Dickinson) was employed as secondary reagents. FITC- (PharMingen, San Diego, CA), PE- (Dako Glostrup, Denmark), Per CP- (Becton Dickinson), APC- (Becton Dickinson) conjugated species- and isotype-matched, mAbs were used to determine the level of background staining.

Elispot Assay

We performed enzyme-linked immunospot (Elispot) assay to know the efficiency of HIV-1 specific T-cell induction by DCs infected with AdV or SeV vector. First, we developed mDCs as described above from PBMCs of two HIV-1-infected patients. On day 7, we infected those mDCs with AdV vector at an MOI of 1,000 or SeV vector at an MOI of 2 for 1 hr, or just added overlapping peptides (*gag*, *env*). We used the overlapping peptides derived from consensus B sequence since both patients were infected with subtype B HIV-1. We did not check the AdV sero-status of these two patients. Both patients were on HAART and have undetectable viral load (<50 copies/ml). CD4 counts of patients 1 and 2 are 408/μl and 336/μl, respectively. We used those mDCs as stimulators in Elispot assay. PBMCs from each patient were used as effectors cells. The protocol of Elispot assay was described previously [Furutsuki et al., 2004].

RESULTS

Sendai Viral Vectors Transduce DCs at Lower MOIs Than Adenoviral Vectors

We infected imDCs with SeVGFP, dF-SeVGFP or AdVGFP at different MOIs (Fig. 1) in order to know which MOI is the best for these three vectors. We stained these cells with PI to evaluate the expression of GFP in viable cells. In SeVGFP, the expression of GFP reached the maximum (32.5%) at an MOI of 2 and the mean fluorescent intensity (MFI) of GFP showed around 1,400 at all MOIs. However, as shown by the fraction of PI-positive cells, SeVGFP killed around 30% of DCs even at an MOI of 0.5. The staining pattern of DCs infected with dF-SeVGFP was similar to that of SeVGFP. In AdVGFP, both the percentage of GFP-positive cells and the MFI of

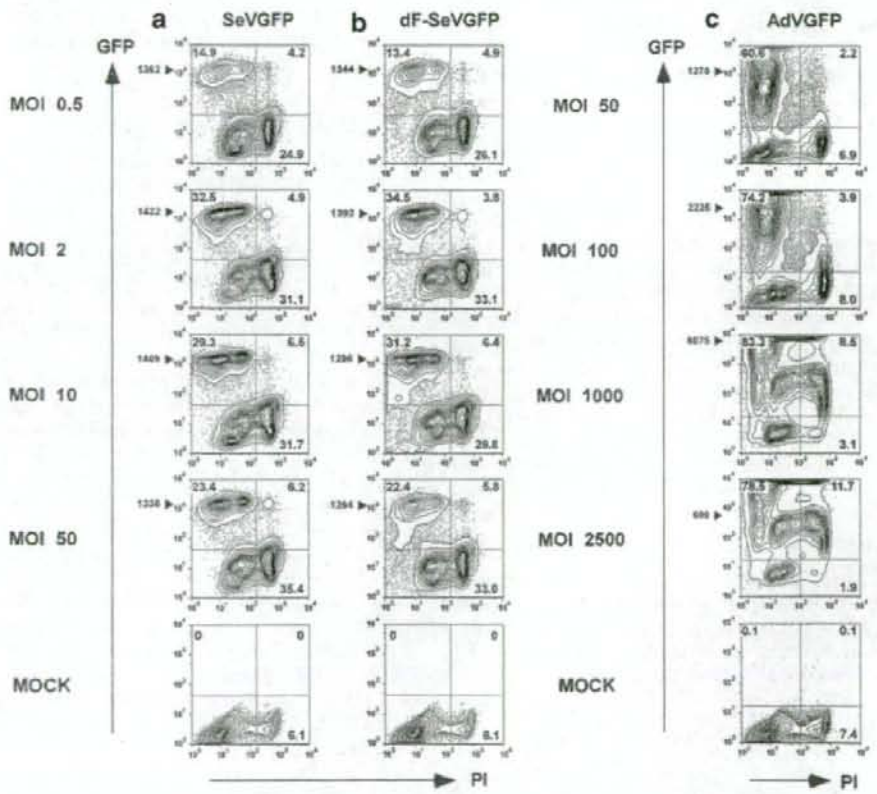

Fig. 1. Comparison of different MOIs for the maximum gene expression by SeV (**a**), dF-SeV (**b**), and AdV (**c**) vectors 48 hr after infection. Cell viability was determined by staining with PI. GFP expression and PI staining were analyzed by flow cytometry. The percentages of GFP- and PI-positive cells are shown on each corner. Arrowheads indicate MFI of GFP-positive cells within PI-negative fraction. The numbers in each panel represent the mean value of three independent experiments.

GFP increased up to an MOI of 1,000. Although PI-positive cells in AdV increased according to MOIs, the percentage of PI-positive cells was less than 14% even at the highest MOI: 2,500. From these results, SeV vector is likely to transduce DCs at much lower MOIs than AdV vector, but kill more DCs than AdV. We chose an MOI of 2 for SeVGFP and dF-SeVGFP, and an MOI of 1,000 for AdVGFP in the subsequent experiments.

Sendai Viral Vectors Showed Maximum Transduction Level Earlier Than Adenoviral Vectors

We next examined the time course of GFP expression (Fig. 2). We detected GFP-positive cells as early as 8 hr after infection in all three vectors. The proportion of GFP-positive cells reached the maximum level (around

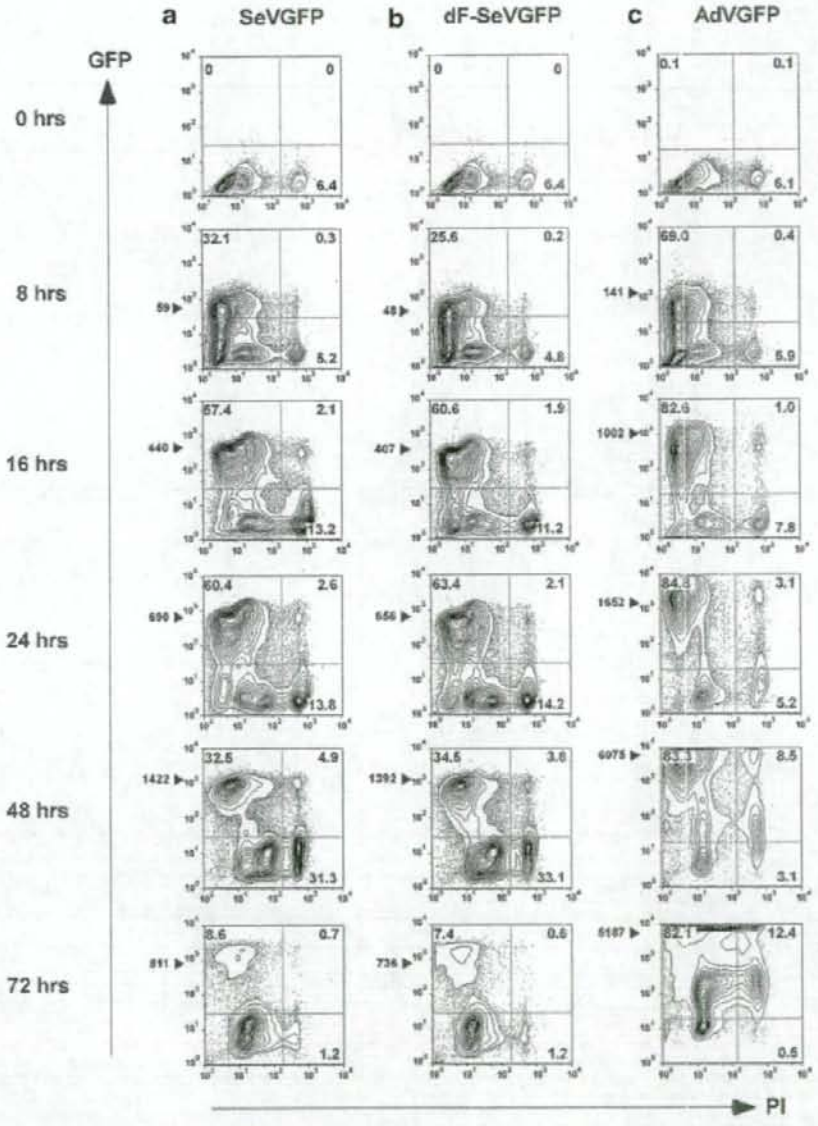

Fig. 2. The time course of the maximum gene expression by SeV (**a**), dF-SeV (**b**), and AdV (**c**) vectors. DCs were infected with SeV and dF-SeV at an MOI of 2 and were infected with AdV at MOI of 1,000 and then cultured for 8–72 hr. Cell viability was determined by staining with PI. GFP expression and PI staining were analyzed by flow cytometry. The percentages of GFP- and PI-positive cells are shown on each corner. Arrowheads indicate MFI of GFP-positive cells within PI-negative fraction. The numbers in each panel represent the mean value of three independent experiments.

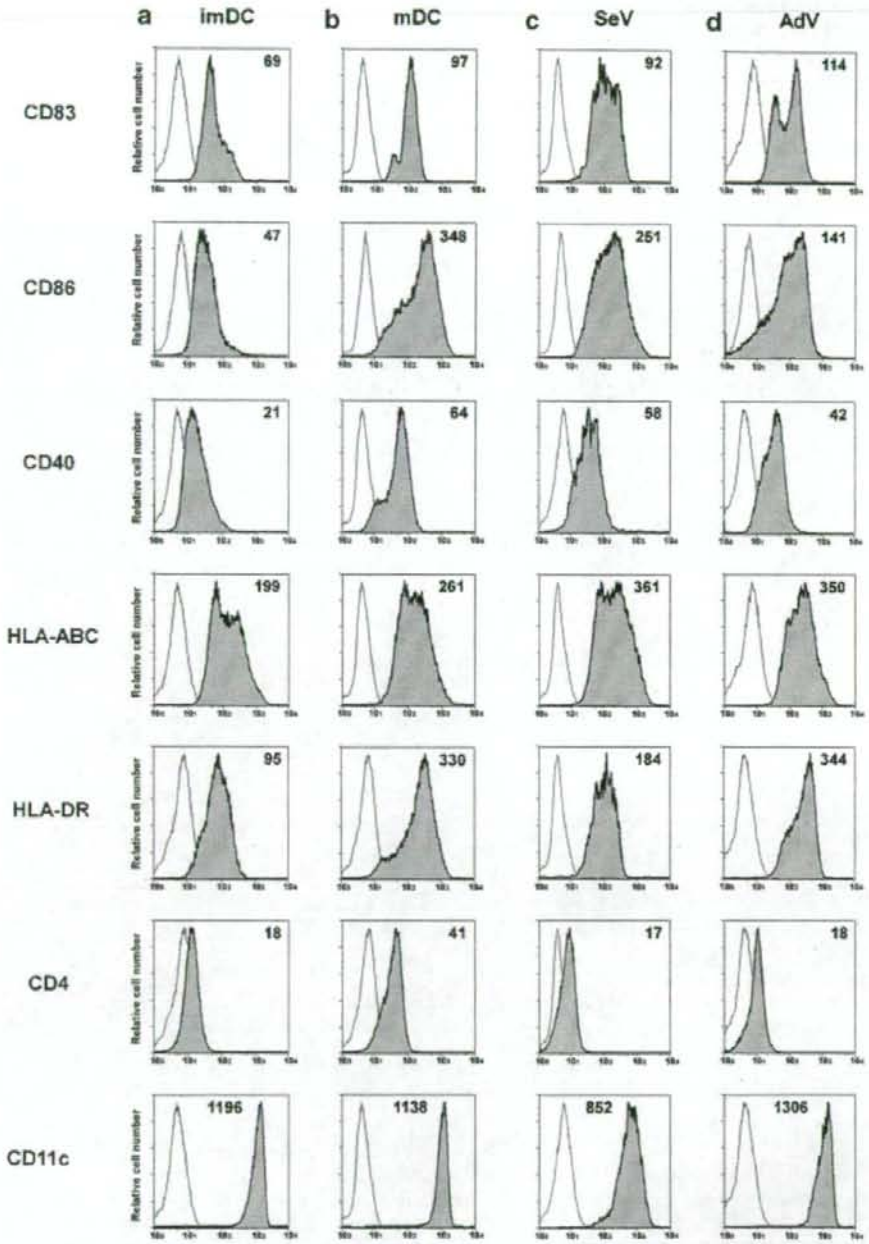

Fig. 3. Infection of DCs with SeV and AdV vectors modified the expression of cell surface markers. DCs infected with SeV vector containing no inserts at an MOI of 2 (**c**) and AdV vector containing no inserts at an MOI of 1,000 (**d**) were maintained in the DC medium for 48 hr (AdV vector) and 24 hr (SeV vector). DCs were incubated with (**b**) or without (**a**) TNF-α for 48 hr, respectively. These DCs were analyzed by flow cytometry with FITC, PE, PerCP, APC-conjugated antibodies for expression of CD86, CD83, HLA-ABC, HLA-DR, CD4, CD40, CD11c. The open profiles represent isotype-matched mAb controls. MFIs are indicated on the right corner in each panel. The background values of all experiments were less than 15. The numbers in each panel represent the mean value of three independent experiments.

60%) 24 hr after infection with SeVGFP or dF-SeVGFP. The proportion of GFP-positive cells decreased to around 30% at 48 hr, although the MFI of GFP showed the maximum at 48 hr. AdVGFP, on the other hand, showed the maximum level of both GFP-positive cells and MFI of GFP 48 hr after infection.

Sendai and Adenoviral Vectors Changed Phenotype of imDCs Following Viral Transduction

In order to determine the effect of transduction on imDCs with these vectors, we examined the surface markers of cells after transduction. The phenotype of imDCs and mDCs are shown in Figure 3a,b, respectively. We infected imDCs with SeV or AdV vectors and cultured them for 24 or 48 hr, respectively (Fig. 3c,d). As compared with the phenotype of uninfected imDCs, DCs infected by SeV and AdV vectors showed up-regulation of a maturation marker CD83, the major histocompatibility complex (MHC) classes I and II molecules (HLA-ABC and HLA-DR), and costimulatory molecules CD40 and CD86. Incubation of DCs in medium and buffers used to prepare vectors did not affect the phenotype of the cells (data not shown). These results indicate that SeV as well as AdV vector infection induced DC maturation in terms of cell surface phenotype.

Both SeV and AdV Vectors Elicited HIV-1 Specific T-Cell Responses

To evaluate protein expressions, we developed five viral vectors carrying HIV-1 structural proteins (Fig. 4a). We infected DCs with these vectors under the optimal conditions we concluded from the results shown above. Gp120 expression by SeV vector was 3.8 times higher than that by AdV vector (Fig. 4b, compare lanes 3–7). Since 3.2 and 5.0 kb are the maximum gene sizes for SeV and AdV vector, respectively [Sakai et al., 1999; the manufacturer's protocol of AdV Expression Kit], we inserted HIV-1 *gag* gene (about 1.5 kb) in SeV and *gag-pol* gene (about 4.9 kb) in AdV vector. Both *cis*-acting RRE sequence and *trans*-acting Rev protein were necessary for Gag protein expression by AdV vector (Fig. 4b, compare lanes 1–2). Rev expression is not required for SeV-mediated Gag or Env expression because SeV replicates in the cytoplasm. In the presence of Rev protein, AdV vector expressed similar levels of Gag protein to SeV vector (Fig. 4b, compare lanes 1–6). Although SeV Gag did not have HIV-1 protease sequence, a band was detected near the size of p24. It was not a nonspecific band derived from SeV because we could not detect the band with other SeV constructs, such as SeV Env (data not shown). Gag might be processed by some proteins of SeV.

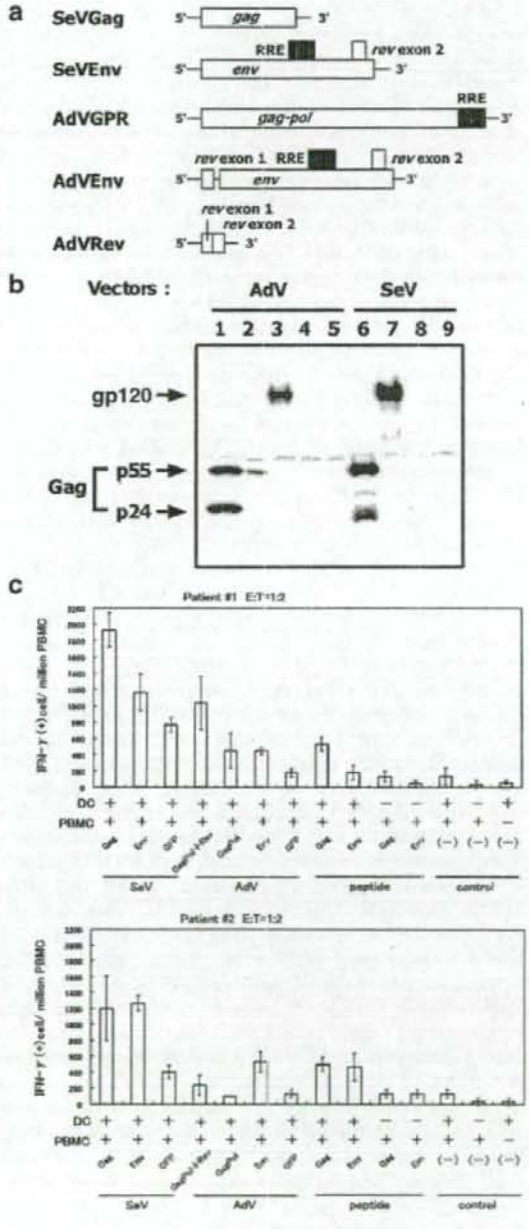

Fig. 4. **a**: Schematic structures of five viral vectors. *gag*, HIV-1 gag gene; *env*, HIV-1 env gene; *gag-pol*, HIV-1 gag and pol genes; *rev*, HIV-1 rev gene; RRE, HIV-1 RRE; GFP, green fluorescent protein. **b**: The expression of HIV-1 structural proteins by AdV and SeV vectors. DCs infected with AdV vector at an MOI of 1,000 and SeV vector at an MOI of 2 were harvested for 48 or 24 hr after infection, respectively. Thirty microgram of lysate was subjected to immunoblot analysis using anti-Gag p24 or anti-Env gp120 mAbs. The other two independent experiments showed similar results. Lane 1, coinfection with AdVGPR and AdVRev; Lane 2, AdVGPR without AdVRev infection; Lane 3, AdVEnv; Lane 4, AdV without inserts; Lane 5, mock; Lane 6, SeVGag; Lane 7, SeVEnv; Lane 8, SeV without inserts; Lane 9, mock. Arrows on the left indicate positions of gp120, Gag p55, and p24. **c**: The results of IFN-γ Elispot assays in two HIV-1 infected patients. Autologous DCs infected with SeV vector or AdV vector, or just added overlapping peptides (*gag*, *env*) were used as stimulators. PBMCs from the same patients were used as effectors cells. Results are shown as mean ± SEM of three independent assays.

After developing mDCs from frozen PBMCs of two HIV-1 infected patients, we infected these mDCs with SeV or AdV vector and used them as stimulators for interferon γ (IFN-γ) Elispot. Both SeV and AdV vectors elicited HIV-1 specific T-cell responses, although some nonspecific responses were also detected (Fig. 4c).

DISCUSSION

DCs are efficient antigen presenting cells that are critical for induction of primary T-cell responses. At present the most useful method for genetic manipulation of DCs is to use viral vectors. As reported previously, AdV vector is efficient at the transduction of DCs [Tan et al., 2005]. SeV is also one of the reliable vectors for immunotherapy and has several unique features, such as cytoplasmic localized replication cycle and brief contact time for cellular uptake. In this study, we analyzed the capacity of SeV as a vector in terms of transducing GFP and HIV-1 genes into human DCs. We showed that SeV vector transduced GFP genes efficiently into monocyte-derived imDCs. DCs infected with SeV and dF-SeV vectors expressed high amount of GFP gene 24 hr after infection at an MOI of 2 (Fig. 2a,b). The expression level of HIV-1 structural gene, *env*, by SeV vector was higher than that by AdV. These results proved the high ability of gene expression by SeV. However, the proportion of GFP positive cells did not increase according to MOI. About 30% of cells were still GFP-negative 48 hr after infection even at an MOI of 50 (Fig. 1a,b). This could be caused by the disruption of sialic acid which is the receptor for SeV.

Both SeV and dF-SeV vectors killed nearly 30% of target DCs at the lowest MOI: 0.5. One of the reasons for this phenomenon is likely to be apoptosis. Several studies reported that SeV is able to induce apoptosis in viral host cells [Tropea et al., 1995; Bitzer et al., 1999]. This cytopathic effect might enhance specific T-cell responses by cross-presentation of DCs. Presentation by DCs derived from virus-infected apoptotic and necrotic cells could activate T-cells efficiently [Arrode et al., 2000; Herr et al., 2000; Larsson et al., 2001; Tabi et al., 2001]. In order to apply SeV in a clinical setting, further studies about cytopathic effect by SeV vector will be required.

AdV vector is known to require high MOI to achieve high transduction rates [Diao et al., 1999]. Our study also demonstrated that much higher MOI was needed in AdV than SeV to transduce DCs. One of the reasons for this phenomenon could be insufficient expression of coxsackievirus and AdV receptor (CAR) [Stockwin et al., 2002] on DCs. CAR is the primary receptor for AdV type 5, and the AdV used in this study was derived from AdV type 5. However, MFI of GFP in AdV vector increased according to MOI. AdV might be able to use other receptors to infect DCs. Several studies have shown that AdV can infect cells through integrins or MHC molecules [Huang et al., 1996; Hong et al., 1997]. Recently, AdV vector containing Ad5/35 chimeric fiber protein was reported as a useful vector for the cells lacking in sufficient CAR expression [Mizuguchi and Hayakawa, 2002]. This chimeric vector would be useful for DCs because the receptor of Ad5/35 vector is CD46, which is expressed on DCs.

When imDCs capture antigens, they maturate while migrating to T-cell areas in the lymph nodes [Banchereau and Steinman, 1998]. DC maturation is critical for strong T-cell binding and stimulation [Lipscomb and Masten, 2002]. Our results showed that SeV vector infection induced DC maturation of human monocyte-derived DCs as well as AdV vector infection. However, the expression levels of CD86 and CD40 were lower as compared to those of mDCs. CD40 expression leads to increased DC survival and stimulates cytokine production [Caux et al., 1994; Wong et al., 1997]. CD86, a ligand for CD28 and cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), stimulates T-cell proliferation and generation of CTL [Lanier et al., 1995]. In order to achieve further up-regulation of those molecules, the addition of a maturation factor, such as TNF-α, should be considered.

DCs infected with SeV as well as AdV elicited HIV-1 specific T-cell responses detected by IFN-γ Elispot (Fig. 4c). Elispot by SeV GFP showed about 800 SFC/million PBMC which was obtained from patient #1. One possibility of this nonspecific response is antigenic cross-reactivity. SeV belongs to the genus *Respirovirus* of the *Paramyxoviridae* family. *Respirovirus* includes human parainfluenza virus type 1 (hPIV-1) and 3 (hPIV-3). hPIV-1 is the most common cause of pediatric laryngotracheobronchitis (croup), which means many people are infected by hPIV-1 in early life. Previous studies showed SeV and hPIV-1 shared sequence homology and antigenic cross-reactivity [Gorman et al., 1990; Lyn et al., 1991; Smith et al., 1994]. The high nonspecific response by SeV GFP could be caused by cross-reactive immunity induced by previous exposure to human hPIV-1.

In conclusion, our results showed that SeV vector had high ability of gene transduction. SeV vector induced the maturation of DCs in terms of their phenotype and stimulated HIV-1 specific T-cell responses, which is beneficial in vaccination. Though further studies will be required to improve vector design, SeV vector has a potential to be used for immuno-genetherapy.

ACKNOWLEDGMENTS

We thank Dr. Izumu Saito and Dr. Yumi Kanegae (University of Tokyo, Japan) for providing AdVGFP and Ax1w1. This work was partly supported by grants for AIDS Research from the Ministry of Health, Labor and Welfare of Japan, The Special Coordination Fund for Promoting Science and Technology of MEXT: Strategic cooperation of control emerging and reemerging infections. This work was supported in part by the Program of Founding Research Centers for Emerging and Reemerging Infectious Diseases of the Ministry of Education, Culture, Sports, Science and Technology (MEXT); Strategic cooperation to control emerging and reemerging infections funded by the Special Coordination Funds for Promoting Science and Technology of MEXT;

(Grants for Research on HIV/AIDS and Research on Publicly Essential Drugs and Medical Devises from the Ministry of Health, Labor, and Welfare of Japan; Grant-in-Aid for Scientific Research (B) from Japan Society for the Promotion of Science (JSPS).

REFERENCES

Agungpriyono DR, Yamaguchi R, Uchida K, Tohya Y, Kato A, Nagai Y, Asakawa M, Tateyama S. 2000. Green fluorescent protein gene insertion of Sendai Virus infection in nude mice: Possibility as an infection tracer. J Vet Med Sci 62:223–228.

Arrode G, Boccaccio C, Lule J, Allart S, Moinard N, Abastado JP, Alam A, Davrinche C. 2000. Incoming human cytomegalovirus pp65 (UL83) contained in apoptotic infected fibroblasts is cross-presented to CD8(+) T cells by dendritic cells. J Virol 74:10018–10024.

Banchereau J, Steinman RM. 1998. Dendritic cells and the control of immunity. Nature 392:245–252.

Bitzer M, Prinz F, Bauer M, Spiegel M, Neubert WJ, Gregor M, Schulze-Osthoff K, Lauer U. 1999. Sendai virus infection induces apoptosis through activation of caspase-8 (FLICE) and caspase-3 (CPP32). J Virol 73:702–708.

Bonini C, Lee SP, Riddell SR, Greenberg PD. 2001. Targeting antigen in mature dendritic cells for simultaneous stimulation of CD4+ and CD8+ T cells. J Immunol 166:5250–5257.

Brander C, Walker BD. 1999. T lymphocyte responses in HIV-1 infection: Implications for vaccine development. Curr Opin Immunol 11:451–459.

Caux C, Massacrier C, Vanbervliet B, Dubois B, Van Kooten C, Durand I, Banchereau J. 1994. Activation of human dendritic cells through CD40 cross-linking. J Exp Med 180:1263–1272.

Diao J, Smythe JA, Smyth C, Rowe PB, Alexander IE. 1999. Human PBMC-derived dendritic cells transduced with an adenovirus vectorinduce cytotoxic T-lym-phocyte responses against a vector-encoded antigen in vitro. Gene Ther 6:845–853.

Engelmayer J, Larsson M, Subklewe M, Chahroudi A, Cox WI, Steinman RM, Bhardwaj N. 1999. Vaccinia virus inhibits the maturation of human dendritic cells: A novel mechanism of immune evasion. J Immunol 163:6762–6768.

Engelmayer J, Larsson M, Lee A, Lee M, Cox WI, Steinman RM, Bhardwaj N. 2001. Mature dendritic cells infected with canarypox virus elicit strong anti-human immunodeficiency virus CD8+ and CD4+ T-cell responses from chronically infected individuals. J Virol 75:2142–2153.

Furutsuki T, Hosoya N, Kawana-Tachikawa A, Tomizawa M, Odawara T, Goto M, Kitamura Y, Nakamura T, Kelleher AD, Cooper DA, Iwamoto A. 2004. Frequent transmission of cytotoxic-T-lymphocyte escape mutants of human immunodeficiency virus type 1 in the highly HLA-A24-positive Japanese population. J Virol 78:8437–8445.

Gorman WL, Gill DS, Scroggs RA, Portner A. 1990. The hemagglutinin-neuraminidase glycoproteins of human parainfluenza virus type 1 and Sendai virus have high structure-function similarity with limited antigenic cross-reactivity. Virology 175:211–221.

Graham FL, Smiley J, Russell WC, Nairn R. 1977. Characteristics of a human cell line transformed by DNA from human adenovirus type 5. J Gen Virol 36:59–74.

Herr W, Ranieri E, Olson W, Zarour H, Gesualdo L, Storkus WJ. 2000. Mature dendritic cells pulsed with freeze-thaw cell lysates define an effective in vitro vaccine designed to elicit EBV-specific CD4(+) and CD8(+) T lymphocyte responses. Blood 96:1857–1864.

Hong SS, Karayan L, Tournier J, Curiel DT, Boulanger PA. 1997. Adenovirus type 5 fiber knob binds to MHC class I alpha2 domain at the surface of human epithelial and B lymphoblastoid cells. EMBO J 16:2294–2306.

Huang S, Kamata T, Takada Y, Ruggeri ZM, Nemerow GR. 1996. Adenovirus interaction with distinct integrins mediates separate events in cell entry and gene delivery to hematopoietic cells. J Virol 70:4502–4508.

Ishii-Watabe A, Uchida E, Iwata A, Nagata R, Satoh K, Fan K, Murata M, Mizuguchi H, Kawasaki N, Kawanishi T, Yamaguchi T, Hayakawa T. 2003. Detection of replication-competent adenoviruses spiked into recombinant adenovirus vector products by infectivity PCR. Mol Ther 8:1009–1016.

Jin X, Bauer DE, Tuttleton SE, Lewin S, Gettie A, Blanchard J, Irwin CE, Safrit JT, Mittler J, Weinberger L, Kostrikis LG, Zhang L, Perelson AS, Ho DD. 1999. Dramatic rise in plasma viremia after CD8(+) T cell depletion in simian immunodeficiency virus-infected macaques. J Exp Med 189:991–998.

Kanegae Y, Makimura M, Saito I. 1994. A simple and efficient method for purification of infectious recombinant adenovirus. Jpn J Med Sci Biol 47:157–166.

Kano M, Matano T, Kato A, Nakamura H, Takeda A, Suzaki Y, Ami Y, Terao K, Nagai Y. 2002. Primary replication of a recombinant Sendai virus vector in macaques. J Gen Virol 83:1377–1386.

Kato A, Sakai Y, Shioda T, Kondo T, Nakanishi M, Nagai Y. 1996. Initiation of Sendai virus multiplication from transfected cDNA or RNA with negative or positive sense. Genes Cells 1:569–579.

Kato M, Igarashi H, Takeda A, Sasaki Y, Nakamura H, Kano M, Sata T, Iida A, Hasegawa M, Horie S, Higashihara E, Nagai Y, Matano T. 2005. Induction of Gag-specific T-cell responses by therapeutic immunization with a Gag-expre-ssing Sendai virus vector in macaques chronically infected with simian-human immunodeficiency virus. Vaccine 23:3166–3173.

Kawana-Tachikawa A, Tomizawa M, Nunoya J, Shioda T, Kato A, Nakayama EE, Nakamura T, Nagai Y, Iwamoto A. 2002. An efficient and versatile mammalian viral vector system for major histocompatibility complex class I/peptide complexes. J Virol 76: 11982–11988.

Lanier LL, O'Fallon S, Somoza C, Phillips JH, Linsley PS, Okumura K, Ito D, Azuma M. 1995. CD80 (B7) and CD86 (B70) provide similar costimulatory signals for T cell proliferation, cytokine production, and generation of CTL. J Immunol 154:97–105.

Larsson M, Fonteneau JF, Somersan S, Sanders C, Bickham K, Thomas EK, Mahnke K, Bhardwaj N. 2001. Efficiency of cross presentation of vaccinia virus-derived antigens by human dendritic cells. Eur J Immunol 31:3432–3442.

Levy JA, Cheng-Mayer C, Dina D, Luciw PA. 1986. AIDS retrovirus (ARV-2) clone replicates in transfected human and animal fibroblasts. Science 232:998–1001.

Li HO, Zhu YF, Asakawa M, Kuma H, Hirata T, Ueda Y, Lee YS, Fukumura M, Iida A, Kato A, Nagai Y, Hasegawa M. 2000. A cytoplasmic RNA vector derived from nontransmissible Sendai virus with efficient gene transfer and expression. J Virol 74:6564–6569.

Lipscomb MF, Masten BJ. 2002. Dendritic cells: Immune regulators in health and disease. Physiol Rev 82:97–130.

Lyn D, Gill DS, Scroggs RA, Portner A. 1991. The nucleoproteins of human parainfluenza virus type 1 and Sendai virus share amino acid sequences and antigenic and structural determinants. J Gen Virol 72:983–987.

Matano T, Shibata R, Siemon C, Connors M, Lane HC, Martin MA. 1998. Administration of an anti-CD8 monoclonal antibody interferes with the clearance of chimeric simian/human immunodeficiency virus during primary infections of rhesus macaques. J Virol 72:164–169.

McMichael AJ, Rowland-Jones SL. 2001. Cellular immune responses to HIV. Nature 410:980–987.

Miyake S, Makimura M, Kanegae Y, Harada S, Sato Y, Takamori K, Tokuda C, Saito I. 1996. Efficient generation of recombinant adenoviruses using adenovirus DNA-terminal protein complex and a cosmid bearing the full-length virus genome. Proc Natl Acad Sci USA 93:1320–1324.

Mizuguchi H, Hayakawa T. 2002. Adenovirus vectors containing chimeric type 5 and type 35 fiber proteins exhibit altered and expanded tropism and increase the size limit of foreign genes. Gene 285:69–77.

Mwau M, Cebere I, Sutton J, Chikoti P, Winstone N, Wee EG, Beattie T, Chen YH, Dorrell L, McShane H, Schmidt C, Brooks M, Patel S, Roberts J, Conlon C, Rowland-Jones SL, Bwayo JJ, McMichael AJ, Hanke T. 2004. A human immunodeficiency virus 1 (HIV-1) clade A vaccine in clinical trials: Stimulation of HIV-specific T-cell responses by DNA and recombinant modified vaccinia virus Ankara (MVA) vaccines in humans. J Gen Virol 85: 911–919.

Nagayama H, Sato K, Morishita M, Uchimaru K, Oyaizu N, Inazawa T, Yamasaki T, Enomoto M, Nakaoka T, Nakamura T, Maekawa T, Yamamoto A, Shimada S, Saida T, Kawakami Y, Asano S, Tani K, Takahashi TA, Yamashita N. 2003. Results of a phase I clinical study using autologous tumour lysate-pulsed monocyte-derived mature dendritic cell vaccinations for stage IV malignant mela-

noma patients combined with low dose interleukin-2. Melanoma Res 13:521–530.

Rea D, Schagen FH, Hoeben RC, Mehtali M, Havenga MJ, Toes RE, Melief CJ, Offringa R. 1999. Adenoviruses activate human dendritic cells without polarization toward a T-helper type 1-inducing subset. J Virol 73:10245–10253.

Rouas R, Uch R, Cleuter Y, Jordier F, Bagnis C, Mannoni P, Lewalle P, Martiat P, Van den Broeke A. 2002. Lentiviral-mediated gene delivery in human monocyte-derived dendritic cells: Optimized design and procedures for highly efficient transduction compatible with clinical constraints. Cancer Gene Ther 9:715–724.

Sakai Y, Kiyotani K, Fukumura M, Asakawa M, Kato A, Shioda T, Yoshida T, Tanaka A, Hasegawa M, Nagai Y. 1999. Accommodation of foreign genes into the Sendai virus genome: Sizes of inserted genes and viral replication. FEBS Lett 456:221–226.

Smith FS, Portner A, Leggiadro RJ, Turner EV, Hurwitz JL. 1994. Age-related development of human memory T-helper and B-cell responses toward parainfluenza virus type-1. Virology 205:453–461.

Stockwin LH, Matzow T, Georgopoulos NT, Stanbridge LJ, Jones SV, Martin IG, Blair-Zajdel ME, Blair GE. 2002. Engineered expression of the Coxsackie B and adenovirus receptor (CAR) in human dendritic cells enhances recombinant adenovirus-mediated gene transfer. J Immunol Methods 259:205–215.

Stubbs AC, Martin KS, Coeshott C, Skaates SV, Kuritzkes DR, Bellgrau D, Franzusoff A, Duke RC, Wilson CC. 2001. Whole recombinant yeast vaccine activates dendritic cells and elicits protective cell-mediated immunity. Nat Med 7:625–629.

Tabi Z, Moutaftsi M, Borysiewicz LK. 2001. Human cytomegalovirus pp65- and immediate early 1 antigen-specific HLA class I-restricted cytotoxic T cell responses induced by cross-presentation of viral antigens. J Immunol 166:5695–5703.

Takeda A, Igarashi H, Nakamura H, Kano M, Iida A, Hirata T, Hasegawa M, Nagai Y, Matano T. 2003. Protective efficacy of an AIDS vaccine, a single DNA priming followed by a single booster with a recombinant replication-defective Sendai virus vector, in a macaque AIDS model. J Virol 77:9710–9715.

Tan PH, Beutelspacher SC, Xue SA, Wang YH, Mitchell P, McAlister JC, Larkin DF, McClure MO, Stauss HJ, Ritter MA, Lombardi G, George AJ. 2005. Modulation of human dendritic-cell function following transduction with viral vectors: Implications for gene therapy. Blood 105:3824–3832.

Tropea F, Troiano L, Monti D, Lovato E, Malorni W, Rainaldi G, Mattana P, Viscomi G, Ingletti MC, Portolani M, et al. 1995. Sendai virus and herpes virus type 1 induce apoptosis in human peripheral blood mononuclear cells. Exp Cell Res 218:63–70.

Tsunetsugu-Yokota Y, Morikawa Y, Isogai M, Kawana-Tachikawa A, Odawara T, Nakamura T, Grassi F, Autran B, Iwamoto A. 2003. Yeast-derived human immunodeficiency virus type 1 p55(gag) virus-like particles activate dendritic cells (DCs) and induce perforin expression in Gag-specific CD8(+) T cells by cross-presentation of DCs. J Virol 77:10250–10259.

Wong BR, Josien R, Lee SY, Sauter B, Li HL, Steinman RM, Choi Y. 1997. TRANCE (tumor necrosis factor [TNF]-related activation-induced cytokine), a new TNF family member predominantly expressed in T cells, is a dendritic cell-specific survival factor. J Exp Med 186:2075–2080.

Yang OO, Kalams SA, Trocha A, Cao H, Luster A, Johnson RP, Walker BD. 1997. Suppression of human immunodeficiency virus type 1 replication by CD8+ cells: Evidence for HLA class I-restricted triggering of cytolytic and noncytolytic mechanisms. J Virol 71: 3120–3128.

Yu D, Shioda T, Kato A, Hasan MK, Sakai Y, Nagai Y. 1997. Sendai virus-based expression of HIV-1 gp120: Reinforcement by the V(−) version. Genes Cells 2:457–466.

Effects of CCR2 and CCR5 Polymorphisms on HIV-1 Infection in Thai Females

Nuanjun Wichukchinda, PhD, Emi E. Nakayama, MD, PhD,† Archawin Rojanawiwat, MD,* Panita Pathipvanich, MD,‡ Wattana Auwanit, PhD,* Suthon Vongsheree, MSc,* Koya Ariyoshi, MD, PhD,§ Pathom Sawanpanyalert, MD, PhD,* and Tatsuo Shioda, PhD†*

Summary: Polymorphisms in *CCR2* and *CCR5* genes reportedly affect HIV-1 transmission and disease progression in HIV-1–infected individuals. In the study presented here, we examined the effects of *CCR2* and *CCR5* polymorphisms on HIV-1 transmission in 74 Thai females who were exposed to HIV but seronegative (ESN) and in 347 HIV-seropositive females. We found that the combination of 2 non-synonymous substitutions, *CCR2 V64I* and *CCR5 G316A*, tended to occur more frequently in ESN females (2 of 74) than in HIV-1–infected females (1 of 347) ($P = 0.08$). This suggested that non-synonymous substitution in the *CCR5* gene also affects HIV-1 transmission in an Asian population in which the *CCR5-Δ32* is very rare.

Key Words: CCR2, CCR5, SNPs, HIV-1 infection, Thai

(*J Acquir Immune Defic Syndr* 2008;47:293–297)

A considerable number of individuals, such as HIV-seronegative commercial sex workers[1] and HIV-seronegative spouses of HIV-seropositive individuals,[2] have been repeatedly sexually exposed to HIV yet remain negative for anti-HIV antibody. Such individuals have been classified as HIV-exposed but seronegative (ESN). Several studies in Thailand demonstrated that some individuals with HIV-seropositive spouses can remain HIV-seronegative; a recent study of ours (not yet published) showed that the majority of these cases could not be explained by their sexual behavior, such as frequency of unprotected sexual contacts, or by the viral load of their infected spouses.

The association of a number of polymorphisms in host genes that are involved in HIV replication and/or immune regulation with HIV infection and disease progression has been documented.[3] We previously reported that a single nucleotide polymorphism (SNP) in the *DC-SIGNR* gene was more prevalent in Thai female ESNs than in HIV-1–infected individuals.[4] In white individuals, the association of a 32-bp deletion of the C-C chemokine receptor 5 (*CCR5-Δ32)* with HIV infection has been demonstrated.[5,6] *CCR5-Δ32* homozygotes are highly resistant to HIV-1 infection, whereas *CCR5-Δ32* heterozygotes delay progression to AIDS by 2 to 3 years.[7,8] However, the *CCR5Δ32* allele is rarely observed in Asians,[5,8–10] including Thais.[11] Moreover, an additional variant in the upstream regulatory region of *CCR5* (*CCR5 P1*) is associated with more rapid progression to AIDS,[12] and a valine-to-isoleucine substitution in the coding region of another C-C chemokine receptor, CCR2 (*CCR2 V64I*), delays HIV disease progression.[13–15]

In the study presented here, we examined polymorphisms in the *CCR2* and *CCR5* genes of Thai ESNs and HIV-1–infected females and found that a combination of *CCR2 V64I* and one of the *CCR5* coding region polymorphisms tended to be more prevalent in Thai female ESNs than in HIV-1–infected individuals.

METHODS

Clinical Specimens

We used samples obtained from previously described 74 ESNs[4] and 246 HIV-1–infected Thai females[16] who visited the HIV clinic in the Day Care Center of Lampang Hospital in north Thailand between July 6, 2000, and July 12, 2001. We also used samples from an additional 101 HIV-1–infected Thai females who visited the clinic after July 12, 2001. Among 74 HIV-seronegative female spouses, the median (interquartile range [IQR]) duration of marriage before they became aware of the HIV status of their husbands was estimated to be 5 (3, 8) years. Median (IQR) frequency of sexual contact before disclosure of their husbands' HIV status was 5 (3, 8) times per month. Sixty-four females (87.7%) reported that they had never used a condom during those sexual contacts.[4] In October 2003, we examined blood samples of the 74 ESNs again to determine whether they were still HIV seronegative. This study was approved by the Ethical Review Committee for Research in Human Subjects, Ministry of Public Health, Thailand. After their written informed consent had been

Received for publication June 12, 2007; accepted November 20, 2007.

From the *National Institute of Health, Department of Medical Sciences, Ministry of Public Health, Nonthaburi, Thailand; †Research Institute for Microbial Diseases, Osaka University, Osaka, Japan; ‡Day Care Center, Lampang Hospital, Lampang, Thailand; and the §Institute of Tropical Medicine, Nagasaki University, Nagasaki, Japan.

Supported by Department of Medical Sciences, Ministry of Public Health, Thailand; the Japan International Cooperation Agency (JICA); the Japanese Foundation for AIDS Prevention (JFAP); the Ministry of Health, Labour and Welfare, Japan; and the Ministry of Education, Culture, Sports, Science, and Technology, Japan.

Correspondence to: Tatsuo Shioda, PhD, Department of Viral Infections, Research Institute for Microbial Diseases, Osaka University, 3-1 Yamadaoka, Suita, Osaka 565-0871 Japan (e-mail: shioda@biken.osaka-u.ac.jp).

obtained, all participants were interviewed by trained study coordinators and clinically examined by 2 designated doctors.

Genotyping

Genomic DNA was extracted and purified from the buffy coat of the samples with the QIAamp mini blood kit (QIAGEN, Hilden, Germany). Each specimen was then analyzed for *CCR2 V64I* and *CCR5 A-2852G* by polymerase chain reaction restriction fragment length polymorphism (PCR-RFLP), as described previously.[17] The G allele at position -2852 of the *CCR5* gene in Thais represents the *CCR5* promoter haplotype (*CCR5 P1*), which has been reported to be associated with rapid progression to AIDS in HIV-1–infected individuals.[17] The entire coding region of *CCR5* was amplified and sequenced with a previously described method.[17]

Expression Levels of CCR5

African green monkey kidney CV1 cells were infected with either Sendai virus (SeV) expressing CCR2A 64I or a parental Z strain of SeV. Nine hours after SeV infection, the cells were superinfected with a recombinant vaccinia virus expressing either the wild type CCR5 or CCR5 316A. After incubation for 5 hrs at 37°C, the cells were stained with T227, a rat monoclonal antibody against the N-terminal extracellular portion of CCR5, followed by staining with fluorescein-5-isothiocyanate (FITC)-conjugated goat antibody directed against rat IgG (Cappel, Aurora, OH) and analyzed by FACScalibur (Becton Dickinson, San Jose, CA). For intracellular staining of CCR5, the cells were permeabilized with 0.05% saponin and 0.2% bovine serum albumin in phosphate buffer saline (PBS) before staining.

RESULTS

Sequence analysis of the entire coding region of *CCR5* genes from the 74 ESNs and 140 of the HIV-1–infected females showed that there were only 2 types of polymorphisms of the *CCR5* coding region. A G-to-A substitution at position 316 was found to convert an uncharged glycine residue into a basic arginine residue at position 106 in the third transmembrane domain of CCR5. This SNP, previously detected in Vietnamese and Chinese subjects, was found to affect surface trafficking of the CCR5.[18,19] A G-to-A substitution at position 668 converts a basic arginine into a glutamine at position 223 in the third cytoplasmic region. This SNP was previously identified in almost all ethnic groups examined.[20] We then determined the nucleotide sequences from positions 280 to 880 of the *CCR5* coding region in the remaining 207 HIV-1–infected females for genotyping their *CCR5 G316A* and *CCR5 G668A*.

Table 1 shows the genotype frequency of *CCR2 V64I*, *CCR5 A–2852G*, *CCR5 G316A*, and *CCR5 G668A* alleles in the 74 ESNs and 347 HIV-1–infected females. There was no significant difference between the 2 groups in either genotype distribution or allele frequency of the 4 alleles, although *CCR5* coding region polymorphisms tended to be more prevalent in ESNs than in HIV-1–infected individuals.

Table 2 shows the relationship among the 4 *CCR2-CCR5* polymorphisms. Haplotype construction by means of Arlequin version 2.01 (Genetics and Biometry Laboratory, Geneva, Switzerland) clearly demonstrated that *CCR2 V64I* alleles never occurred together with *CCR5 G–2852A*. This result confirmed our previous observation in Japanese and Thai subjects.[17] We also confirmed that *CCR5 G668A* alleles were in strong linkage disequilibrium with *CCR2 V64I* ($D' = 1$).[17] In contrast, *CCR5 G316A* was always associated with *CCR5 A–2852G* but never with *CCR2 V64I*.

TABLE 1. Frequency of *CCR2 V64I*, *CCR5 A-2852G*, *CCR5 G316A*, and *CCR5 G668A* Alleles in 74 Female ESNs and 347 HIV-1–Infected Females at Lampang Hospital

Genotype	ESN	HIV-1 Infected	*P*
CCR2 64I			
Homozygotes	4	13	
Heterozygotes	21	95	
Wild type	49	239	
Allele frequency	0.196	0.174	0.533*
CCR5 G-2852A			
Homozygotes	3	14	
Heterozygotes	25	97	
Wild type	46	236	
Allele frequency	0.209	0.180	0.404*
CCR5 G316A			
Homozygotes	0	0	
Heterozygotes	3	7	
Wild type	71	340	
Allele frequency	0.020	0.010	0.392†
CCR5 G668A			
Homozygotes	0	0	
Heterozygotes	5	12	
Wild type	69	335	
Allele frequency	0.034	0.017	0.199†
CCR2 V64I and *CCR5 G316A*	2	1	
Others	72	346	
Frequency	0.027	0.003	0.081†
Homozygous *CCR5 G-2852A* and *CCR5 G316A*	0	3	
Others	74	344	
Frequency	0	0.009	1†
Homozygous *CCR2 V64I* and *CCR5 G668A*	1	2	
Others	73	345	
Frequency	0.014	0.006	0.441†

*χ^2 test.
†Fisher exact test.

The presence of 2 alternatively spliced CCR2 isoforms, CCR2A and CCR2B, has been reported in a freshly isolated human monocyte, THP-1, and in MonoMac 6 leukemia cell lines.[21,22] We previously reported that the CCR2A isoform with V64I substitution could impair cell surface trafficking of CCR5 more strongly than could wild type CCR2A.[23] As mentioned above, *CCR5 G316A* was also found to reduce levels of cell surface CCR5 expression. We therefore further examined the individuals carrying both *CCR2 V64I* and *CCR5 G316A* (Table 1) and found that 2 of the 74 ESNs carried both alleles, whereas only 1 of the 347 HIV-1–infected individuals carried them. There was thus a clear trend for this combination to occur more frequently in ESNs than in HIV-1–infected individuals, although this trend did not reach statistical

TABLE 2. Linkage Disequilibrium Among *CCR2 V64I, CCR5 A-2852G, CCR5 G316A,* and *CCR5 G668A*

	CCR5 A-2852G			CCR5 G316A			CCR5 G688A		
	Homozygotes	Heterozygotes	Wild Type	Homozygotes	Heterozygotes	Wild Type	Homozygotes	Heterozygotes	Wild Type
CCR2 V64I									
Homozygotes	0	0	17	0	0	17	0	3	14
Heterozygotes	0	22	94	0	3	113	0	14	102
Wild type	17	100	171	0	7	281	0	0	288
CCR5 A-2852G									
Homozygotes	—	—	—	0	3	14	0	0	17
Heterozygotes	—	—	—	0	7	115	0	1	121
Wild type	—	—	—	0	0	282	0	16	266

significance ($P = 0.081$, Fisher exact test). The 2 ESNs were able to maintain their HIV-seronegative status for at least 27 more months after the first blood samples were obtained. In contrast, the combination of disease-accelerating *CCR5 A-2852G* and *CCR5 G316A* (homozygous *CCR5 A-2852G* with *CCR5 G316A*) was observed only in HIV-1–infected individuals and not in ESNs (Table 1). These findings suggest that the combination of *CCR2* and *CCR5* polymorphisms in 2 chromosomes affects heterosexual transmission in Thai females. No such trend was observed with respect to *CCR5 G668A* alleles ($P = 0.441$). It should be noted that neither of the ESNs with *CCR2 V64I* and *CCR5 G316A* carried an A allele at SNP VS2277998 of *DC-SIGNR*, which was more frequently found in the ESNs than in the HIV-1–infected Thai females,[4] indicating that the association of the *CCR2-CCR5* genotype with HIV-1–seronegative status is independent from that of the previously published *DC-SIGNR* genotypes.

Next, we examined whether cell surface expression of the CCR5 316A protein was also impaired by coexpression with the CCR2A 64I protein. CV1 cells were infected with either SeV expressing CCR2A 64I or with a parental Z strain of SeV. Nine hours after SeV infection, the cells were superinfected with a recombinant vaccinia virus expressing either the wild type CCR5 or CCR5 316A. As shown in Figure 1,

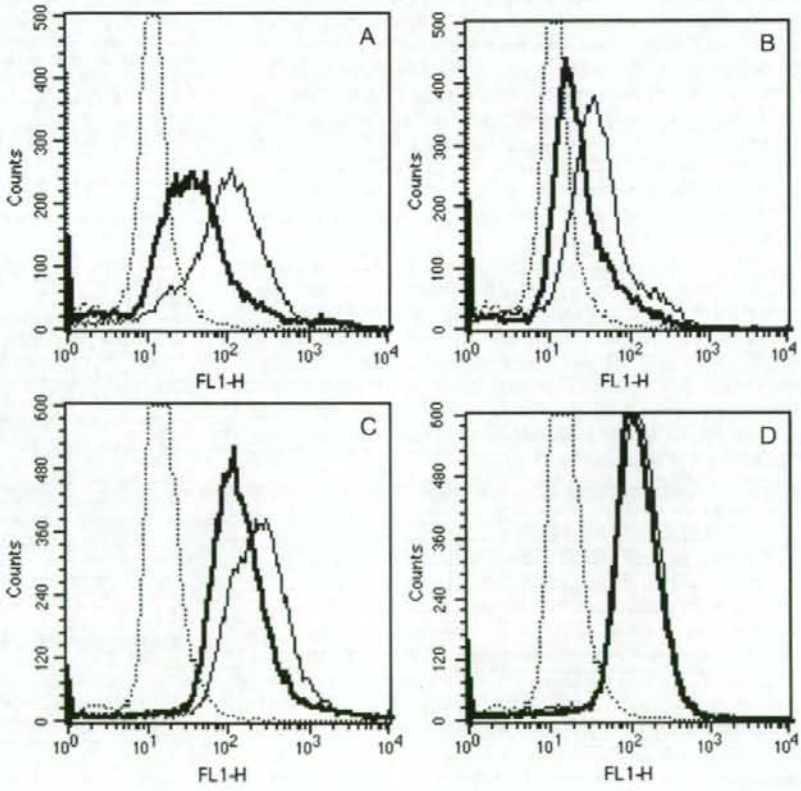

FIGURE 1. Cell surface and intracellular expression of CCR5. African green monkey kidney CV1 cells were infected with the Sendai virus (SeV) expressing CCR2 64I (bold lines) or a parental Z strain of SeV (solid lines). Nine hours after SeV infection, cells were superinfected with a recombinant vaccinia virus expressing either the wild type CCR5 (A and C) or CCR5 316A (B and D). After incubation for 5 hours at 37°C, cells were permeabilized (C and D) or not (A and B) with saponin. The cells were then stained with T227, a rat monoclonal antibody against the N-terminal extracellular portion of CCR5, followed by staining with FITC-conjugated goat antibody against rat IgG and analyzing with a flow cytometer. Dotted lines denote cells stained with the second antibody as negative control. Representative results of 6 independent experiments with similar results are shown.

CCR2A 64I greatly impaired surface expression of the wild type CCR5 (Fig. 1A) and that of CCR5 316A (Fig. 1B). Because the level of cell surface expression of CCR5 316A was very low, coexpression of CCR2A 64I resulted in nearly complete elimination of cell surface CCR5 expression (Fig. 1B). When the cells were permeabilized, however, in cells expressing both CCR5 316A and CCR2A 64I we observed nearly equal levels of CCR5 to those in cells expressing the wild type CCR5 alone (Fig. 1C) or CCR5 316A alone (Fig. 1D). These findings confirmed that the very low levels of surface expression of CCR5 316A in the presence of CCR2A 64I are the result of severe impairment of its cell surface trafficking.

DISCUSSION

Previously, 2 alleles of *CCR5 G316A* were detected in 87 HIV-1–negative Vietnamese, but not in 45 HIV-1–positive Vietnamese, suggesting that this allele could be protective against HIV-1 transmission.[18] In the study of larger numbers of Thais reported here, we detected substantial numbers of this allele in HIV-1–infected Thai females. However, the combination of this allele with the other protective allele, *CCR2 V64I*, tended to occur more frequently in ESNs than in HIV-1–infected individuals. Surface expression of CCR5 316A was almost completely eliminated by coexpression with CCR2A 64I. These findings suggest that *CCR5 G316A* has a protective effect on HIV-1 transmission only in the presence of the other protective allele, *CCR2 V64I*. The lack of protection by *CCR5 G316A* without *CCR2 V64I* against HIV-1 transmission despite its poor surface trafficking may be due to its complete linkage disequilibrium with *CCR5 A-2852G*, which represents the disease-accelerating *CCR5 P1*. However, the combination of *CCR2 V64I* and *CCR5 G316A* was rare even in ESNs, suggesting that factors other than *CCR2* and *CCR5* polymorphisms may contribute to their seronegative status.

Reduced cell surface expression of CCR5 was previously reported on primary CD4 T lymphocytes obtained from heterozygous individuals for *CCR5Δ32*[24] or *CCR5 893(-)*.[25] Although we have clearly shown that surface expression of CCR5 316A was almost completely eliminated by coexpression with CCR2A 64I in our experimental system, it is not clear whether primary cells from individuals with *CCR5 G316A* and *CCR2 V64I* indeed show reduced surface levels of CCR5. It is thus necessary to examine surface expression levels of CCR5 of various cells obtained from those individuals.

We previously reported that all the *CCR2 V64I* alleles and nearly all the *CCR5* coding region polymorphisms in Japanese, and all the *CCR5-Δ32* alleles in the French ALT, IMMUNOCO, and SEROCO cohorts occurred in the CCR5 promoter haplotypes with higher promoter activity.[17] We therefore hypothesized the existence of a certain selective pressure favoring low levels of CCR5 expression during human evolution. Because the disease-accelerating *CCR5 P1* also carries the CCR5 promoter with higher promoter activity, our finding reported here further supports our hypothesis, as both the *CCR5 G316A* and *CCR5 G668A* alleles identified in Thai subjects were also associated with the CCR5 haplotypes with higher promoter activity

ACKNOWLEDGMENTS

We are grateful to all the patients who participated in the Lampang cohort and the blood donors who allowed us to use their blood for this study. We also thank the medical staff of Lampang Hospital for their cooperation.

REFERENCES

1. Fowke KR, Nagelkerke NJ, Kimani J, et al. Resistance to HIV-1 infection among persistently seronegative prostitutes in Nairobi, Kenya. *Lancet*. 1996;348:1347–1351.
2. Mann JM, Quinn TC, Francis H, et al. Prevalence of HTLV-III/LAV in household contacts of patients with confirmed AIDS and controls in Kinshasa, Zaire. *JAMA*. 1986;256:721–724.
3. O'Brien SJ, Nelson GW. Human genes that limit AIDS. *Nat Genet*. 2004; 36:565–574.
4. Wichukchinda N, Kitamura Y, Rojanawiwat A, et al. The polymorphisms in *DC-SIGNR* affect susceptibility to HIV-1 infection. *AIDS Res Hum Retroviruses*. 2007;23:686–692.
5. Samson M, Libert F, Doranz BJ, et al. Resistance to HIV-1 infection in caucasian individuals bearing mutant alleles of the CCR-5 chemokine receptor gene. *Nature*. 1996;382:722–725.
6. Liu R, Paxton WA, Choe S, et al. Homozygous defect in HIV-1 coreceptor accounts for resistance of some multiply-exposed individuals to HIV-1 infection. *Cell*. 1996;86:367–377.
7. Dean M, Carrington M, Winkler C, et al. Genetic restriction of HIV-1 infection and progression to AIDS by a deletion allele of the CKR5 structural gene. Hemophilia Growth and Development Study, Multicenter AIDS Cohort Study, Multicenter Hemophilia Cohort Study, San Francisco City Cohort, ALIVE Study. *Science*. 1996;273:1856–1862.
8. Huang Y, Paxton WA, Wolinsky SM, et al. The role of a mutant CCR5 allele in HIV-1 transmission and disease progression. *Nat Med*. 1996;2: 1240–1243.
9. Martinson JJ, Chapman NH, Rees DC, et al. Global distribution of the CCR5 gene 32-basepair deletion. *Nat Genet*. 1997;16:100–103.
10. Jiang JD, Wang Y, Wang ZZ, et al. Low frequency of the ccr5delta32 HIV-resistance allele in mainland China: identification of the first case of ccr5delta32 mutation in the Chinese population. *Scand J Infect Dis*. 1999; 31:345–348.
11. Ruchusatsawat N, Vongsheree S, Thaisri H, et al. The first report of CCR5 delta 32 mutant in Thai injecting drug users. *Asian Pac J Allergy Immunol*. 2000;18:93–98.
12. Martin MP, Dean M, Smith MW, et al. Genetic acceleration of AIDS progression by a promoter variation of CCR5. *Science*. 1998;282:1907–1911.
13. Smith MW, Carrington M, Winkler C, et al. CCR2 chemokine receptor and AIDS progression. *Nat Med*. 1997;3:1052–1053.
14. Kostrikis LG, Huang Y, Moore JP, et al. A chemokine receptor CCR2 allele delays HIV-1 disease progression and is associated with a CCR5 promoter mutation. *Nat Med*. 1998;4:350–353.
15. Mulherin SA, O'Brien TR, Ioannidis JP, et al. Effects of CCR5-Delta32 and CCR2-64I alleles on HIV-1 disease progression: the protection varies with duration of infection. *AIDS*. 2003;17:377–387.
16. Wichukchinda N, Nakayama EE, Rojanawiwat A, et al. Protective effects of IL4-589T and RANTES-28G on HIV-1 disease progression in infected Thai females. *AIDS*. 2006;20:189–196.
17. Liu H, Nakayama EE, Theodorou I, et al. Polymorphisms in CCR5 chemokine receptor gene in Japan. *Int J Immunogenet*. 2007;34: 325–335.
18. Capoulade-Metay C, Ma L, Truong LX, et al. New CCR5 variants associated with reduced HIV coreceptor function in southeast Asia. *AIDS*. 2004;18:2243–2252.
19. Ma L, Dudoit Y, Tran T, et al. Biochemical and HIV-1 coreceptor properties of K26R, a new CCR5 Variant in China's Sichuan population. *J Acquir Immune Defic Syndr*. 2005;39:38–43.
20. Ansari-Lari MA, Liu XM, Metzker ML, et al. The extent of genetic variation in the CCR5 gene. *Nat Genet*. 1997;16:221–222.

21. Charo IF, Myers SJ, Herman A, et al. Molecular cloning and functional expression of two monocyte chemoattractant protein 1 receptors reveals alternative splicing of the carboxyl-terminal tails. *Proc Natl Acad Sci U S A*. 1994;91:2752–2756.
22. Wong LM, Myers SJ, Tsou CL, et al. Organization and differential expression of the human monocyte chemoattractant protein 1 receptor gene. Evidence for the role of the carboxyl-terminal tail in receptor trafficking. *J Biol Chem*. 1997;272:1038–1045.
23. Nakayama EE, Tanaka Y, Nagai Y, et al. A CCR2-V64I polymorphism affects stability of CCR2A isoform. *AIDS*. 2004;18:729–738.
24. Wu L, Paxton WA, Kassam N, et al. CCR5 levels and expression pattern correlate with infectability by macrophage-tropic HIV-1, in vitro. *J Exp Med*. 1997;185:1681–1691.
25. Shioda T, Nakayama EE, Tanaka Y, et al. Naturally occurring deletional mutation in the C-terminal cytoplasmic tail of CCR5 affects surface trafficking of CCR5. *J Virol*. 2001;75:3462–3468.

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

Akihide Ryo[a,b,c], Naomi Tsurutani[d], Kenji Ohba[b,e], Ryuichiro Kimura[e,f], Jun Komano[b], Mayuko Nishi[a], Hiromi Soeda[a], Shinichiro Hattori[b], Kilian Perrem[g], Mikio Yamamoto[h], Joe Chiba[f], Jun-ichi Mimaya[i], Kazuhisa Yoshimura[j], Shuzo Matsushita[j], Mitsuo Honda[b], Akihiko Yoshimura[k], Tatsuya Sawasaki[l], Ichiro Aoki[a], Yuko Morikawa[d], and Naoki Yamamoto[b,c]

[a]Department of Pathology, Yokohama City University School of Medicine, 3-9 Fuku-ura, Kanazawa-ku, Yokohama 236-0004, Japan; [b]AIDS Research Center, National Institute of Infectious Diseases, 1-23-1 Toyama, Shinjuku-ku, Tokyo 162-8640, Japan; [d]Kitasato Institute for Life Sciences, Kitasato University, Shirokane 5-9-1, Minato-ku, Tokyo 108-8641, Japan; [e]Department of Molecular Virology, Graduate School of Medicine, Tokyo Medical and Dental University, 1-5-45 Yushima, Bunkyo-ku, Tokyo 113-8519, Japan; [f]Molecular Oncology Laboratory, Department of Pathology, Royal College of Surgeons in Ireland, Smurfit Building, Beaumont Hospital, Dublin 9, Ireland; [h]Department of Biochemistry II, National Defense Medical College, 3-2 Namiki, Tokorozawa-shi, Saitama 359-8513, Japan; [g]Department of Biological Science and Technology, Science University of Tokyo, 2641 Yamazaki, Noda, Chiba 278-8510, Japan; [i]Division of Hematology and Oncology, Shizuoka Children's Hospital, 860 Urushiyama, Aoi-ku, Shizuoka 420-8660, Japan; [j]Division of Clinical Retrovirology and Infectious Diseases, Center for AIDS Research, Graduate School of Medical Sciences, Kumamoto University, Kumamoto 860-0811, Japan; [k]Division of Molecular and Cellular Immunology, Medical Institute of Bioregulation, Kyushu University, Fukuoka 812-8582, Japan; and [l]Cell Free Science and Research Center, Ehime University, Ehime 790-8577, Japan

Edited by Robert C. Gallo, University of Maryland, Baltimore, MD, and approved November 19, 2007 (received for review May 24, 2007)

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-

Author contributions: A.R. and N.T. contributed equally to this work; A.R., A.Y., Y.M., and N.Y. designed research; A.R., N.T., K.O., R.K., M.N., H.S., S.H., T.S., I.A., and Y.M. performed research; J.K., S.H., M.Y., J.C., J.-I.M., K.Y., S.M., M.H., and A.Y. contributed new reagents/analytic tools; A.R., N.T., K.O., M.N., H.S., K.P., M.Y., K.Y., S.M., T.S., I.A., Y.M., and N.Y. analyzed data; and A.R., K.P., and N.Y. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

Freely available online through the PNAS open access option.

[c]To whom correspondence may be addressed. E-mail: aryo@nih.go.jp or nyama@nih.go.jp.

This article contains supporting information online at www.pnas.org/cgi/content/full/0704831105/DC1.

www.pnas.org/cgi/doi/10.1073/pnas.0704831105

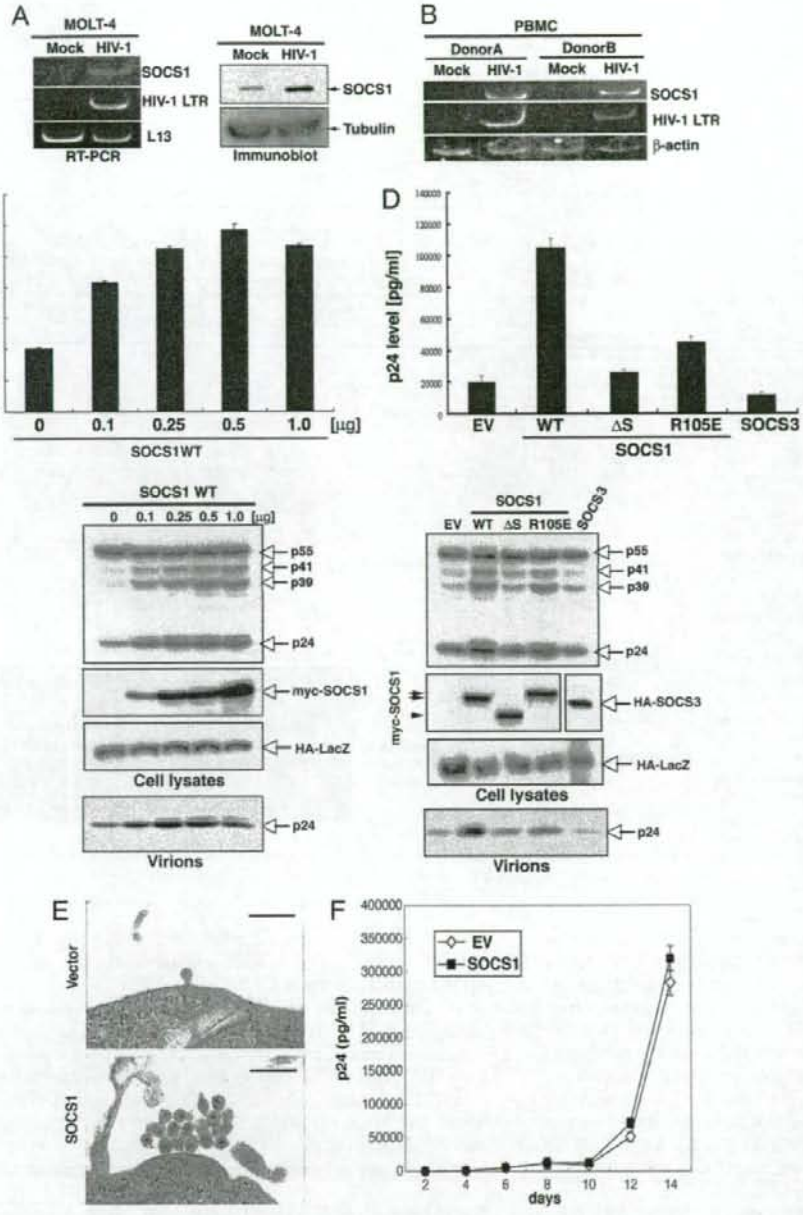

Fig. 1. SOCS1 is induced upon HIV-1 infection and enhances HIV-1 particle production. (*A*) MOLT-4 cells were mock-infected or infected with HIV-1_{NL4-3}, and then total RNA and protein extracts derived from these cells were subjected to semiquantitative RT-PCR (*Left*) and immunoblotting (*Right*),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

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

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

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

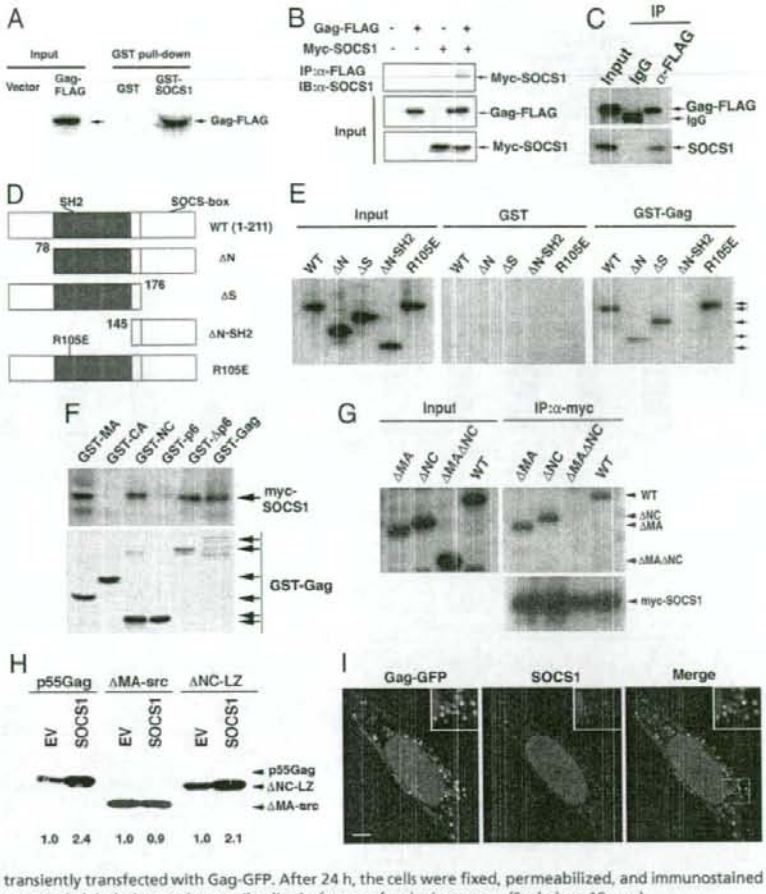

Fig. 2. SOCS1 interacts with HIV-1 Gag. (*A*) Extracts of 293T cells transfected with either empty vector or Gag-FLAG were subjected to pull-down analyses using glutathione–agarose beads with GST-SOCS1 in the presence of 10 ng/ml RNase followed by immunoblotting with anti-FLAG antibodies. (*B*) Extracts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

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

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

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

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

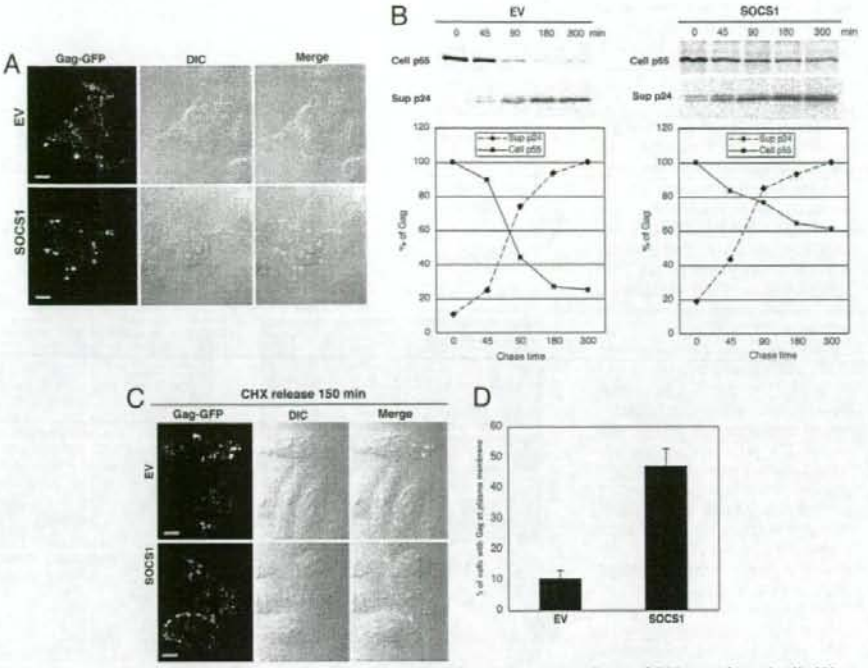

Fig. 3. SOCS1 enhances both the stability and trafficking of HIV-1 Gag. (*A*) HeLa cells cotransfected with pNL4-3 and either control vector (EV) or SOCS1 were immunostained with antibodies targeting anti-p24 (CA). Confocal microscopy with differential interference contrast (DIC) was then performed.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

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

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

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

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

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

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

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

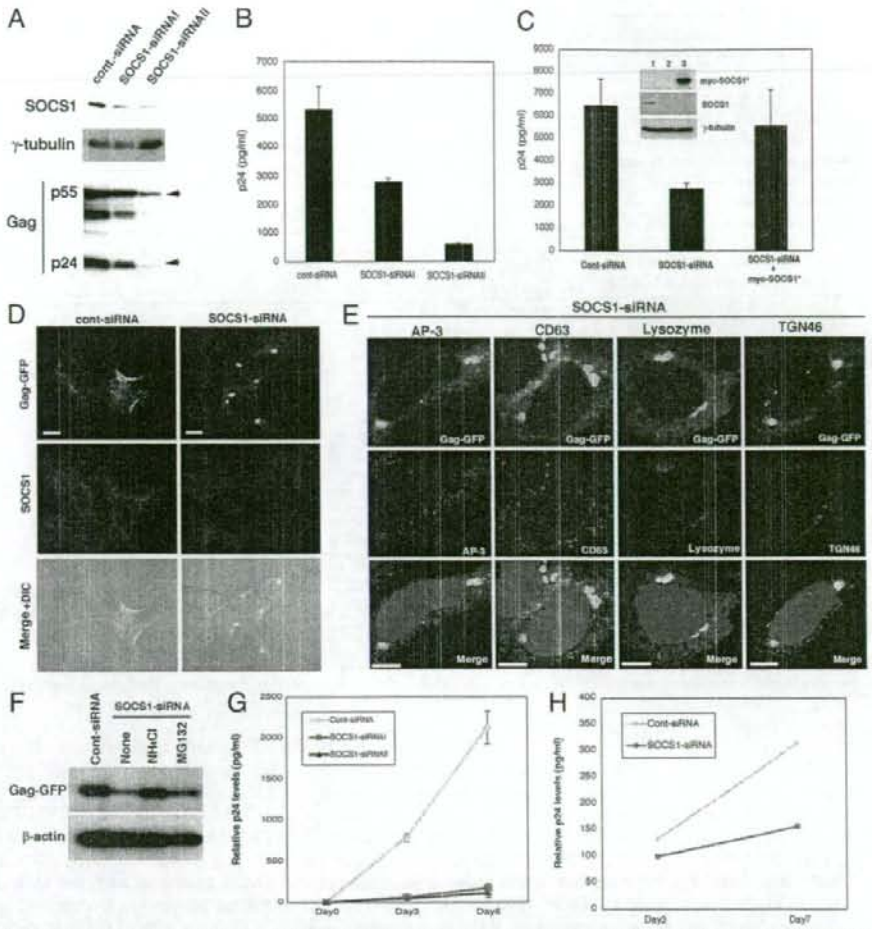

Fig. 4. The targeted inhibition of SOCS1 suppresses Gag trafficking and HIV-1 particle production and enhances Gag degradation in lysosomes. (*A* and *B*) 293T cells were transfected with either control siRNA or two different SOCS1-specific siRNAs (I or II) together with pNL4-3. At 48 h after transfection,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

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

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

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

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

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

Discussion

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

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

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

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

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

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

Materials and Methods

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

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

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

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

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

Published online 29 October 2008

Nucleic Acids Research, 2008, Vol. 36, No. 21 **6859–6871**
doi:10.1093/nar/gkn802

The antiretroviral potency of APOBEC1 deaminase from small animal species

Terumasa Ikeda[1,2], Takeo Ohsugi[3], Tetsuya Kimura[1], Shuzo Matsushita[4], Yosuke Maeda[2], Shinji Harada[2] and Atsushi Koito[1,*]

[1]Department of Retrovirology and Self-Defense, Faculty of Medical and Pharmaceutical Sciences, [2]Department of Medical Virology, Faculty of Medical and Pharmaceutical Sciences, [3]Center for Animal Resources and Development, Institute of Resource Development and Analysis and [4]Center for AIDS Research, Kumamoto University, Kumamoto 860-8556, Japan

Received August 28, 2008; Revised October 7, 2008; Accepted October 11, 2008

ABSTRACT

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

INTRODUCTION

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

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

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

*To whom correspondence should be addressed. Tel: +81 96 373 5133; Fax: +81 96 373 5132; Email: akoito@kumamoto-u.ac.jp

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

MATERIALS AND METHODS

Molecular cloning of A1s

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

Generation of catalytic site-mutated rabbit A1 expression vectors

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

Amino acid alignments and phylogenetic analysis

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

Viral preparation and infectivity assay

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

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