup, Denmark) for 1 h at room temperature. For a tertiary probe, we used horseradish peroxidase (HRP)-streptavidine (GE Healthcare) if necessary. Signals were visualized with an LAS3000 imager (Fujifilm, Tokyo, Japan) after treating the membranes with the Lumi-Light Western Blotting Substrate (Roche Diagnostics GmbH, Mannheim, Germany).

Reporter assay

Luciferase activity was measured 48 h after transfection or infection using a DualGlo assay kit (Promega) according to the manufacturer's protocol. The beta-galactosidase activity was measured using a LumiGal assay kit (BD Biosciences/Clontech, San Jose, California, USA) according to the manufacturer's protocol. The

chemiluminescence was detected with a Veritas luminometer (Promega).

Monitoring viral replication

To monitor HIV-1 replication, the culture supernantants were subjected to either a reverse transcriptase assay [16] or an enzyme-linked immunosorbent assay (ELISA) to detect p24 antigens using a Retro TEK p24 antigen ELISA kit according to the manufacturer's protocol (Zepto Metrix, Buffalo, New York, USA). For simian immunodeficiency virus (SIV) a p27 antigen ELISA kit was used according to the manufacturer's protocol (Zepto Metrix). The signals were measured with a Multiskan Ex microplate photometer (ThermoLabsystems, Helsinki, Finland). For vaccinia virus, adenovirus, and herpes simplex virus (HSV)-1, the activity of reporter genes was measured as previously described [13].

Generating viruses

To produce HIV-1 and SIV, 293T cells were transfected with plasmids encoding proviral DNA of HIV-1 (pHXB2) or pSIVmac239ΔnefLuc and culture supernatants containing viruses were collected at 48 h post-transfection. Murine leukemia virus (MLV) and lentiviral vectors pseudotyped with VSV-G were produced as described previously by cotransfecting 293T cells with either the pNL-Luc and pVSV-G vectors or the pMD*gag-pol*, pVSV-G, and pCMMP vectors [13]. Green fluorescent cells were sorted by fluorescence activated cell sorter (FACS) Aria (Becton Dickinson, San Jose, California, USA).

Reverse transcriptase-polymerase chain reaction

Total RNA was isolated with an RNeasy kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instruction. The reverse transcriptase (RT)-polymerase chain reaction (PCR) assay was performed with a One Step RNA PCR Kit (Takara, Otsu, Japan), imaged by a Typhoon scanner 9400 (GE Healthcare), and quantified with Image Quant software (GE Healthcare). For the amplification of endogenous HEXIM1, the forward primer 5′-ACCACACGGAGAGCCTGCAGAAC-3′ and the reverse primer 5′-TAGCTAAATTTACGAAACCAAAGCC-3′ were used. For the amplification of HEXIM1-f, the forward primer 5′-GTACCTGGAACTGGAGAAGTGCCC-3′ and the reverse primer 5′-CAATTGCTAGTCGTCATCGTCTTTGTAGTC-3′ were used. For cyclophilin A, the forward primer 5′-CACCGCCACCATGGTCAACCCCACCGTGTTCTTCGAC-3′ and the reverse primer 5′-CCCGGGCCTCGAGCTTTCGAGTTGTCCACAGTCAGCAATGG-3′ were used.

Quantitative real time polymerase chain reaction

The real time PCR reaction was performed in a DNA Engine Opticon 2 Continuous Fluorescence Detection System (Bio-Rad, Hercules, California, USA). The cellular genomic DNA and total RNA were extracted 48 h post-infection with a DNeasy kit (Qiagen) and RNeasy kit (Qiagen), respectively, according to the manufacturer's instruction. For the reagents, we used QuantiTect SYBR Green PCR and RT-PCR Kits (Qiagen). To estimate the amount of integrated HIV-1 DNA, Alu-LTR PCR was performed according to the method described previously using the following primers: for the first PCR, 5′-AACTAGGGAACCCACTGCTTAAG-3′ and 5′-TGCTGGGATTACAGGCGTGAG-3′, and for the second PCR, 5′-AACTAGGGAACCCACTGCTTAAG-3′ and 5′-CTGCTAGAGATTTTCCACACTGAC-3′ [17]. The beta-globin primers have been described previously [18]. To estimate the amount of HIV-1 RNA, the second PCR primers for the Alu-LTR PCR were used. The primers for cyclophilin A are described above.

Results and discussion

The HEXIM1 cDNA tagged with a FLAG epitope at either the amino terminus (f-HEXIM1) or the carboxy terminus (HEXIM1-f) was cloned in a mammalian expression plasmid (Fig. 1a). A luciferase assay revealed that the Tat-dependent enhancement of transcription from the HIV-1 LTR was reduced by co-transfecting HEXIM1-expressing plasmids, whereas neither Tat-independent basal transcription from the HIV-1 LTR nor CMV promoter-driven transcription was affected (Fig. 1b). An oncogenic retrovirus human T cell leukemia virus type 1 (HTLV-1) encodes for *tax*, a functional homologue of HIV-1's *tat*, that utilizes P-TEFb to enhance transcription from the LTR promoter [19]. However, *tax*-dependent enhancement of transcription was not affected by HEXIM1 in similar experimental conditions (Fig. 1c). To monitor the effect of HEXIM1 on HIV-1 replication, we introduced HEXIM1-expressing plasmids into HeLa-CD4 cells along with pNL4-3, which produces replication-competent HIV-1, and measured the RT activity in the culture supernatant 1 week post-transfection. Transfecting HEXIM1-expressing plasmids decreased the RT activity in a dose-dependent manner (Fig. 1d). Next, we asked whether the inhibition of viral replication was specific to HIV-1 by examining vaccinia virus, adenovirus, and HSV-1 replication. We found that the propagation of these three viruses was not inhibited by HEXIM1-f expression (Fig. 1e–g), suggesting that the inhibition of viral replication by HEXIM1 was HIV-1-specific.

To examine whether HEXIM1 negatively affects lentiviral replication in the physiologically relevant host, we isolated human T cell lines constitutively expressing HEXIM1-f. We cloned HEXIM1-f cDNA into a pCMMP (MLV retroviral vector plasmid (Fig. 2a). The plasmid encoded an internal ribosomal entry site (IRES)-mediated green fluorescent protein (GFP) expression cassette, so that MLV vector-infected cells

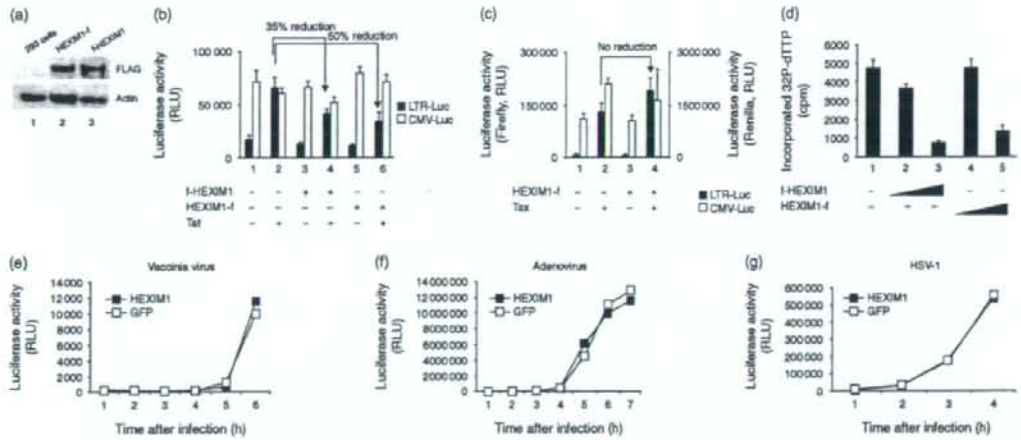

Fig. 1. Expression of hexamethylene bisacetamide-induced protein 1 (HEXIM1) specifically inhibits HIV-1 replication. (a) Detection of HEXIM1 cDNA tagged with a FLAG epitope at either the amino terminus (f-HEXIM1) or the carboxy terminus (HEXIM1-f) by western blot analysis in transiently transfected 293 cells (upper panel, approximately 65 kD). A western blot against actin is shown as a loading control (lower panel). (b) Expressing FLAG-tagged HEXIM1 decreased the luciferase activity driven by HIV-1 long terminal repeat (LTR) promoter in the presence of Tat (lanes 4 and 6, LTR-Luc, solid bars). However, FLAG-tagged HEXIM1 did not affect the expression of renilla luciferase from co-transfected plasmid driven by the cytomegalovirus (CMV) promoter (CMV-Luc, open bars). Representative data from three independent experiments done in triplicate are shown. Cells were transfected with 0.8 μg HEXIM1-expressing plasmid for the indicated lanes, 0.1 μg of pSV*tat* for the indicated lanes, and 0.1 μg of pLTR-Luc and 0.5 μg for phRL/CMV for all lanes. (c) Expressing FLAG-tagged HEXIM1 did not decrease the luciferase activity driven by HTLV-1 LTR promoter in the presence of Tax (lanes 2 and 4, LTR-Luc, solid bars) as well as renilla luciferase driven by the CMV promoter (CMV-Luc, open bars). Representative data from three independent experiments done in triplicate are shown. Cells were transfected with 0.8 μg of HEXIM1-expressing plasmid for the indicated lanes, 0.1 μg of pCGtax for the indicated lanes, and 0.1 μg of pHTLV LTR Luc and 0.5 μg for phRL/CMV for all lanes. (d) The dose-dependent reduction of HIV-1 production by transfection of HEXIM1-encoding plasmids (0.1 μg for lanes 2 and 4, 0.4 μg for lanes 3 and 5) along with a plasmid producing infectious HIV-1 (pNL4-3, 0.1 μg) in HeLa-CD4 cells. (e–g) Expressing HEXIM1-f did not limit the replication of vaccinia virus (e), adenovirus (f), or HSV-1 (g) in 293T cells. The *y*-axis represents the reporter gene activity, which reflects viral replication. Representative data from three independent experiments are shown. GFP, green fluorescent protein; RLU, relative light unit.

could be readily identified by the green fluorescence. Human T cell lines, including SUP-T1, MOLT-4, CEM, Jurkat, and M8166 were infected with MLV pseudotyped with vesicular stomatitis virus glycoprotein (VSV-G), and GFP-positive cells were collected with a FACS (Fig. 2a). For the negative control, we used MLV expressing GFP only. The successful introduction of HEXIM1-f into the cells was verified by RT-PCR and Western blot analysis (Fig. 2b and c). The total HEXIM1 protein expression in HEXIM1-f-transduced cells was approximately 3.7-, 1.5-, 2.0-, 4.8-, and 1.8-fold higher than in GFP-transduced cells in the CEM, Jurkat, MOLT-4, SUP-T1, and M8166 cell lines, respectively (Fig. 2c). To our surprise, the HEXIM1-f-expressing T cell lines remained GFP-positive, and therefore HEXIM1-f-positive, for more than 6 months and proliferated at rates almost indistinguishable from GFP-expressing cells. The expression levels of cyclin T1, cyclin T2, actin, and Bip/GRK78 in HEXIM1-f-expressing cells were almost identical to those in GFP-expressing cells, suggesting that the gene expression did not compensate the upregulated HEXIM1 (Fig. 2b and c). Expression of cyclin T2 was undetectable in M8166 cells (Fig. 2c). Similarly, HEXIM1-f expression did not affect the cell surface levels of the HIV-1 receptors CD4 and CXCR4 as demonstrated by FACS analysis (data not shown). These data indicate that the expression of HEXIM1-f did not reach levels where the physiological regulation of P-TEFb blocked cellular gene transcription.

The replication kinetics of HIV-1 or SIV was monitored by measuring the accumulation of viral capsid antigen in the culture medium. Strikingly, HIV-1 replicated more slowly in cells of all four T cell lines expressing HEXIM1-f than in cells expressing GFP (Fig. 2d–g). Similarly, HEXIM1-f-expressing M8166 cells supported SIV replication less efficiently than did GFP-expressing M8166 cells (Fig. 2h). Interestingly, the magnitude of HIV-1 replication delay was the most substantial in SUP-T1 cells, in which the levels of endogenous HEXIM1 were the lowest among the four cell lines tested for HIV-1 replication (Fig. 2c). Similar observations were made when the HIV-1 infection experiments were repeated, indicating that the expression of functional HEXIM1-f did not change over the course of the replication monitoring. We tested whether the viruses emerged in

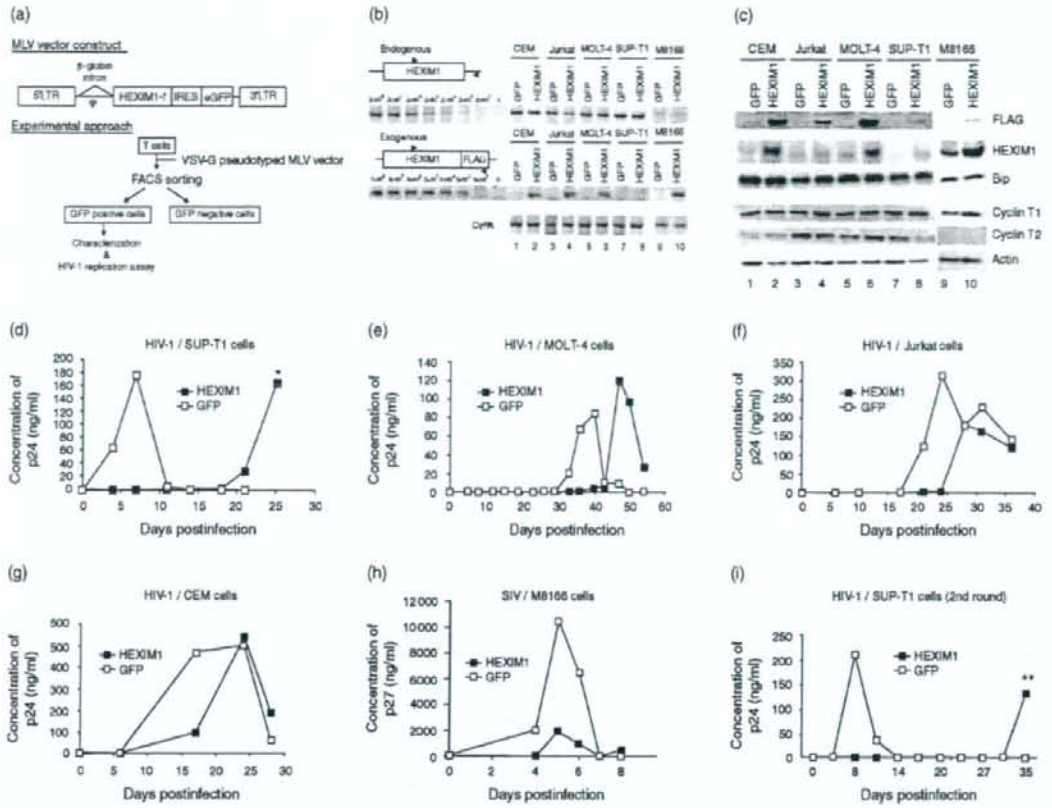

Fig. 2. Lentiviral replication is inhibited in various T cell lines constitutively expressing hexamethylene bisacetamide-induced protein 1 (HEXIM-1) cDNA tagged with a FLAG epitope at the carboxy terminus (HEXIM1-f). (a) The genomic organization of the retroviral vector expressing HEXIM1-f and a schematic representation of the experimental approach. (b) Detection of endogenous HEXIM1 and murine leukemia virus (MLV)-transduced HEXIM1-f (exogenous) mRNA by reverse transcriptase-polymerase chain reaction in green fluorescent protein (GFP)- and HEXIM1-f-expressing cells. The primer design is drawn schematically. Amplification efficiency was examined by using a known number of templates as standards for HEXIM1. Cyclophilin A (CyPA) was amplified to ensure the quality of the RNA. (c) Western blot analysis demonstrating expression of HEXIM1-f (denoted FLAG), endogenous HEXIM1 (HEXIM1), Bip, cyclin T1, cyclin T2, and actin in isolated T cell lines. (d–g) Replication profiles of HIV-1 (HXB2) in SUP-T1 (d), MOLT-4 (e), Jurkat (f), and CEM (g) cells either expressing HEXIM1-f or GFP alone. Representative data from two or three independent experiments are shown. (h) Replication profile of SIV in M8166 cells either expressing HEXIM1-f or GFP alone. Representative data from two independent experiments are shown. (i) The replication profiles of HIV-1 recovered from SUP-T1/HEXIM1-f cells (asterisk in Fig. 2d) in fresh SUP-T1/GFP or SUP-T1/HEXIM1-f. LTR, long terminal repeat.

HEXIM1-f-expressing cells were 'revertants' that might be able to replicate in HEXIM1-f-expressing cells as fast as in GFP-expressing cells. To address this, we recovered virus-containing culture supernatants from SUP-T1/HEXIM1-f cells at the peak of replication kinetics (asterisk, Fig. 2d). Then, both fresh SUP-T1/GFP and SUP-T1/HEXIM1-f were infected with the recovered virus and the replication kinetics was monitored. However, HIV-1 still replicated in SUP-T1/HEXIM1-f cells more slowly than in SUP-T1/GFP cells (Fig. 2i), akin to the original profiles (Fig. 2d), and the nucleotide sequences of LTR and *tat*, the primary targets of HEXIM1, remained unchanged (double asterisk in Fig. 2i). In addition, no mutations were found in viruses propagated in GFP-expressing SUP-T1 cells. Similar observations were made in MOLT-4 cells (data not shown). These data provide direct evidence that the expression of HEXIM1 inhibits lentiviral replication in human T cell lines.

Based on our experimental observations as well as the reported functions of HEXIM1, we assumed that the ability of HEXIM1 to limit HIV-1 replication was mostly due to the inhibition of Tat/P-TEFb-dependent transcriptional elongation. However, it was possible that HEXIM1 might also have targeted other viral replication

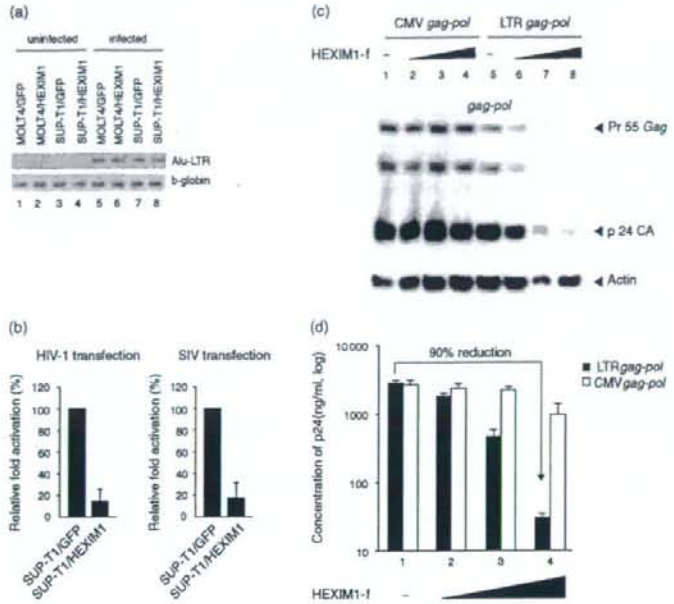

Fig. 3. Hexamethylene bisacetamide-induced protein 1 (HEXIM1) cDNA tagged with a FLAG epitope at the carboxy terminus (HEXIM1-f) does not affect the efficiency of viral integration or post-translational processes. (a) The Alu-long terminal repeat (LTR) and beta-globin polymerase chain reaction products from VSV-G-pseudotyped HIV-1-infected MOLT-4 and SUP-T1 cells expressing either green fluorescent protein (GFP) or HEXIM1-f alone were separated in an agarose gel and photographed. (b) The luciferase activities in SUP-T1/GFP or SUP-T1/HEXIM1-f cells electroporated with 10 μg of a plasmid encoding LTR-driven firefly luciferase plus 1 μg of phRL/cytomegalovirus (CMV). The firefly luciferase activity normalized to renilla luciferase activity in SUP-T1/GFP cells was set to 100%. The error bars represent the standard deviation of three independent experiments. (c) Western blot analysis showing Gag and its cleaved products expressed from either CMV promoter- or LTR promoter-driven *gag-pol* expression plasmid in the presence of pSVtat (0.1 μg, all lanes) and increasing amounts of HEXIM1-f (0.2 μg for lanes 2 and 6, 0.6 μg for lanes 3 and 7, and 2.0 μg for lanes 4 and 8). (d) The amount of p24 produced in the culture supernatant from cells analyzed in Fig. 3c was measured by enzyme-linked immunosorbent assay. Representative data from three independent experiments done in triplicate are shown. SIV, simian immunodeficiency virus.

steps. To test this possibility, we examined the viral entry and production processes separately. The efficiency of viral entry was analyzed by measuring the efficiency of viral integration. SUP-T1/GFP or SUP-T1/HEXIM1-f cells were infected with a replication-incompetent HIV-1 vector pseudotyped with VSV-G that expresses luciferase upon successful infection. We conducted an Alu-LTR PCR assay to detect the integrated viral genome. PCR products were detected only from HIV-1-infected cells (Fig. 3a). The signal intensities of Alu-LTR PCR products from GFP- and HEXIM1-f-expressing cells were similar. To compare the efficiency of viral infection as well as transcription quantitatively, we employed a real time PCR technique. Some infected cells were collected for an Alu-LTR PCR assay to quantify the amount of integrated viral genome, and the rest were processed to measure the amount of viral transcript as well as the luciferase activity. The amount of Alu-LTR PCR product from SUP-T1/HEXIM1-f cells was 3.5- and 3.3-fold more to that from SUP-T1/GFP cells from two independent experiments, respectively (Table 1). These data suggest that the efficiency of viral integration was not inhibited in HEXIM1-f-expressing SUP-T1 cells. In contrast, the relative abundance of HIV-1 transcript expressed in SUP-T1/HEXIM1-f cells was substantially decreased to 0.03 and 2.9% relative to SUPT1/GFP cells (Table 1). Furthermore, the luciferase activities were 200-fold lower in SUP-T1/HEXIM1-f cells than in SUP-T1/GFP cells (Table 1). Similar data was obtained from MOLT-4 cells infected with HIV-1 pseudotyped with VSV-G (data not shown). The transfection of plasmids encoding reporter viral DNA can bypass the viral entry and make it possible to measure the effect of HEXIM1 on LTR-driven transcription and translation. Consistent with above data, transfecting pNL-Luc into SUP-T1/HEXIM1-f cells gave significantly lower luciferase activities than SUP-T1/GFP cells (Fig. 3b, left). Similar data were obtained using pSIVmac239ΔnefLuc (Fig. 3b, right). These data strengthen the possibility that HEXIM1 targets post-integration processes.

Table 1. Effect of hexamethylene bisacetamide-induced protein 1 (HEXIM1) cDNA tagged with a FLAG epitope at the carboxy terminus (HEXIM1-f) on viral entry and transcription in SUP-T1 cells examined by quantitative real time polymerase chain reaction.

		Integrated HIV-1 genome			HIV-1 transcript			Luciferase activity	
Exp.	Transduced gene	Alu-LTR (copy)	β-globin (copy)	Normalized[a] (%)	HIV-1 RNA (copy)	CyPA (copy)	Normalized[b] (%)	RLU[c]	Normalized[d] (%)
1	GFP	5.2×10^5	6.7×10^6	100.0	1.6×10^6	6.8×10^7	100.0	3.2×10^5	100.0
	HEXIM1-f	2.0×10^6	7.4×10^6	351.3	6.7×10^1	1.0×10^8	0.03	1.5×10^3	0.5
2	GFP	4.6×10^6	1.8×10^7	100.0	3.1×10^8	8.9×10^7	100.0	7.1×10^5	100.0
	HEXIM1-f	1.6×10^7	1.9×10^7	333.2	9.4×10^6	9.3×10^7	2.9	3.4×10^3	0.5

[a]The number of Alu-long terminal repeat (LTR) products divided by the number of beta-globin products in SUP-T1/GFP is set to 100%. The abundance of Alu-LTR products in SUP-T1/HEXIM1-f relative to SUP-T1/green fluorescent protein (GFP) is shown.
[b]The number of HIV-1 RNA transcripts in SUP-T1/GFP divided by the number of cyclophilin A (CyPA) transcripts is set to 100%. The abundance of HIV-1 RNA in SUP-T1/HEXIM1-f relative to SUP-T1/GFP is shown.
[c]The luciferase activity is shown by relative light unit (RLU).
[d]The luciferase activity in SUP-T1/GFP is set to 100%. The luciferase activity in SUP-T1/HEXIM1-f relative to SUP-T1/GFP is shown.

To test this further, we analyzed the efficiency of post-transcriptional processes with a transient transfection assay measuring the amount of Pr55 Gag, a viral gene product, and virus-like particles (VLPs) produced in the culture supernatants. For this purpose, we used the CMV promoter-driven *gag-pol* expression plasmid, because HEXIM1-f did not affect CMV-driven transcription (Fig. 1b). At the levels of HEXIM1-f where LTR-driven Tat-dependent transcription was drastically inhibited (Fig. 3c, lanes 7, 8), the amount of CMV promoter-driven Gag expression was almost identical to that in the absence of HEXIM1-f (Fig. 3c, lanes 1–4). Furthermore, the processing pattern of Pr55 Gag in the presence of HEXIM1-f was identical to that in its absence (Fig. 3c). These data indicate that HEXIM1-f did not inhibit the transcription from a Tat-independent promoter, the translation of viral protein, or the protease activity of HIV-1. Finally, the potential effect of HEXIM1 on viral budding was examined. To do this, the amount of p24 CA in the culture supernatant of transfected cells was quantified as a representation of the amount of VLP. Expressing HEXIM1-f reduced VLP production from cells co-transfected with pLTR*gag-pol* and pSVtat at levels comparable to the protein expression levels (Fig. 3c and d). In contrast, expressing HEXIM1-f did not reduce the amount of VLP produced by cells co-transfected with pCMV*gag-pol* and pSVtat in conditions in which Tat-dependent LTR transcription was substantially inhibited (Fig. 3c and d). Taken together, this indicates that HEXIM1-f lowers the efficiency of Tat-dependent transcription from LTR promoter but does not block the efficiency of the late phase of the viral life cycle including translation, Gag's assembly, and budding. Thus, it is likely that HEXIM1 primarily targets Tat/P-TEFb-dependent transcription to inhibit HIV-1 replication.

Our findings demonstrated that HEXIM1, a cellular P-TEFb inhibitor, is a specific negative regulator of lentiviral replication in human T cell lines. The replication of vaccinia virus, adenovirus, and HSV-1 were not affected by HEXIM1-f expression; however, the *Tat*-dependent transcription of the LTR promoter of both HIV-1 and SIV was reduced by HEXIM1-f. HEXIM1 limited replication of HIV-1 dramatically at levels where it did not visibly affect cell physiology (as little as a 5-fold increase over the endogenous levels), nor were revertants immediately selected in HEXIM1-f-expressing cells. These data support the feasibility of developing HIV-1 inhibitors targeting the processes in which HEXIM1 is involved. For example, it is conceivable to hunt for a non-toxic chemical inducer for HEXIM1 since expression of HEXIM1 is induced by hexamethylene bisacetamide (HMBA) that is considerably toxic for cells [20].

P-TEFb has been shown to support transcription of the *c-myc* and CIITA transcription factors (reviewed in [21,22]). The functions of these transactivators are critical for cell proliferation, but in this study constitutive expression of HEXIM1-f, which reduces P-TEFb activity, did not affect the cell proliferation of human T cell lines, the human epithelial cell lines HEK293 or the NP2 glioblastoma cell lines (data not shown). How can this be explained? Very recently, a high-molecular-weight bromodomain protein, Brd4, was found to function as a 'cellular *tat*' [23,24]. Interestingly, it was shown that Brd4 binds not only to cyclin T1 but also to cyclin T2, a widely expressed variant of cyclin T, to which HEXIM1 binds but Tat does not [23–25]. We hypothesize that Brd4 might be able to recruit and activate P-TEFb more efficiently than does Tat, leaving cellular transcription unaffected by the upregulated expression of HEXIM1 from the retroviral vector. An alternative possibility comes from the fact that HEXIM1 does not interact with the ubiquitously expressed cyclin K, which functions as a P-TEFb component. It is possible that Tat is not able to utilize P-TEFb consisting of CDK9 and cyclin K but Brd4 can, such that cyclin K may substitute for cyclin T1 to support Brd4-mediated cellular gene transcription.

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

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

AIDS | pathogenesis | drug target | lysozyme

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

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

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

Results

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

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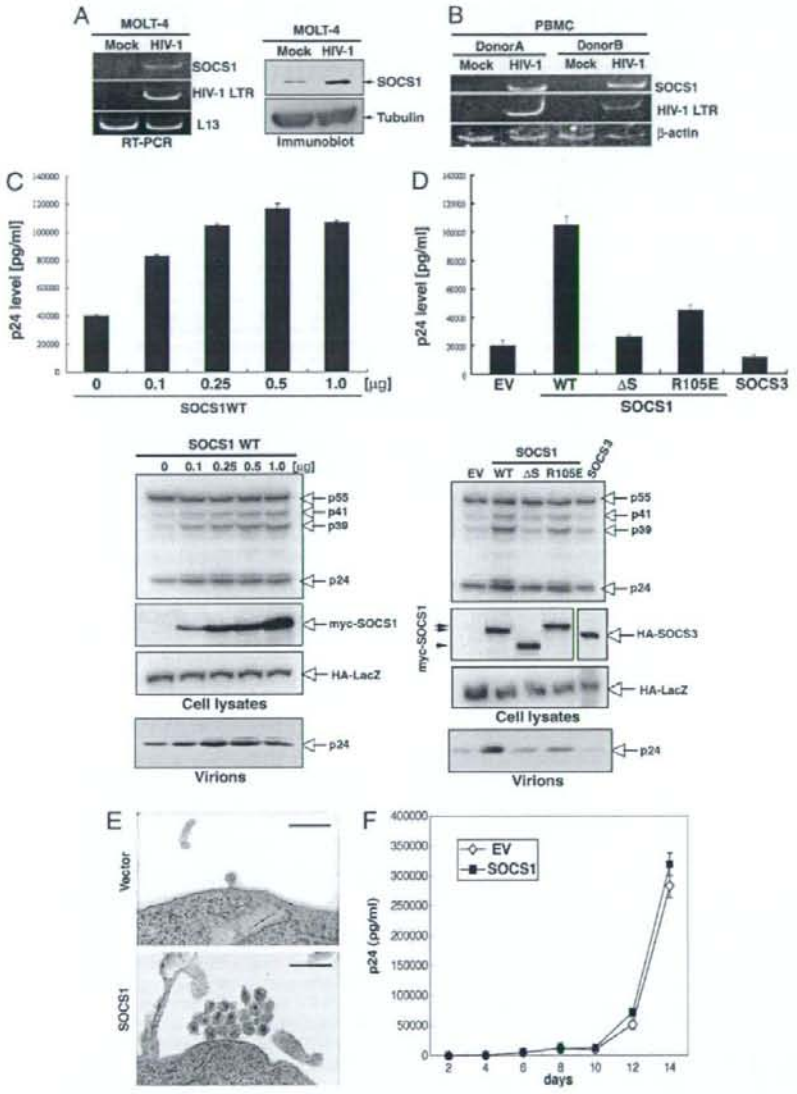

Fig. 1. SOCS1 is induced upon HIV-1 infection and enhances HIV-1 particle production. (A) MOLT-4 cells were mock-infected or infected with HIV-1_{NL4-3}, and then total RNA and protein extracts derived from these cells were subjected to semiquantitative RT-PCR (Left) and immunoblotting (Right), respectively.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

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

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

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

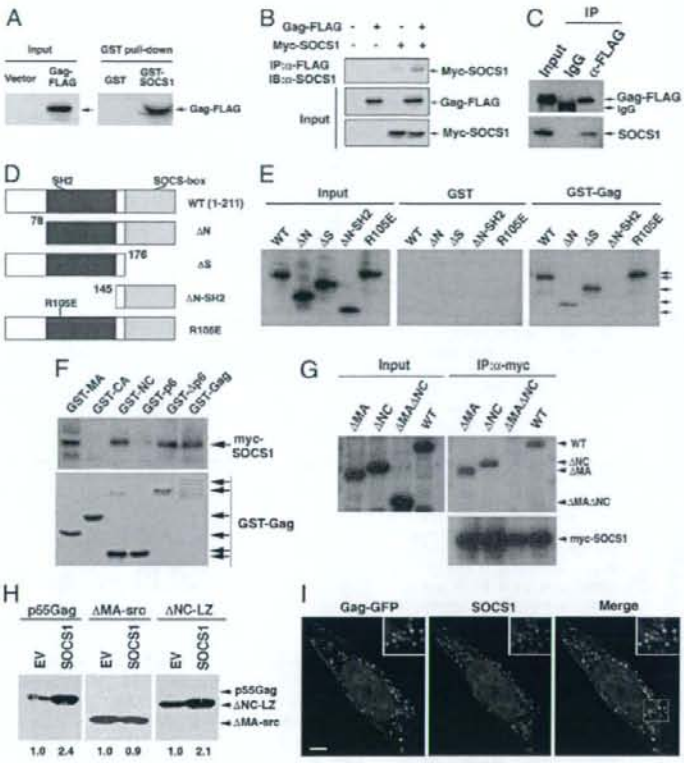

Fig. 2. SOCS1 interacts with HIV-1 Gag. (A) Extracts of 293T cells transfected with either empty vector or Gag-FLAG were subjected to pull-down analyses using glutathione–agarose beads with GST-SOCS1 in the presence of 10 ng/ml RNase followed by immunoblotting with anti-FLAG antibodies. (B) Extracts of 293T cells transiently expressing myc-SOCS1 and Gag-FLAG were subjected to immunoprecipitation (IP) with anti-FLAG monoclonal antibodies in the presence of 10 ng/ml RNase followed by immunoblotting (IB) analysis with either anti-FLAG or anti-myc polyclonal antibodies. (C) 293T cells were transiently transfected with Gag-FLAG, and cell lysates were then subjected to immunoprecipitation with anti-FLAG antibodies followed by immunoblotting with an antibody directed against endogenous SOCS1. (D and E) 293T cells expressing various myc-tagged SOCS1 mutants (schematically depicted in D) were analyzed by GST pull-down analysis with either GST or GST-Gag recombinant protein (E). (F) GST fusion proteins of the indicated regions of Gag were bound to glutathione beads and incubated with cell lysates from 293T cells expressing myc-SOCS1 in the presence of 10 ng/ml RNase followed by immunoblotting with anti-myc antibodies. (G) SOCS1 binds p55 Gag via either its MA or NC domains. 293T cells were transfected with myc-SOCS1 and cotransfected with Gag-FLAG, GagΔMA-FLAG, GagΔNC-FLAG, or GagΔMAΔNC-FLAG. At 24 h after transfection, cell lysates treated with 10 μg/ml RNase were subjected to coimmunoprecipitation with anti-myc monoclonal antibodies followed by immunoblotting with anti-FLAG or anti-myc polyclonal antibodies. (H) Functional interaction of SOCS1 with MA but not NC. 293T cells were transfected with wild-type Gag, ΔMA-src, or ΔNC-LZ (Z_{IP}-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. 1E). 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. 1E). 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. 1F). 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. 2A). 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. 2D, further demonstrated that a mutant lacking the both N-terminal and SH2 domain (ΔN-SH2) could not bind

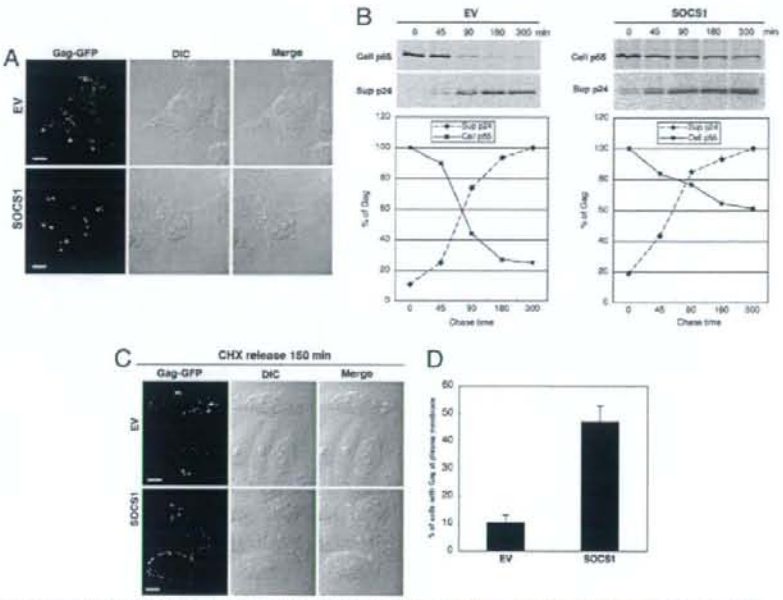

Fig. 3. SOCS1 enhances both the stability and trafficking of HIV-1 Gag. (A) HeLa cells cotransfected with pNL4-3 and either control vector (EV) or SOCS1 were immunostained with antibodies targeting anti-p24 (CA). Confocal microscopy with differential interference contrast (DIC) was then performed. (Scale 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. 2E). 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. 2E), 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. 2F), 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. 2G). 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. 2H), 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. 2I). 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. 3A). Furthermore, the levels of cytoplasmic Gag were found to be much lower in the SOCS1-expressing cells compared with the control cells (Fig. 3A). 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. 3B). 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. 3B). This finding again suggests that SOCS1 facilitates the intracellular trafficking of newly synthesized Gag proteins to PM.

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

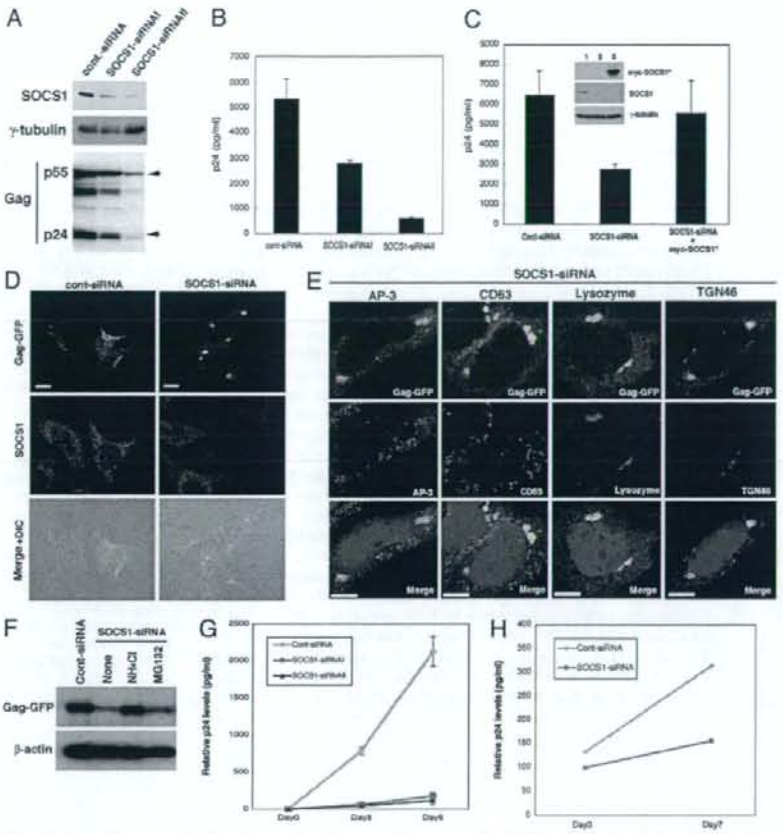

Fig. 4. The targeted inhibition of SOCS1 suppresses Gag trafficking and HIV-1 particle production and enhances Gag degradation in lysosomes. (A and B) 293T cells were transfected with either control siRNA or two different SOCS1-specific siRNAs (I or II) together with pNL4-3. At 48 h after transfection,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

this time point, Gag-GFP was found to localize predominantly in a perinuclear region in the control cells (Fig. 3C), whereas almost half of the SOCS1-transfected cells exhibited Gag-GFP localization on PM (Fig. 3D). 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. 4C), 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. 4D). 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. 4E). 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. 4F), further indicating that the perinuclear clusters of Gag will undergo lysosomal degradation rather than proteasomal degradation when optimal Gag transport to PM is suppressed by the inhibition of SOCS1.

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

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

Discussion

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

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

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

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

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

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

Materials and Methods

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

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

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

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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

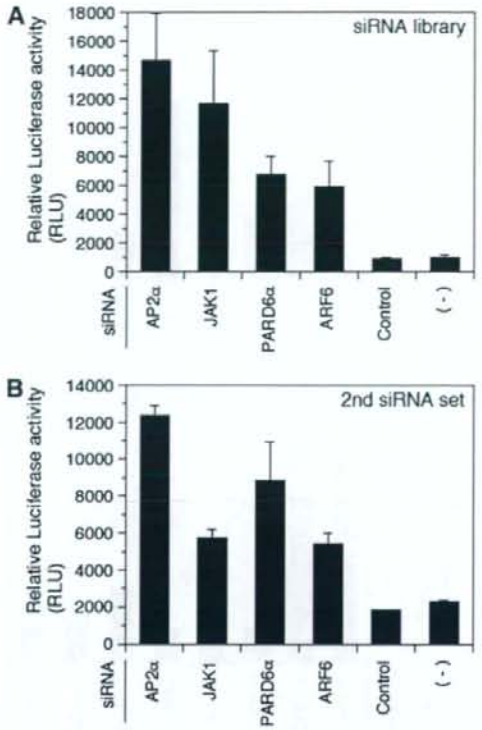

Fig. 1. siRNAs against AP2α, JAK1, PARD6α, and ARF6 enhanced the HIV-1 replication at the early phase(s) of the viral life cycle. J111 cells were transfected with the siRNAs included in siRNA mini-library (A) or the second set of siRNAs (B) directed against the indicated target gene. Forty-eight hours 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 ($0.15\% \pm 0.04\%$) 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

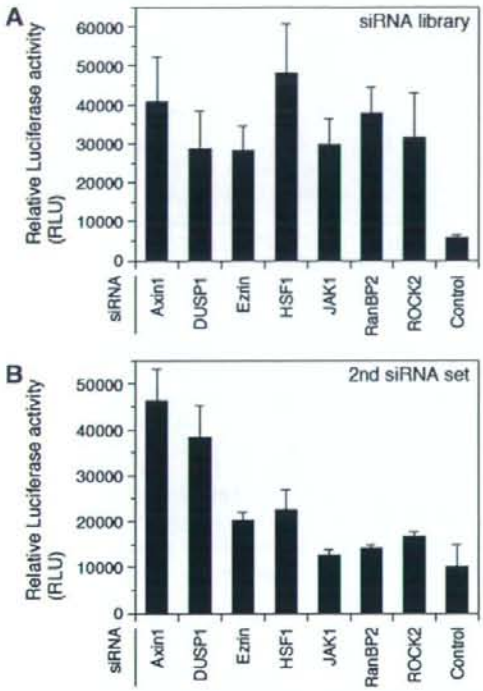

Fig. 2. siRNAs against Axin1 and DUSP1 reproducibly enhanced the HIV-1 gene expression. J111 cells were transfected with the siRNAs included in siRNA mini-library (A) or the second set of siRNAs (B) directed against the indicated target gene. Forty-eight hours after siRNA-transfection, the cells were 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.

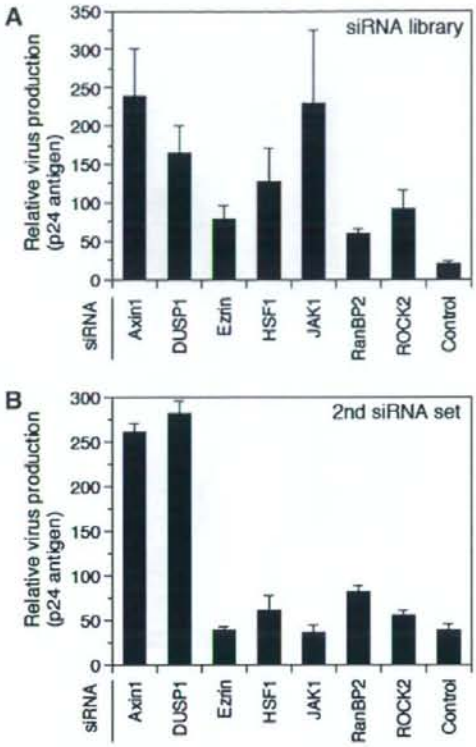

Fig. 3. siRNAs against Axin1 and DUSP1 reproducibly enhanced the HIV-1 replication at the late phase(s) of viral life cycle. J111 cells were transfected with siRNA, and then transfected with pNL-Luc-envCT and pRL-TK, as described in the legend of Fig. 2. Forty-eight hours after the transfection of HIV-1 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

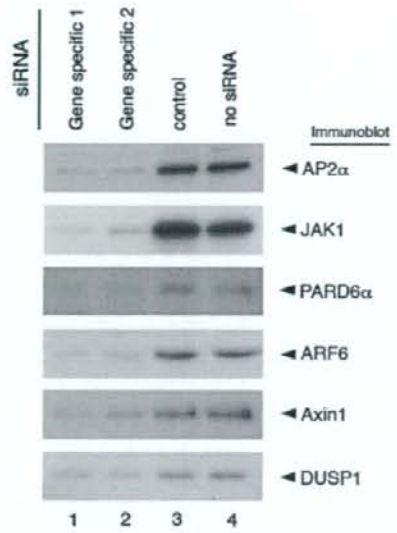

Fig. 4. Two sets of siRNAs against AP2α, ARF6, PARD6α, JAK1, Axin1, and DUSP1 suppressed the expression levels of target gene products. J111 cells were transfected with two sets of siRNA against indicated target gene (lanes 1 and 2), control siRNA (lane 3) or MOCK-transfected (lane 4). Then, the cells 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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