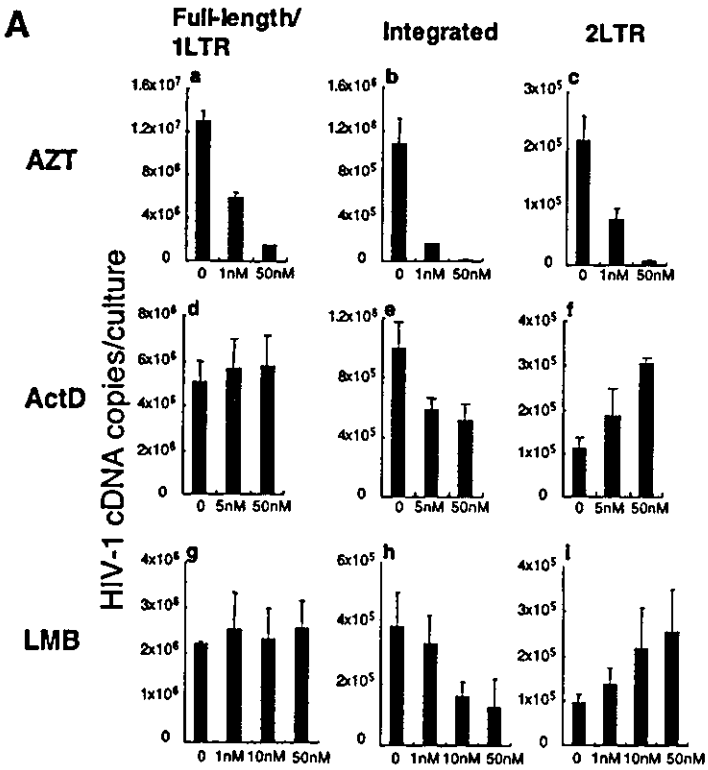

Fig. 2. Measurement of nuclear entry and integration of HIV-1 cDNA in cells treated with inhibitors. (A) Inhibition of HIV-1 cDNA appearance with chemical treatment. 293T cells were infected with HIV-1 vector at a m.o.i. of 0.5–1 and cultured in the presence of AZT, ActD, or LMB at indicated doses. HIV-1 cDNA copy numbers in AZT-treated (a, b, c), ActD-treated (d, e, f) and LMB-treated (g, h, i) cultures are indicated. Left panels (a, d, g) indicate full-length/1LTR circle, middle panels (b, e, h) indicate integrated forms, and right panels (c, f, i) indicate 2LTR circle, respectively. (B) Inhibition of nuclear entry of HIV-1 cDNA with VSV M transduction. Full-length/1LTR circle, 2LTR circle and integrated forms of HIV-1 cDNA in VSV M-transduced cultures are indicated. 293T cells were transfected with VSV M- or VSV M(D)-expressing DNA (lane 3, 4). pcDNA3.1/Zeo(+) was used as control (lane 2). The VSV M-transduced cells were also transfected together with Nup98-expressing DNA (lane 5). Then, these cells were infected with HIV-1 vector 24 h later. Total DNA was extracted 24 h after infection. Results are mean ± S.D. of three independent experiments (A and B). $^*P < 0.05$ was judged as a significant difference using Welch's *t*-test. (C) Binding of Nup98 to VSV M protein. Western blotting using anti-GFP antibody (whole lysate) indicates VSV M or VSV M(D) expression. Binding of ectopically expressed Nup98 to VSV M protein was shown by immunoprecipitation with anti-HA antibody (anti-HA IP) and Western blotting using anti-Nup98 or anti-GFP antibodies. Results of one representative experiment from three independent experiments are shown.

assays with nuclear localization signal (NLS) of SV40 or M9 import signal (mediated by importin α/β and importin β2, respectively), while the ability of the mutant pore to import ribosomal protein L23a (mediated by either importin β, importin β2, importin β3 or importin 7) [28] and splice some protein U1A (independent of cytosolic transport factors) was intact [27]. To examine the import ability of Nup98-siRNA-targeted pores, we performed a set of import assays. These experiments were performed in a transport buffer containing specific soluble transport receptors, an energy-regenerating system, and a DsRed-labeled protein acting as a substrate for import into the nuclei of digitonin-permeabilized cells [29]. The nuclear import with an SV40 NLS import signal was substantially lower in the Nup98-depleted cells than in control cells. In contrast, translocation of rpL23 or U1A was similar in Nup98-depleted and control cells (Fig. 2E, F). These results indicated that protein import pathways were similarly impaired in both Nup98-depleted human cells using siRNA and Nup98-knockout cells, although the level of impairment of NLS in the Nup98-depeleted cells using siRNA seems to be lower than that in knockout cells.

3.4. A role of Nup98 in HIV-1 cDNA import

Finally, Nup98-depleted cells using a siRNA-expressing plasmid DNA were infected with HIV-1 vector, and the levels of integrated, 2LTR, and full-length/1LTR forms of HIV-1 cDNA were measured. Obvious reductions of integrated and 2LTR but not full-length DNA were noted in the Nup98-depleted culture. In contrast, in culture transfected with a control siRNA-expressing DNA targeted for luciferase (si-Luc), the levels of integrated, 2LTR and full-length DNA were still high (Fig. 3G). These findings suggest that Nup98 in the NPC participates in the nuclear entry of HIV-1 cDNA.

4. Discussion

The major finding of the present study is that Nup98 has an important role in the nuclear import of HIV-1 cDNA, based on a series of experiments using an inhibitor (VSV M protein) and the siRNA technique for Nup98. The role of

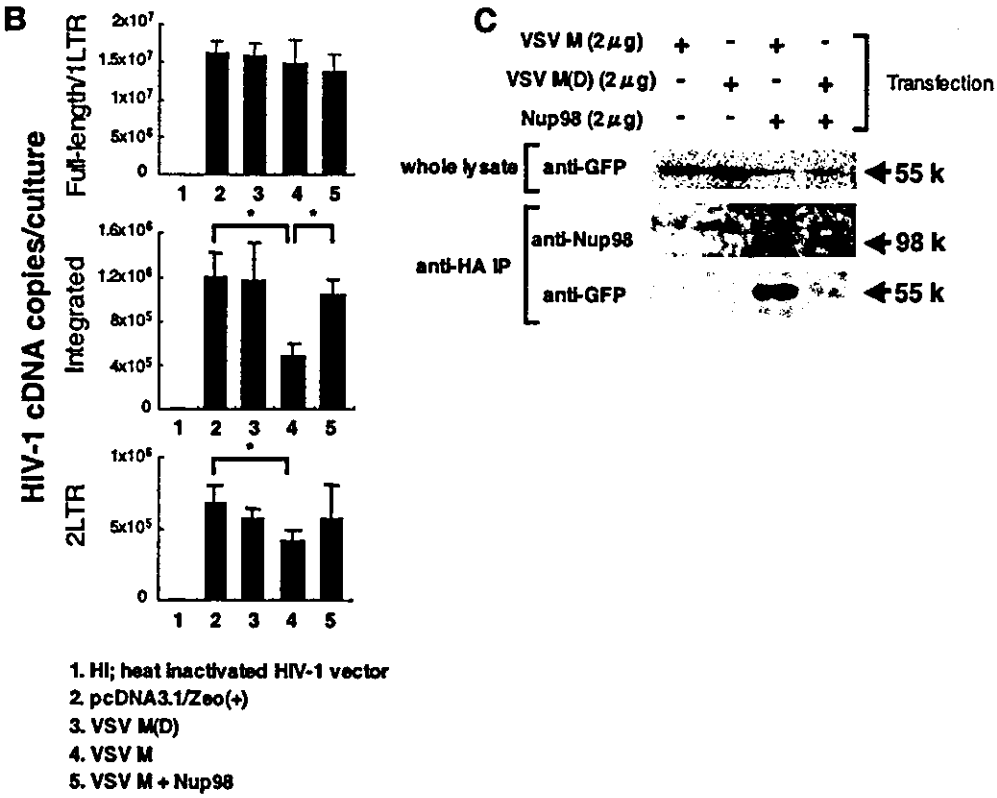

Fig. 2. *(continued)*

NPC in virus replication has been shown previously in other virus families [30]. It was reported that the import process of adenovirus type 2 DNA through NPC involves Nup214 as well as histone H1 [31]. Adenovirus capsid docks on Nup214, probably involving recruitment of importin 7/β heterodimer to the capsid, and effective disassembly at NPC activates the entry of viral DNA into nuclei [31]. Herpes simplex virus capsid docks on NPC, probably mediated by importin β alone, and undergoes a conformational shift that results in extrusion of viral DNA genome into nucleus through NPC [32]. It is possible that specific molecular events may occur at NPC during the nuclear entry of HIV-1 cDNA [15].

We recently transduced a siRNA-expressing DNA for Nup98 using lentivirus vector coexpressed H-2K^k, as described above, and at this time the culture was challenged with replication-competent EGFP-expressing HIV-1. Flow cytometric analysis indicated that numbers of HIV-1-infected cells were obviously inhibited in Nup98-siRNA-expressing T cells but not in control luciferase-specific siRNA-expressing cells. Significant reduction of HIV-1 p24^{gag} (five to sixfold) was also noted in the culture supernatant (data not shown). It was reported that Nup98 and Nup214 are required for Rev-dependent export of HIV-1 RNA [24]. Thus, inhibition of Nup98 expression by functional impairment of Nup98 by siRNA may induce inhibition of HIV-1 gene expression through inhibition of the Rev-dependent export. Some degree of the inhibitory effect of HIV-1 infection by the siRNA may be partly caused by this inhibitory effect of Rev function. But quantitative analysis of HIV-1 cDNA in single-round infection demonstrated that the inhibition of nuclear import of HIV-1 cDNA (Fig. 3G) was apart from gene expression. These findings suggest the involvement of a specific import pathway in the nuclear entry of HIV cDNA through NPC.

Most of the transport receptors identified to date are members of a large family of RanGTP binding proteins, which exhibit a limited sequence similarity to the Ran binding domain of importin β. The interaction of these receptors with Nups is regulated by small GTPase Ran. Ran is a small GTPase that cycles between a GDP-bound form (RanGDP) and a GTP-bound form (RanGTP) and plays an important role in both import and export [8]. The directional active nuclear transport is controlled by the different RanGDP and RanGTP concentration gradients within the cell. In the cytoplasm, a much higher concentration of RanGDP to RanGTP is maintained, and conversion of RanGDP to RanGTP occurs by exchanging the entire nucleotide and is catalyzed by the guanine nucleotide exchanging factor (RCC1) [33]. The exchange of the nucleotide and disassembly of importin β2 complex at a site on Nup98 was reported [13]. The VSV M-mediated inhibition of nuclear traffic is due to the inhibition of RanGDP to RanGTP conversion [13]. Therefore, in the presence of VSV M expression or inhibition of Nup98 expression should induce the disruption of the RanGDP and RanGTP concentration gradients. Our data strongly suggest

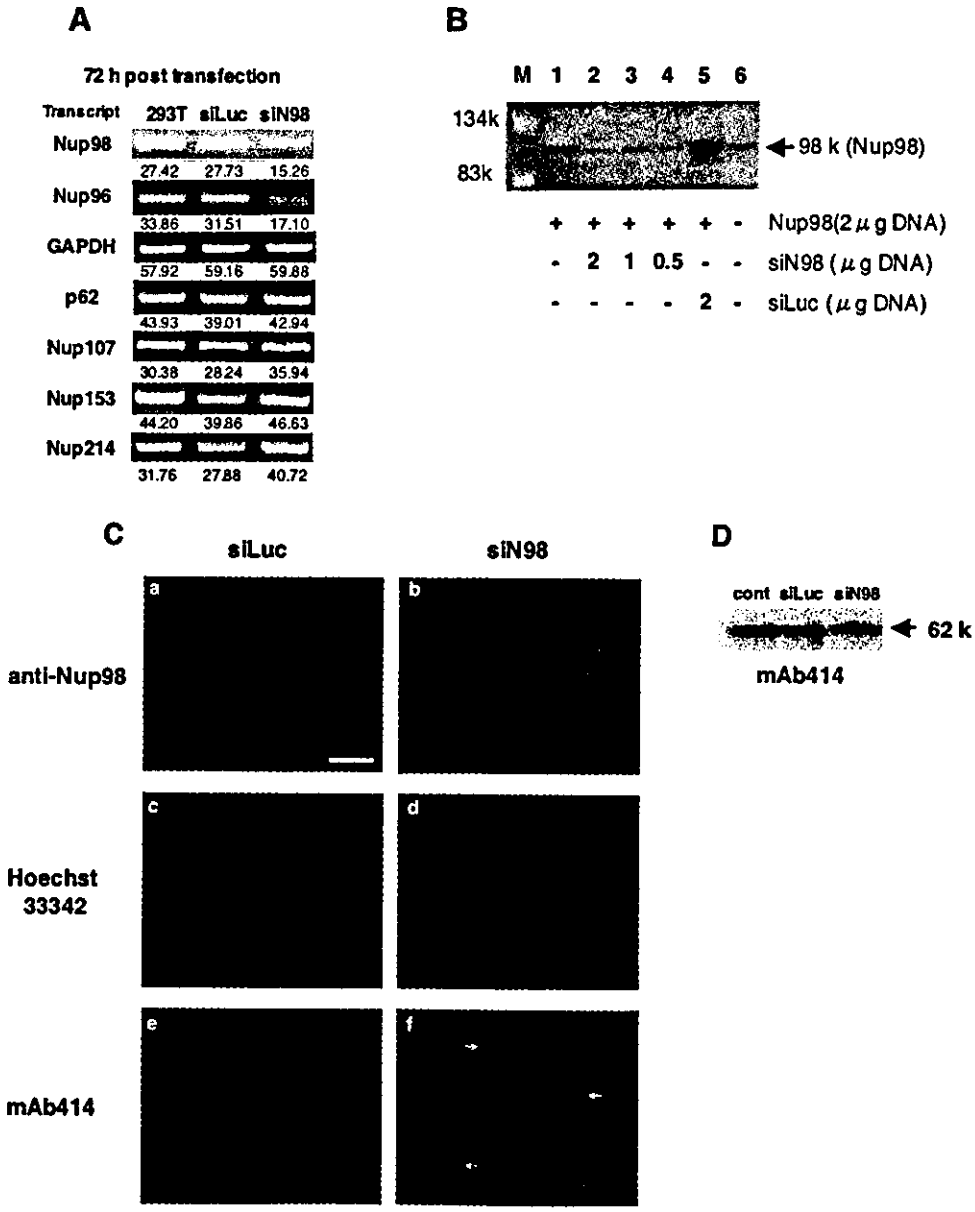

Fig. 3. Depletion of Nup98 by siRNA impairs specific NPC function. (A) Inhibition of Nup98 and Nup96 mRNA expression with Nup98-targeted siRNA. 293T cells were transfected with siRNA-expressing plasmid DNA targeted for Nup98 or luciferase (siN98 or siLuc, respectively) and mRNA levels of Nup98, Nup96, p62, Nup107, Nup153, Nup214 or GAPDH were determined by RT-PCR. The numbers under the band indicate intensity of each band digitalized by using NIH image. (B) Inhibition of Nup98 expression with siRNA-expressing plasmid DNA. Western blotting analysis using an anti-Nup98 antibody in siLuc or siN98-transfected 293T cells is indicated. Nup98-expressing plasmid (Nup98 as indicated) was transfected without (lane 1) or with (lane 2-5) siRNA-expressing plasmid as indicated. The expression level of endogenous Nup98 is indicated in lane 6. Size marker (M) is indicated. (C) Expression of Nup98 and localization of FG-repeat nucleoporins in siRNA-transduced cultures. Immunofluorescence analysis of HeLa cells infected with siLuc (a, c, e)- or siN98 (b, d, f)-expressing lentivirus vector using an anti-Nup98 (upper panels) or mAb414 antibody (lower panels) are shown. Hoechst 33342 staining (middle panels) indicates nucleus. Blue arrow in bottom panel indicates weak signal on nuclear rim, and white arrow indicates a shift labeling to the cytoplasm, probably annulate lamellae form. Calibration bar = 30 μm. (D) Western blotting analysis of HeLa cells infected with siRNA-expressing lentivirus vector using mAb414. The level of p62 protein expression is indicated. (E) Nuclear import assay of Nup98-depeleted HeLa cells. Cells were infected with the siLuc (upper panels)- or siN98 (lower panels)-expressing lentivirus vector, and 3 days later, nuclear import for these depleted cells after permealization with digitonin was examined using DsRed-labeled SV40 NLS (a, d), U1A (b, e), and rpL23a (c, f), respectively. Import of SV40 NLS is dependent on importin α/β and that of rpL23a is dependent on importin β3. Import of U1A is independent of active transfer machinery. The reaction was performed at 30 °C and stopped after 30 min by fixation. Calibration bar = 30 μm. Results of one representative assay from three independent experiments are shown (A, B, C, D, E). (F) Quantification of nuclear import. Cells with nuclear import were counted in five randomly selected visual fields. Data are expressed as percentage of imported cells (mean ± S.D. of five fields). *$P < 0.05$, compared with siLuc by Welch's t-test. (G) Measurement of newly synthesized HIV-1 cDNA in siLuc- or siN98-transfected cells. 293T cells were transfected with siN98- or siLuc-expressing plasmid DNA, and 72 h later, EGFP-expressing HIV-1 vector was used for infection. DNA was extracted 24 h after infection and DNA was subjected to real-time PCR assay. The number of HIV-1 cDNA copies per culture is indicated. A heat inactivated HIV-1 vector (HI) was used as negative control. Results are mean ± S.D. of six independent experiments. *$P < 0.05$, compared with siLuc by Welch's t-test.

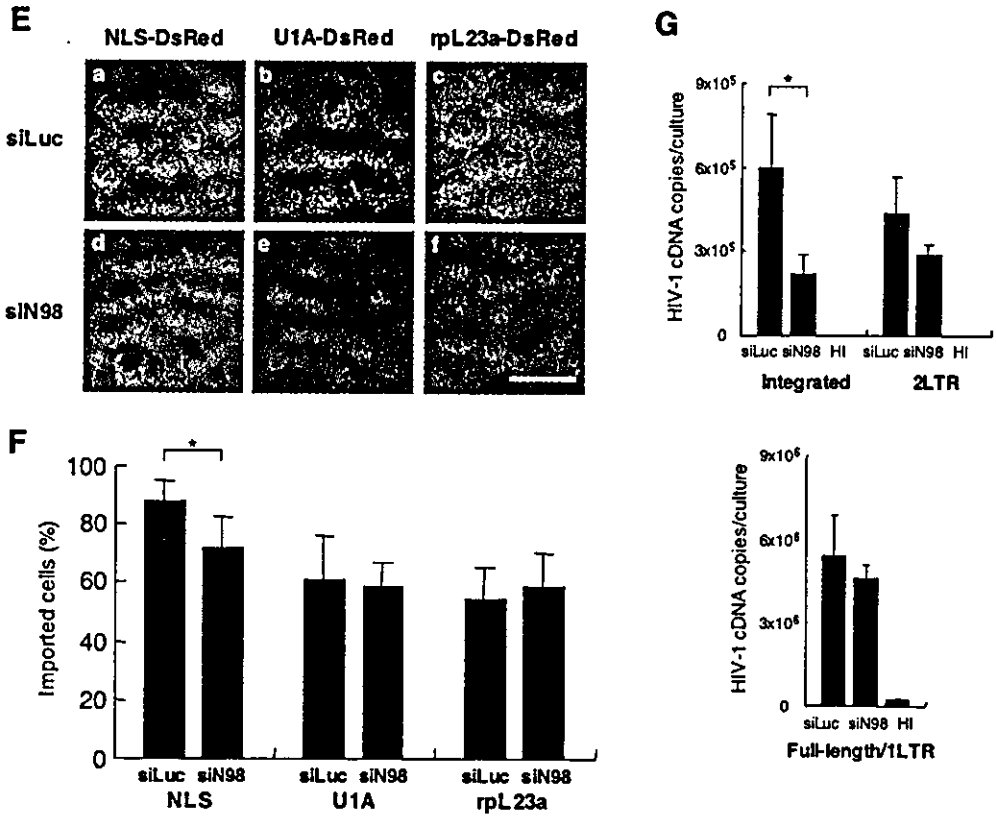

Fig. 3. *(continued)*

that these RanGTP/GDP gradients and localization of Nups are important for the directional active transport of HIV-1 cDNA. In fact, it has been recently reported that importin 7 is a major transport receptor for nuclear import of HIV-1 PIC [15]. Fassati et al. showed that PIC isolated from HIV-1-infected macrophages efficiently entered the nucleus mediated by importin 7 in a Ran- and energy-dependent manner.

Importin 7 is also known as a transport receptor for ribosomal proteins and histone H1 [28,34]. In our experiments, Nup98 depletion hampered the nuclear entry ability mediated by importin α/β, which was examined by NLS. In contrast, the nuclear entry abilities predominantly mediated by importin β3, which was examined by rpL23a, and these transporter-independent pathways, which were examined by U1A, were not affected in the Nup98-depleted NPC. Import of rpL23a was mediated alternatively by multiple transport receptors such as importin β, importin β2 (transportin), importin β3 and importin 7 [28]. It was reported that the mutant pore of Nup98-knockout cells has reduced affinity for many specific transport receptors, especially importin 7 [27]. Thus, it is possible that the functional association of importin 7 and Nup98 may disrupt and Nup98 depletion may induce impairment of the nuclear import pathway predominantly mediated by importin 7. In the present study, we did not succeed in establishing in vitro nuclear import assay of HIV-1 PIC using Nup98-depleted NPC. It will be very interesting to examine whether Nup98 is directly involved in the nuclear import of PIC. Further biochemical analyses, such as experiments showing large complex formation with PIC, importin 7, and Nup98 in infected cells are required.

In conclusion, we have demonstrated in the present study the role of Nup98 in the nuclear entry of HIV-1 cDNA in cultured cells. Our results suggest that a similar mechanism may be operative in HIV-1-infected individuals. Therefore, inhibition of Nup98 function could be a potentially important target for therapeutic intervention in patients with acquired immunodeficiency syndrome.

Acknowledgements

We thank Dr. H. Miyoshi, K. Taira and E. Izaurralde for providing several reagents used in our study. This work was supported by a Grant-in-Aid for Scientific Research on Priority Areas from the Ministry of Education, Culture, Sports, Science, and Technology of Japan; by grants for Research on HIV/AIDS and Health Sciences from the Ministry of Health, Labor and Welfare of Japan. Y. Koyanagi was also supported by the Naito Foundation.

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JOURNAL OF VIROLOGY, Aug. 2004, p. 8654–8662 Vol. 78, No. 16
0022-538X/04/$08.00+0 DOI: 10.1128/JVI.78.16.8654–8662.2004

Spirodiketopiperazine-Based CCR5 Inhibitor Which Preserves CC-Chemokine/CCR5 Interactions and Exerts Potent Activity against R5 Human Immunodeficiency Virus Type 1 In Vitro

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Received 6 January 2004/Accepted 31 March 2004

We identified a novel spirodiketopiperazine (SDP) derivative, AK602/ONO4128/GW873140, which specifically blocked the binding of macrophage inflammatory protein 1α (MIP-1α) to CCR5 with a high affinity (K_d of ≈3 nM), potently blocked human immunodeficiency virus type 1 (HIV-1) gp120/CCR5 binding and exerted potent activity against a wide spectrum of laboratory and primary R5 HIV-1 isolates, including multidrug-resistant HIV-1 (HIV-1_{MDR}) (50% inhibitory concentration values of 0.1 to 0.6 nM) in vitro. AK602 competitively blocked the binding to CCR5 expressed on Chinese hamster ovary cells of two monoclonal antibodies, 45523, directed against multidomain epitopes of CCR5, and 45531, specific against the C-terminal half of the second extracellular loop (ECL2B) of CCR5. AK602, despite its much greater anti-HIV-1 activity than other previously published CCR5 inhibitors, including TAK-779 and SCH-C, preserved RANTES (regulated on activation normal T-cell expressed and secreted) and MIP-1β binding to $CCR5^+$ cells and their functions, including CC-chemokine-induced chemotaxis and CCR5 internalization, while TAK-779 and SCH-C fully blocked the CC-chemokine/CCR5 interactions. Pharmacokinetic studies revealed favorable oral bioavailability in rodents. These data warrant further development of AK602 as a potential therapeutic for HIV-1 infection.

Highly active antiretroviral therapy has had a major impact on the AIDS epidemic in industrially advanced nations (5, 20); however, eradication of human immunodeficiency virus type 1 (HIV-1) appears to be currently impossible, in part due to the viral reservoirs remaining in blood and infected tissues (6, 27). The limitation of antiviral therapy of AIDS is exacerbated by complicated regimens, the development of drug-resistant HIV-1 variants (11), and a number of inherent adverse effects. Successful antiviral drugs, in theory, exert their virus-specific effects by interacting with viral receptors, virally encoded enzymes, viral structural components, viral genes, or their transcripts without disturbing cellular metabolism or function (20). However, at present, no antiretroviral drugs or agents are likely to be completely specific for HIV-1 or to be devoid of toxicity or side effects in the therapy of AIDS, which has been a critical issue because patients with AIDS and its related diseases will have to receive antiretroviral therapy for a long period of time, perhaps for the rest of their lives (6, 27). Thus, the identification of new antiretroviral drugs which have unique mechanisms of action and produce no or minimal side effects remains an important therapeutic objective (20). In this respect, it has been thought that certain chemokine receptor inhibitors might produce no or minimal toxicity.

In the present study, we designed, synthesized, and identified a novel small nonpeptidic CCR5 inhibitor, AK602/ONO4128/GW873140, and related compounds which showed high binding affinity to CCR5, potently inhibited CCR5 gp120 interactions, and had potent HIV-1-specific antiviral activity against laboratory and clinical strains of HIV-1, including highly drug-resistant HIV-1 variants. We describe here the pharmacological characteristics of AK602/ONO4128/GW873140 and its unique feature that, despite the compound's much greater anti-HIV-1 activity compared to previously published CCR5 inhibitors, AK602/ONO4128/GW873140 preserves RANTES and MIP-1β binding to $CCR5^+$ cells and their functions.

MATERIALS AND METHODS

Reagents. Two newly designed and synthesized spirodiketopiperazine (SDP) derivatives, AK530 [(3*S*)-1-but-2-yn-1-yl-3-[(1*S*)-cyclohexylhydroxymethyl]-9-(3,5-dimethyl-1-phenyl-1H-pyrazol-4-ylmethyl)-1,4,9-triazaspiro[5.5]undecane-2,5-dione dihydrochloride] and AK602 [4-[4-[(3*R*)-1-butyl-3-[(1*R*) cyclohexylhydroxymethyl]-2,5-dioxo-1,4,9-triazaspiro[5.5]undec-9-yl methyl]phenoxy]benzoic acid hydrochloride], are discussed in the present report. The methods for their synthesis and physicochemical profiles will be described elsewhere. The structures of these two compounds are shown in Fig. 1. A previously reported prototypic SDP derivative, E913 (17), was used as a reference compound. E921 and

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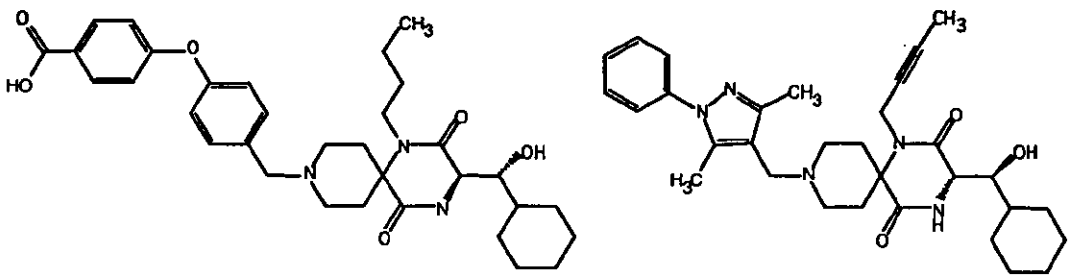

AK602 Mr. 614.2 AK530 Mr. 604.6

FIG. 1. Structures of AK602 and AK530.

AK671, which have the same structures as CCR5 inhibitors TAK-779 and SCH-351125 (SCH-C), respectively, were synthesized as previously described by others (1, 28).

Zidovudine was purchased from Sigma (St. Louis, Mo.). Nelfinavir and saquinavir were provided by Japan Energy (Tokyo, Japan) and Roche Products (Welwyn Garden City, United Kingdom), respectively.

^{125}I-labeled chemokines macrophage inflammatory protein-1α (MIP-1α), macrophage inflammatory protein-1β (MIP-1β), and RANTES were purchased from Amersham Pharmacia Biotech (Little Chalfont, United Kingdom) and PerkinElmer Life Sciences, Inc. (Boston, Mass.), and three corresponding unlabeled chemokines (MIP-1α, MIP-1β, and RANTES) were purchased from PeproTech Inc. (Rocky Hill, N.J.). Recombinant HIV-1_{YU2} gp120 (rgp120) and human soluble CD4 (sCD4) were purchased from Immuno Diagnostics, Inc. (Woburn, Mass.).

Cells, viruses, and anti-HIV-1 assay. Chinese hamster ovary (CHO) cells expressing CCR5 (17) were maintained in Ham's F-12 medium (Gibco-BRL, Rockville, Md.) supplemented with 10% fetal calf serum (JRH Biosciences, Lenaxa, Kans.), 50 U of penicillin per ml, and 50 μg of streptomycin per ml in the presence of 5 μg of blasticidin S hydrochloride per ml. Peripheral blood mononuclear cells were isolated from buffy coats of HIV-1-seronegative individuals with Ficoll-Hypaque density gradient centrifugation and cultured at a concentration of 10^6 cells/ml in RPMI 1640-based culture medium supplemented with 10% fetal calf serum and antibiotics with 10 μg of phytohemagglutinin per ml for 3 days prior to use (phytohemagglutinin-peripheral blood mononuclear cells). Cell line CCR5$^+$ MOLT4 (18) was a kind gift from Yosuke Maeda, Kumamoto University, Japan.

A panel of HIV-1 strains was employed for drug susceptibility assays: HIV $1_{Ba\text{-}L}$ (8), HIV-$1_{JR\text{-}FL}$ (13) HIV-$1_{NL4\text{-}3}$ (34), a wild-type HIV-1_{MOKW} isolated from a drug-naive AIDS patient (17), and two multidrug-resistant (HIV-1_{MDR}) primary HIV-1 strains (HIV-1_{JSL} and HIV-1_{MM}) (36). All primary HIV-1 strains were passaged once or twice in phytohemagglutinin-peripheral blood mononuclear cell cultures, and the culture supernatants were stored at −80°C until use. Antiviral assays with phytohemagglutinin-peripheral blood mononuclear cells were conducted as previously reported (12, 17, 26).

HIV-1 gp120 binding inhibition assays. CCR5$^+$ CHO cells were incubated with rgp120 (5 μg/ml) and sCD4 at 5 μg/ml, biotinylated with EZ-link sulfo-NHS-SS-biotin (Pierce, Rockford, Ill.) in the presence of the indicated concentrations of a CCR5 inhibitor for 1 h at 37°C. Cells were washed, and the binding of the rgp120-sCD4 complex to CCR5$^+$ CHO cells was determined with phycoerythrin-conjugated streptavidin (BD PharMingen, San Diego, Calif.). Nonspecific binding was determined based on the mean florescence intensity of phycoerythrin-conjugated streptavidin with sCD4 but without rgp120. Drug concentrations that brought about 50% inhibition (IC_{50}) of mean fluorescence intensity were then determined.

Generation of ^{3}H-labeled CCR5 inhibitors. Five CCR5 inhibitors, AK530, AK602, E913, E921/TAK-779, and AK671/SCH-C, were tritiated by reductive amination with sodium triacetoxyborotritide (10), methylation with [^{3}H]methyliodide, and heterogeneous catalytic exchange with tritium gas (4). Detailed description of the radiosynthesis of the inhibitors will be presented by J.M. elsewhere. In brief, [^{3}H]E913, [^{3}H]AK530, and [^{3}H]AK602 were prepared by reductive amination of the corresponding aldehyde with piperidine-containing components of each inhibitor with an excess of sodium triacetoxyborotritide, and the tritium label was positioned selectively into the methylene group connecting the two components, generating inhibitors with specific activities of 10.2 Ci/mmol, 17.5 Ci/mmol, and 8.3 Ci/mmol, respectively. [^{3}H]E921/TAK-779 was prepared by methylating the *N*-methyl precursor of E921/TAK-779 with [^{3}H]methyliodide, generating [^{3}H]E921/TAK-779, with a specific activity of 6.1 Ci/mmol. For the preparation of [^{3}H]AK671/SCH-C, methyl-2,4-dimethylpyridine-3-carboxylate was tritiated by an exchange with tritium gas, catalyzed by palladium on carbon in ethanol and triethylamine. Its conversion to N-oxide and alkaline hydrolysis of the resulting ester provided [^{3}H]2,4-dimethyl-pyridine-3-carboxylic acid. Its condensation with *N-tert*-butoxycarbonyl precursor provided [^{3}H]AK671/SCH-C, with a specific activity of 5 Ci/mmol.

Saturation binding assay. CCR5$^+$ CHO cells (1.5×10^5 cells/well) were plated onto 48-well, flat-bottomed culture plates, incubated for 24 h, rinsed with Ham's F-12 medium containing 20 mM HEPES and 0.5% bovine serum albumin (Sigma), exposed to various concentrations of each ^{3}H-labeled CCR5 inhibitor, washed thoroughly with cold phosphate-buffered saline, and lysed with 0.5 ml of 1 N NaOH, and the radioactivity in the lysates was measured. The nonspecific binding of a radiolabeled compound was determined based on the radioactivity detected in the CCR5$^+$ CHO cell-plated wells containing the same amount of the ^{3}H-labled CCR5 inhibitor and a 200-fold greater amount of the corresponding non radiolabeled compound. The K_d (dissociation) values of CCR5 inhibitors and the maximal binding values (B_{max} = number of CCR5/cell) were calculated based on their specific radioactivity with Graphpad Prism software (Intuitive Software for Science, San Diego, Calif.). All assays were performed in duplicate, and the values shown in this report are the arithmetic means (±1 standard deviation) of 3 to 10 independently conducted assays.

Chemokine binding inhibition and chemotaxis inhibition assays. CCR5$^+$ CHO cells (1.5×10^5) were plated onto 48-well microculture plates, incubated for 24 h, rinsed, exposed to 3 nM [^{125}I]MIP-1α, [^{125}I]MIP-1β, or [^{125}I]RANTES in the presence of various concentrations of a CCR5 inhibitor at room temperature for 1 h, thoroughly washed with phosphate-buffered saline, and lysed with 0.5 ml of 1 N NaOH, and their radioactivity was counted. The nonspecific binding of the labeled chemokine to the cells was determined based on the radioactivity detected in the wells plated with the same number of CCR5-negative CHO (CHO-K1) cells exposed to each radiolabeled chemokine (3 nM).

Chemotaxis inhibition assays were conducted with CCR5$^+$ MOLT4 cells and the ChemTx System (Neuro Probe, Inc., Gaithersburg, Md.). In brief, CCR5$^+$ MOLT4 cells were exposed to various concentrations of each CCR5 inhibitor for 30 min, thoroughly rinsed, plated onto the upper chamber of the ChemTx System, exposed to 0.5 nM RANTES contained in the lower chamber, and incubated for 4 h at 37°C, and the number of the cells which migrated from the upper chamber to the lower chamber was determined. Percent chemotaxis was determined with the formula 100 × [(number of CCR5 inhibitor-exposed cells which migrated to the lower chamber in the presence of RANTES) − (number of CCR5 inhibitor-unexposed cells which migrated to the lower chamber in the absence of RANTES)]/[(number of CCR5 inhibitor-unexposed cells which migrated to the lower chamber in the presence of RANTES) − (number of CCR5 inhibitor-unexposed cells which migrated to the lower chamber in the absence of RANTES)].

FACS analysis. Fluorescence-activated cell sorting (FACS) analysis was performed as previously described (17) with minor modifications. Briefly, CCR5$^+$ CHO cells (3×10^5) were stained with a phycoerythrin- or fluorescein isothiocyanate-conjugated anti-CCR5 monoclonal antibody 2D7 (BD PharMingen, San Diego, Calif.) or 45523 or 45531 (R&D Systems, Minneapolis, Minn.), with or without a test CCR5 inhibitor, washed, and examined with an Epics XL (Beckman Coulter, Fullerton, Calif.).

RESULTS

Potent activity of AK602 against R5 wild-type and multidrug-resistant R5 HIV-1. We have previously reported that a prototypic SDP derivative, E913, was active against R5 HIV-1 in vitro, with IC_{50} values of 30 to 60 nM as tested in target phytohemagglutinin-treated peripheral blood mononuclear cells (17). Following optimization for increased potency against R5 HIV-1 and favorable pharmacokinetic features, we identified AK602 as the most potent agent among newly designed and synthesized SDP derivatives. AK602 exerted potent activity against three wild-type R5 HIV-1 strains (HIV-$1_{Ba\text{-}L}$, HIV $1_{JR\text{-}FL}$ and HIV-1_{MOKW}) with IC_{50} values of 0.1 to 0.4 nM (Table 1). It was of note that AK602 was substantially more potent than two previously published CCR5 inhibitors, E921/TAK-779 and AK671/SCH-C (1, 28).

During the extended study of the antiviral activity of the prototypic E913, we noted that its activity against R5 HIV-$1_{Ba\text{-}L}$ in vitro varied substantially; the range of IC_{50} values spanned from 14 to 650 nM (Fig. 2). When we tested the activity of E921/TAK-779 in phytohemagglutinin-treated peripheral blood mononuclear cells from multiple seronegative donors, its variability was also substantial: its IC_{50} values varied from 2 to 200 nM. However, when we tested AK602, the variability of AK602's anti-HIV-1 activity was limited and similar to that seen for zidovudine. The difference in the range of the CCR5 inhibitor's IC_{50} values seems to correlate with the potency of the inhibitor examined. Indeed, we have seen a greater variability in the antiviral activity of the prototypic E913 (Fig. 2). Moreover, AK602 suppressed the infectivity and replication of two HIV-1_{MDR} variants, HIV-1_{MM} and HIV-1_{JSL} (36), at extremely low concentrations (IC_{50} values of 0.4 to 0.6 nM), while these two R5 HIV-1 variants were less susceptible to zidovudine, nelfinavir, and saquinavir (IC_{50} values were greater by factors of 10 to 36, >83, and 27 to 32, respectively, compared to those against HIV-$1_{Ba\text{-}L}$). As expected, none of these CCR5 inhibitors suppressed the infectivity and replication of X4 HIV-$1_{NL4\text{-}3}$ in vitro. Although certain CC-chemokines reportedly enhance the replication of X4 HIV-1 (19, 22), no such enhancement of X4 HIV-1 replication was seen with the CCR5 inhibitors examined in this study at concentrations of up to 1 μM (data not shown).

CCR5 binding properties of SDP derivatives. We determined the CCR5 binding profiles of SDP derivatives and compared them with those of previously published CCR5 inhibitors in saturation binding assays employing ^{3}H-labeled compounds. Figure 3A depicts the CCR5 binding profile of AK602, showing that it binds with high affinity to CCR5. The K_d values thus determined for AK602, E913, E921/TAK-779, and AK671/SCH-C were 2.9 ± 1.0 (Fig. 3A), 111.7 ± 3.5, 32.2 ± 9.6, and 16.0 ± 1.5 nM (data not shown), respectively.

We also asked whether the SDP derivatives blocked the binding to CCR5 of rgp120 following exposure to sCD4. As shown in Fig. 3B, AK602 potently blocked rgp120/sCD4 binding to CCR5 with an IC_{50} value of 2.7 nM, followed by E921/TAK-779 and AK-671/SCH-C, with IC_{50} values of 12.0 and 16.5 nM, respectively. When we asked whether AK602 blocked the intracellular Ca^{2+} mobilization induced by MIP-1α, MDC, SDF-1α, and MCP-1, whose primary receptors are CCR5, CCR4, CXCR4, and CCR2, respectively, with the method we

TABLE 1. Anti-HIV-1 activity of SDP derivatives

Compound	Mean IC_{50} (IC_{90}) ± SD in p24 assay (nM)						CC_{50}[a] (μM)
	HIV-$1_{Ba\text{-}L}$ (R5)	HIV-$1_{JR\text{-}FL}$ (R5)	HIV-1_{MOKW}[b] (R5)	HIV-1_{MM}[b] ($R5_{MDR}$)	HIV-1_{JSL}[b] ($R5_{MDR}$)	HIV-$1_{NL4\text{-}3}$ (X4)	
AK602	0.4 ± 0.3 (12 ± 10)	0.1 ± 0.1 (4 ± 2)	0.2 ± 0.1 (5 ± 3)	0.6 ± 0.2 (11 ± 2)	0.4 ± 0.3 (7 ± 2)	>1,000	50
AK530	32 ± 27 (324 ± 120)	13 ± 4 (144 ± 60)	NT[c]	ND	ND	>1,000	60
E913	82 ± 58 (709 ± 256)	81 ± 46 (>1,000)	51 ± 14 (941 ± 201)	61 ± 28 (>1,000)	64 ± 30 (713 ± 405)	>1,000	50
E921/TAK-779	28 ± 32 (256 ± 169)	5 ± 1 (237 ± 25)	11 ± 7 (194 ± 168)	14 ± 8 (352 ± 180)	7 ± 4 (316 ± 151)	>1,000	50
AK671/SCH-C	4 ± 2 (79 ± 52)	2 ± 0.5 (56 ± 57)	2 ± 1 (54 ± 20)	3 ± 0.5 (138 ± 25)	2 ± 0.3 (84 ± 18)	>1,000	>100
Zidovudine	7 ± 4 (48 ± 21)	10 ± 9 (157 ± 72)	6 ± 5 (47 ± 20)	250 ± 98 (>1,000)	70 ± 64 (>1,000)	11 ± 5 (181 ± 90)	>100
Nelfinavir	12 ± 8 (105 ± 48)	ND	14 ± 8 (82 ± 56)	>1,000	>1,000	20 ± 7 (75 ± 52)	ND
Saquinavir	11 ± 5 (60 ± 21)	ND	5 ± 2 (49 ± 40)	300 ± 65 (>1,000)	350 ± 105 (>1,000)	10 ± 4 (48 ± 2)	ND

[a] Cytotoxic concentrations of a compound that reduces the number of cells by 50% (CC_{50}) were determined as previously reported (17).
[b] HIV-1_{MOKW} was isolated from a drug-naive AIDS patient (17), while HIV-1_{MM} and HIV-1_{JSL} were from patients who received antiretroviral therapy for a long period and whose virus acquired a number of mutations in the RT- and PR-encoding HIV-1 genes (36).
[c] ND, not determined

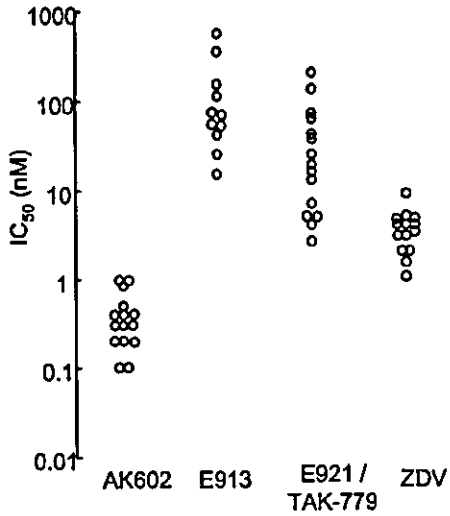

FIG. 2. Variability of anti-HIV-1 activity of AK602 in phytohemagglutinin-peripheral blood mononuclear cells. The range of IC_{50} values of E913 and E921/TAK-779 against HIV-1_{Ba-L} varied substantially when examined in multiple phytohemagglutinin-peripheral blood mononuclear cells as target cells, 14 to 650 nM (n = 11) and 2 to 200 nM (n = 15), respectively, while that of AK602 was relatively narrow, 0.1 to 1 nM (n = 15), similar to that of zidovudine (ZDV), 1 to 9 nM (n = 14).

published previously (17), AK602 completely blocked MIP-1α-induced Ca^{2+} mobilization at 0.1 μM and beyond; however, it failed to block Ca^{2+} mobilization induced with MDC, SDF-1α, and MCP-1 (data not shown).

We also attempted to illustrate where AK602 binds on the CCR5 molecule by employing several monoclonal antibodies known to bind to different domains of CCR5. FACS analyses revealed that there was no AK602 inhibition of the binding of monoclonal antibody 2D7, known to bind to the N-terminal half (or domain A) of the second extracellular loop of CCR5 (14) (Fig. 4). In contrast, AK602 competitively blocked the binding of two different monoclonal antibodies, 45523, reportedly directed against multidomain epitopes of CCR5, and 45531, which is known to be specific against the C-terminal half (or domain B) of the second extracellular loop (ECL2B) of CCR5 (14), as examined with $CCR5^+$ CHO cells (Fig. 4). These data suggest that the potent activity of AK602 against R5 HIV-1 stems from its binding to ECL2B and/or its vicinity with high affinity, resulting in inhibition of gp120/CD4 binding to CCR5. It was of note, however, that another SDP derivative, AK530, whose antiviral activity was moderate (the IC_{50} value against HIV-1_{Ba-L} was 32 nM; Table 1), whose rgp120/sCD4 binding inhibition was the lowest among the inhibitors examined (IC_{50}, 280 nM; Fig. 3B), and had only a moderate effect on the binding of monoclonal antibody 45531 to $CCR5^+$ cells (data not shown), had the highest binding affinity to CCR5 (K_d value, 0.4 nM; data not shown) among the SDP derivatives, suggesting that the binding pocket (or subsite) of certain SDP derivatives (such as AK530) does not quite overlap that of AK602.

SDP derivatives bind to CCR5 but permit RANTES and MIP-1β to bind to CCR5. We asked whether SDP derivatives blocked the binding of CC-chemokines to CCR5 expressed on the surface of CHO cells with [^{125}I]RANTES, [^{125}I]MIP-1α, and [^{125}I]MIP-1β and CCR5 inhibitors AK602, AK530, E921/TAK-779, and AK671/SCH-C. As shown in Fig. 5A, the concentrations of E921/TAK-779 and AK671/SCH-C which

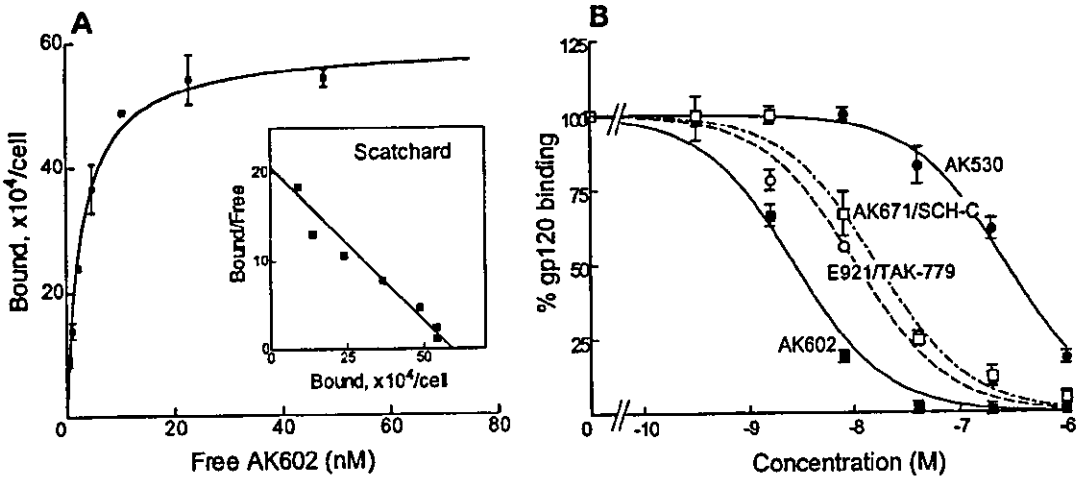

FIG. 3. CCR5 binding profiles and rgp120 binding blocking of various CCR5 inhibitors. (A) Binding affinity of AK602 to CCR5. $CCR5^+$ CHO cells were incubated with the ^{3}H-labeled CCR5 inhibitors AK530, AK602, E913, E921/TAK-779, and AK671/SCH-C for 1 h. Following thorough washing, cells were lysed, the radioactivity in the lysates was determined, and B_{max} and K_d values were calculated. The K_d values thus obtained were 0.4 ± 0.4, 2.9 ± 1.0, 111.7 ± 3.5, 32.2 ± 9.6, and 16.0 ± 1.5 nM, respectively. All assays were independently performed 3 to 10 times, and the values represent the arithmetic means ± 1 standard deviation. (B) AK602 potently blocks the binding of rgp120/sCD4 to CCR5. $CCR5^+$ CHO cells were incubated with rgp120 (5 μg/ml) and sCD4 (5 μg/ml) in the presence or absence of the indicated concentrations of CCR5 inhibitors, and the binding of rgp120/sCD4 complex to $CCR5^+$ CHO cells was determined. The 50% binding inhibition (EC_{50}) value was determined based on the mean fluorescence intensity values obtained with or without CCR5 inhibitors. EC_{50} values for AK602, AK530, E921/TAK-779, and AK671/SCH-C were 2.7, 280, 12.0, and 16.5 nM, respectively.

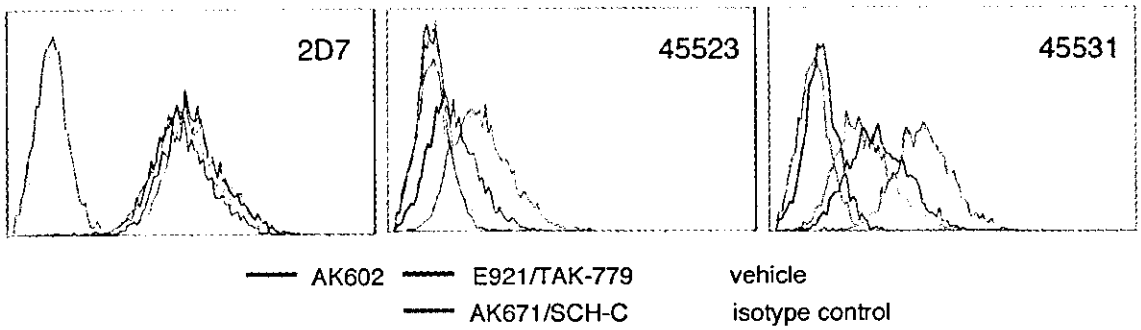

FIG. 4. AK602 binds to the second extracellular loop of CCR5. AK602 at 100 nM almost completely inhibited the binding of two monoclonal antibodies, 45523, directed against multidomain epitopes of CCR5, and 45531, recognizing ECL2B of CCR5. In contrast, E921/TAK-779 and AK671/SCH-C moderately blocked the binding of 45523 and 45531. Note that there was no AK602 inhibition of the binding of a monoclonal antibody 2D7, which is known to bind to domain A of ECL2 of CCR5.

blocked RANTES binding to CCR5 by 50% (IC_{50}) were 110 and 40 nM, respectively, and RANTES binding was completely blocked in the presence of ≥10 μM E921/TAK-779 or AK671/SCH-C. In contrast, AK602 only partially blocked RANTES binding to CCR5 by 40% even at 10 μM (Fig. 5A). The binding of MIP-1β to CCR5 was also completely blocked by E921/TAK-779 and AK671/SCH-C; however, AK602 failed to completely block MIP-1β binding (Fig. 5B). The MIP-1β binding value in the presence of 10 μM AK602 was 10%, and no further blockade occurred at higher concentrations up to 40 μM (data not shown). AK530 also failed to completely block the binding of RANTES and MIP-1β to CCR5.

These data suggest that the binding pockets (or subsites) of CCR5 for SDP derivatives only partially overlap the CC-chemokine binding sites of CCR5 or that the conformational changes ensuing the binding of SDP derivatives to CCR5 have only moderate effects on the binding of RANTES and MIP-1β. In the initial search for CCR5 inhibitors, lead compounds were sought as those inhibiting MIP-1α binding to CCR5 and MIP-1α-driven cytosolic Ca^{2+} flux, and thus, as expected, AK602 blocked MIP-1α binding to CCR5 although AK530 was substantially less potent in blocking MIP-1α binding (Fig. 5C). E921/TAK-779 and AK671/SCH-C were also found to completely block MIP-1α binding to CCR5 (Fig. 5C).

AK602 and RANTES bind simultaneously to CCR5. As described above, AK602 and AK530 only partially inhibited RANTES binding to $CCR5^+$ CHO cells; however, it was not clear whether those SDP derivatives and RANTES bound simultaneously to CCR5. Therefore, competitive binding assays employing ^3H-labeled and unlabeled AK602 and ^{125}I-labeled and unlabeled RANTES were conducted. As shown in Fig. 6A, the binding of [^3H]AK602 (10 nM) to CCR5 was only partially inhibited by ≥4 nM RANTES. Also, the binding of [^{125}I]RANTES at 8 nM was only inhibited by up to 20% in the presence of 10 nM AK602 (Fig. 6B).

The interpretation that AK602 and RANTES bind simultaneously to CCR5 was corroborated by another experiment in which a lower concentration of [^3H]AK602 and much higher concentrations of RANTES were used (Fig. 6A, inset). The radioactivity counted for [^3H]AK602 (5 nM) bound to $CCR5^+$ CHO cells was only moderately blocked in the presence of 100 and 1,000 nM RANTES, by 32 and 46%, respectively (Fig. 6A, inset). These data suggest that the SDP derivatives, in particular AK602, and RANTES bind simultaneously to CCR5, al-

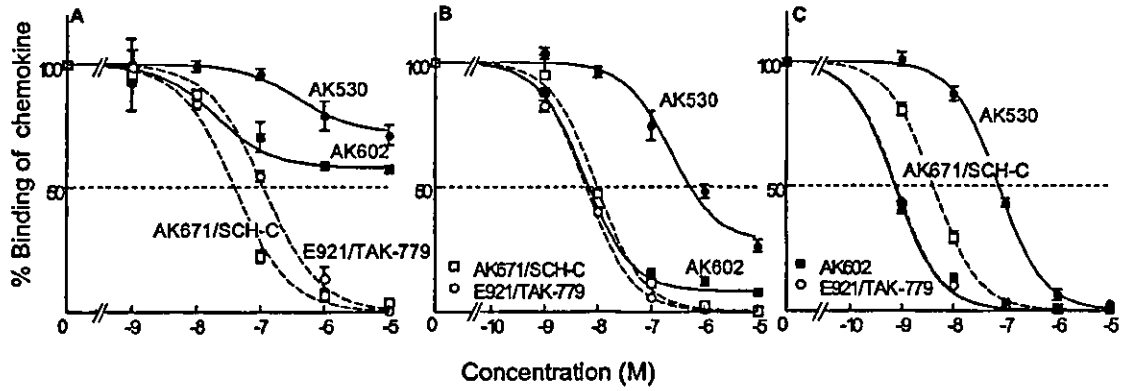

FIG. 5. Inhibition of CC-chemokine binding to CCR5 by various CCR5 inhibitors. $CCR5^+$ CHO cells were incubated with 3 nM [^{125}I]RANTES (A), [^{125}I]MIP 1 β (B), or [^{125}I]MIP-1 α (Pnel C) in the presence and absence of various concentrations of CCR5 inhibitors. Note that while AK671/SCH-C and E921/TAK-779 completely inhibited the binding of [^{125}I]RANTES, [^{125}I]MIP-1α, and [^{125}I]MIP-1β to CCR5, SDP derivatives partially blocked RANTES (A) and MIP-1β (B) binding, although they completely blocked MIP-1α binding (C).

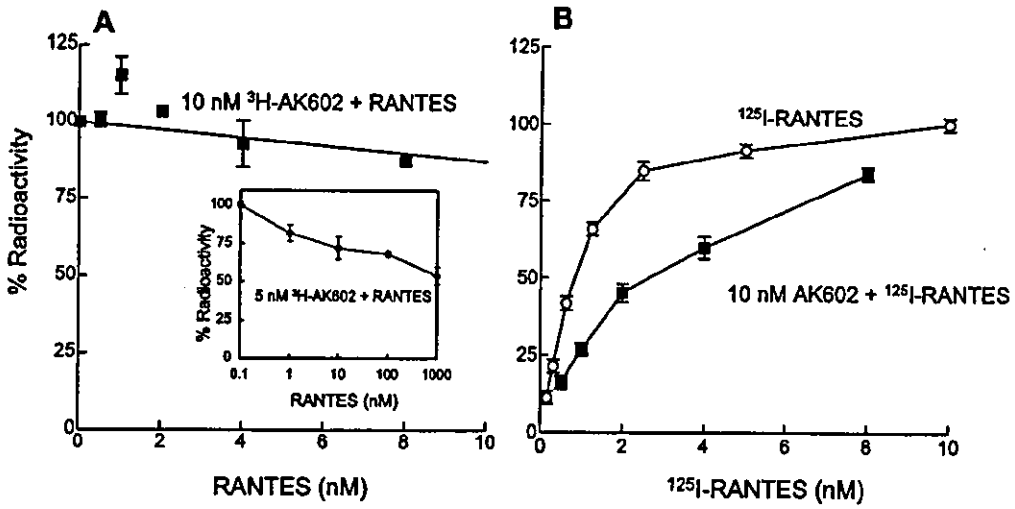

FIG. 6. AK602 and RANTES bind simultaneously to CCR5. (A) CCR5$^+$ CHO cells were exposed to 10 nM [^{3}H]AK602 and various concentrations of unlabeled RANTES. After 1 h of incubation, the cells were washed, and the [^{3}H]AK602 bound to the cells was measured. Note that 100% radioactivity on the ordinate denotes the radioactivity of cell-bound [^{3}H]AK602 without RANTES and that ≈90% of CCR5 molecules are bound to AK602 at 10 nM (Fig. 3A). (B) CCR5$^+$ CHO cells were exposed to 10 nM unlabeled AK602 and various concentrations of [^{125}I]RANTES. After 1 h of incubation, the cells were washed, and the [^{125}I]RANTES bound to the cells was measured. The binding profile of [^{125}I]RANTES alone is illustrated by open circles. Note that 100% radioactivity is equated to the radioactivity of cell-bound [^{125}I]RANTES at 10 nM. The K_d values of RANTES in the presence and absence of 10 nM AK602 were 4.5 and 0.6 nM, respectively.

though conformational changes potentially caused by either of the two might have occurred. Indeed, 15 to 25% inhibition was seen at nearly equimolar concentrations of AK602 and RANTES, which may reflect the involvement of the conformational changes caused by either of the two agents or an overlap in their binding sites (or domains).

AK602 permits RANTES-induced chemotaxis and CCR5 internalization at anti-HIV-1 activity-exerting concentrations. We next asked whether AK602 allowed RANTES-induced chemotaxis and CCR5 internalization with CCR5$^+$ MOLT4 cells and CCR5$^+$ CHO cells at its anti-HIV-1 activity-exerting concentrations. As shown in Fig. 7A, AK671/SCH-C most potently blocked chemotaxis, followed by E921/TAK-779. The chemotaxis values at the IC_{50}s against R5 HIV-1_{Ba-L} of AK671/SCH-C and E921/TAK-779 (4 and 24 nM, respectively: Table 1) were low, 18 and 8%, respectively, suggesting that these two inhibitors considerably blocked chemotaxis at their anti-HIV-1 IC_{50} concentrations as determined in peripheral blood mononuclear cells. In contrast, the chemotaxis seen at the IC_{50} level of AK602, 0.4 nM (see Table 1), was considerable, with 70% retained (Fig. 7A), while that seen AK530 was much less (30%).

In order to corroborate the modest chemotaxis inhibition seen with AK602, the inhibition of RANTES-induced CCR5 internalization was also examined. In the absence of CCR5 inhibitors, ≈50% of CCR5 molecules were internalized from the surface of CCR5$^+$ CHO cells incubated for 1 h at 37°C in the presence of 10 nM RANTES; however, AK671/SCH-C and E921/TAK-779 at 100 nM considerably blocked internalization, and only 19 and 6%, respectively, of CCR5 molecules were internalized. In the presence of higher concentrations of AK671/SCH-C and E921/TAK-779, 300 and 1,000 nM, virtually no CCR5 internalization occurred (Fig. 7B). In contrast, AK530 and AK602 at 100 nM allowed RANTES-induced CCR5 internalization of 46 and 30%, respectively, and even at 300 and 1,000 nM, 10 to 34% CCR5 internalization occurred (Fig. 7B).

DISCUSSION

A novel SDP derivative, AK602/ONO4128/GW873140, exhibited high affinity to CCR5, blocked rgp120/sCD4 complex binding to CCR5, and exerted potent activity against a wide spectrum of laboratory and primary R5 HIV-1 isolates, including HIV-1_{MDR}. We recently examined AK602 against several non-clade B R5 HIV strains and found that in general AK602 is comparably active against such non-clade B strains (data not shown). It is of note that several small-molecule CCR5 inhibitors have been reported in the literature, including SCH-D (D. Schurmann et al., Abstr. 11th Conf. Retroviruses Opportunistic Infections, 2004, abstr. 140LB), UK427,857 (A. L. Pozniak et al. Abstr. 43rd Intersci. Conf. Antimicrob. Agents Chemother., 2003, abstr. H-443), CMPD167 (32), and TAK-220 (Y. Iizawa et al., Abstr. 10th Conf. Retroviruses Opportunistic Infections, 2003, abstr. 11).

In the present study, we also demonstrated that AK602 potently blocked rgp120/sCD4 complex binding to CCR5. With respect to gp120/CD4 binding to CCR5, Olson et al. previously reported no correlation between fusion with and entry into the target cell of HIV-1 and inhibition of rgp120/sCD4 complex binding to CCR5, based on data with various anti-CCR5 monoclonal antibodies (24). However, with all small-molecule SDP derivatives examined in the present study, inhibition of HIV-1 infectivity and replication generally correlated with inhibition of the rgp120/sCD4 complex binding to CCR5, strongly suggesting that the anti-HIV-1 activity of SDP

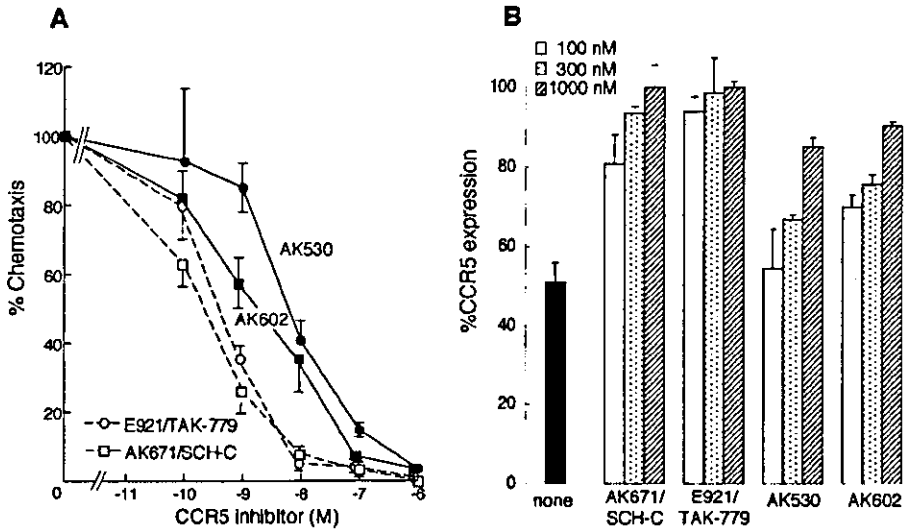

FIG. 7. AK602 allows RANTES-induced chemotaxis and CCR5 internalization. (A) CCR5$^+$ MOLT4 cells were exposed to various concentrations of AK530, AK602, E921/TAK-779, or AK671/SCH-C, thoroughly washed, plated onto the upper chamber of the ChemTx System, exposed to 0.5 nM RANTES contained in the lower chamber, and incubated for 4 h; the number of the cells which migrated to the lower chamber was determined, and chemotaxis was calculated. (B) CCR5$^+$ CHO cells were exposed to 10 nM RANTES in the presence or absence of various concentrations of each CCR5 inhibitor and washed with acidic solution for removal of the cell-bound RANTES (21). The amount of cell surface CCR5 was subsequently determined with monoclonal antibody 3A9 (BD PharMingen), which recognizes the N terminus of CCR5 and competes with none of the CCR5 inhibitors tested. In panel A, the level of chemotaxis suppression by TAK-779 and SCH C was greater than that by AK530 and AK602 at four concentrations examined, although complete suppression was seen only at the highest concentration of the AK compounds, 1 μM. However, in panel B, the level of CCR5 internalization suppression by TAK-779 and SCH-C was greater than that of the AK compounds at all three concentrations examined.

derivatives stems from their inhibition of gp120 binding to CCR5, as reported for other CCR5 inhibitors such as TAK-779 (3), although the binding pocket (or subsite) of CCR5 for certain SDP derivatives (such as AK530) apparently does not quite overlap the rgp120/sCD4 complex binding site of CCR5 (Fig. 3B). It is also possible that the conformational changes ensuing upon AK602's binding to CCR5 could differ from that ensuing upon AK530's binding to CCR5, thereby producing differences in gp120/sCD4 binding and anti-HIV activity.

It is generally noted that although the determination of any binding sites with antibodies provides "indirect" evidence, in many cases it gives good insights (14). Indeed, SCH-C has been reported to induce conformational changes in CCR5 and bind to its transmembrane (TM) domain, thereby blocking HIV-gp120 binding to CCR5. In our data, SCH-C completely blocked the binding of the "multidomain"-reactive monoclonal antibody 45523, which reportedly causes conformational changes in CCR5, while it only moderately blocked the binding of the ECL2B-specific monoclonal antibody 45531 (Fig. 4). In contrast, AK602 completely blocked the binding of both 45523 and 45531. Considering that monoclonal antibody 45531's CCR5 binding is closely linked to amino acids 184 to 189 of ECL2B, as shown by Lee and colleagues (14), it was thought that the binding site of AK602 includes ECL2B or is vicinal to it. Indeed, our recent analysis with the alanine-scanning algorithm showed that AK602 totally failed to bind to a CCR5 mutant when a K191A substitution was introduced (Maeda et al., unpublished data), corroborating and extending the idea that AK602's binding site involves the ECL2B domain.

It is noted that the IC_{50} of AK602 against HIV-1 as determined in peripheral blood mononuclear cells (0.4, 0.1, and 0.2 nM against HIV-1_{Ba-L}, HIV-1_{JR-FL}, and HIV-1_{MOKW}, respectively: Table 1) are substantially lower than the K_d of AK602 (2.9 nM) and the IC_{50} of AK602 for its inhibition of rgp120/sCD4 complex binding to CCR5 (2.7 nM). The anti-HIV-1 IC_{50}s of AK602 are also lower than the IC_{50}s of AK602 for its inhibition of MIP-1α-induced Ca^{2+} influx (39.8 nM; unpublished data) and that for its inhibition of CCR5 internalization (~300 nM; unpublished data).

One possible explanation for these inconsistencies is the different cell lines employed for each assay. However, it is of note that when we determined the IC_{50} values against several R5 HIV strains and K_d values of AK602 in MAGI/CCR5 cells (18), AK602's IC_{50}s (~0.2 nM) were reproducibly lower than AK602's K_d (3.8 nM) (data not shown). Thus, one can postulate that for the inhibition of HIV-1 infection by CCR5 inhibitors, not all CCR5 molecules might have to be occupied. In this regard, our studies with ^{3}H-labeled AK602 and CD4$^+$ target cells expressing CCR5, MAGI/CCR5 (18) and U373-MAGI (34), have shown that less than 30% of HIV-1 infection occurred when approximately 50% of CCR5 molecules were bound by AK602, and at its anti-HIV-1 IC_{50} concentration, AK602 was found to bind to 5 to 20% of CCR5 molecules on the target cells (Maeda et al., unpublished data). These data suggest that when one of the multimerized CCR5 molecules is bound or occupied by AK602, inhibition of the cell is likely to be blocked, although further stoichiometric analyses need to be conducted.

It has been thought that individuals carrying a gene encoding a mutant form of CCR5 called Delta32 are resistant to HIV-1

infection and apparently do not have significant health problems (2, 15, 23, 25). One can assume that individuals with homozygous CCR5-Delta32 might inherently have certain defenses which could compensate for the deficiency of CCR5. In this regard, there has been a report that individuals carrying homozygous CCR5-Delta32 have longer survival of renal transplants than those with other genotypes, suggesting that such individuals might have compromised graft rejection immunity (7). Moreover, Woitas et al. have reported that individuals with homozygous CCR5-Delta32 have significantly higher levels of hepatitis C virus in blood than their counterparts who have wild-type CCR5, suggesting that the CCR5-Delta32 mutation may be an adverse host factor in hepatitis C virus infection (35), although others have recently argued against a role of CCR5 in susceptibility to hepatitis C virus infection or response to antiviral therapy (9). Thus, sustained, long-term suppression of the effect of CC-chemokines/CCR5 interactions, in particular in those who carry wild-type CCR5 and might not have a possible compensatory mechanism for the absence of CCR5, might produce adverse effects, and caution should be used in the development of chemokine receptor antagonists as potential therapeutics for HIV-1 infection.

In this respect, SDP derivatives such as AK602 can preserve CC-chemokine/CCR5 interactions at their anti-HIV activity-exerting concentrations; they allow RANTES and MIP-1β binding to $CCR5^+$ cells and their functions at anti-HIV-1 concentrations. In contrast, two previously published CCR5 inhibitors, TAK-779 and SCH-C, fully blocked CC-chemokine/CCR5 interactions (Fig. 5 and 7). It is of note that AK602's complete inhibition of the binding of MIP-1α was not surprising because in the initial search of lead compounds, we sought compounds that blocked the binding of ^{125}I-labeled MIP-1α to $CCR5^+$ CHO cells and MIP-1α-elicited cellular Ca^{2+} mobilization, as described previously (17).

In support of the above observation, the results of competitive biding assays with [^{3}H]AK602 and [^{125}I]RANTES and their corresponding unlabeled agents clearly indicated that AK602 and RANTES bind simultaneously to CCR5 (Fig. 6). Moreover, AK602 allowed $CCR5^+$ MOLT4 cells to undergo RANTES-elicited chemotaxis (Fig. 7A) and $CCR5^+$ CHO cells to internalize CCR5 in response to RANTES (Fig. 7B) at concentrations much greater than AK602's anti-HIV-1 activity-exerting concentration in peripheral blood mononuclear cells. However, it is worth noting that although AK602 blocked the binding of [^{125}I]RANTES to $CCR5^+$ CHO cells only by ≈40% at micromolar concentrations (Fig. 5A), it virtually completely blocked the RANTES-induced chemotaxis at micromolar concentrations, as examined in $CCR5^+$ MOLT4 cells (Fig. 7A). This apparent inconsistency could be explained by the different cell lines employed for each assay and the fact that the number of CCR5 molecules in $CCR5^+$ CHO cells (≈5 × 10^5/cell) is substantially different from that of $CCR5^+$ MOLT4 cells (≈1 × 10^5/cell), and thus, AK602 could more efficiently block the chemotaxis of MOLT4 cells. It is also possible that AK602 may more effectively block CCR5 multimerization, which is reportedly important for the functionality of the G protein-coupled receptor (29), rather than the RANTES binding block to CCR5 per se. However, it is not clear yet whether AK602's unique profile that AK602 partially allows RANTES and MIP-1β to bind to CCR5 despite AK602's tight binding to CCR5 brings about a clinical advantage. This can be examined only in the setting of clinical trials and careful clinical investigation in long-term treatment with such an agent.

Several HIV-1 variants which acquired resistance to CC-chemokines, including MIP-1α and CCR5 inhibitors, have been reported. Trkola et al. described that when HIV-1 was passaged in the presence of increasing concentrations of a CCR5-specific, structurally SCH-C-related CCR5 inhibitor, AD101, an escape mutant which contained 22 amino acid substitutions in the gp120 subunits emerged as early as after 19 passages (31). This escape mutant showed a >20,000-fold resistance to AD101 and was similarly resistant to SCH-C compared with wild-type HIV-1, suggesting that HIV-1 can acquire the capability of using CCR5 bound to certain classes of CCR5 inhibitors for its entry into the target cell (31). Maeda et al. reported that HIV-$1_{JR\text{-}FL}$, following in vitro selection against MIP-1α over 3 months, acquired amino acid substitutions in the V2 and V3 regions of HIV-1 gp120 and became four- to sixfold more resistant to MIP-1α, MIP-1β, and RANTES (18). In this regard, as of this writing, we have passaged HIV-$1_{Ba\text{-}L}$ in $CD4^+$ $CCR5^+$ PM1 cells (16) in the presence of moderately increasing concentrations of AK602 in one selection experiment and aggressively increasing concentrations of AK602 in another selection experiment over 22 months (45 passages); however, the virus has acquired no detectable resistance to AK602 and no significant amino acid substitutions (Nakata et al., unpublished data).

It is worth noting that the anti-HIV-1 activity of AK602 is virtually unaffected by the presence of human serum proteins. For instance, the IC_{50} of AK602 against HIV-$1_{Ba\text{-}L}$ in the presence of 10% fetal calf serum in culture medium was 0.4 ± 0.3 nM, while those of AK602 with 10 μM α1-acid glycoprotein and 45% human serum added to the culture medium were 0.8 ± 0.3 and 0.7 ± 0.7 nM, respectively. AK602 failed to induce Ca^{2+} flux, chemotaxis, or CCR5 internalization in $CCR5^+$ cells (Maeda et al., unpublished data). As far as the sensitivities of our methods used in the present work, AK602 is to be categorized as a nonagonist or antagonist. The phase 1 clinical trial of AK602 in HIV-1-seronegative individuals has recently been concluded, and no significant adverse effects have been documented. Considering that AK602 potently inhibited the replication of HIV-1 in vitro and in a nonobese diabetic-SCID mouse model (Nakata et al., unpublished data) and that AK602 has a favorable oral bioavailability in rodents, averaging 20 to 30% (unpublished data), the present data strongly suggest that AK602 is a promising CCR5 inhibitor as a potential therapeutic for HIV-1 infection.

ACKNOWLEDGMENTS

We thank Steve LaFon, Larry Boone, Jim Demarest, Eddy Arnold, Shigeyoshi Harada, Kazuhisa Yoshimura, and Yosuke Maeda for helpful discussion and critical reading of the manuscript.

This work was supported in part by a grant from the Research for the Future Program (JSPS-RFTF 97L00705) of the Japan Society for the Promotion of Science, a Grant-in-Aid for Scientific Research (Priority Areas) from the Ministry of Education, Culture, Sports, Science, and Technology of Japan (Monbu-Kagakusho), and a Grant for the Promotion of AIDS Research from the Ministry of Health, Welfare, and Labor of Japan (Kosei-Rohdosho).

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JOURNAL OF VIROLOGY, Oct. 2004, p. 11352–11359
0022-538X/04/$08.00+0 DOI: 10.1128/JVI.78.20.11352–11359.2004

Vol. 78, No. 20

A Lentiviral cDNA Library Employing Lambda Recombination Used To Clone an Inhibitor of Human Immunodeficiency Virus Type 1-Induced Cell Death

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Received 17 November 2003/Accepted 27 May 2004

Expression cloning technology of cDNAs is a suitable tool for identifying novel functional properties of genes. Here, we generated a lentiviral cDNA library-expressing system for human T cells based on a site-specific recombination system of phage lambda for transferring cDNA libraries with a minimum loss of its complexity. The library-transduced $CD4^+$ T cells were challenged with wild-type human immunodeficiency virus type 1 (HIV-1), and the cells that acquired resistance to HIV-1-induced cytopathic effect (CPE) were selected. From these cells, CD14 was isolated and proved to inhibit the entry of HIV-1 and the HIV-1-induced CPE. This cloning system allows rapid identification of genes encoding novel properties in human T cells and probably other mammalian cells.

A number of screening systems from genetic libraries have been developed to identify novel functional properties of the genes. A successful screening with mammalian cells is dependent on the efficiency of the transduction system into the appropriate target cells. A plasmid-based expression system has been generally used (1, 2, 17). However, this system has a limit due to the inefficient transfection into particular cells, such as nonadherent cells. In addition, the introduced genes are expressed only transiently. Therefore, it is desirable to develop a new technology that can efficiently achieve long-lasting expression of genetic information in the nonadherent cells, especially human lymphocytes. Retrovirus vectors appear to overcome these limits (16). Retrovirus infects a wide range of mammalian cell types, including lymphocytes, with a high efficiency. The library-inserted retrovirus vector can integrate into the host's chromosome and is expressed permanently. These properties have been utilized for a gene delivery system for lymphocytes (16). However, the prototype murine leukemia virus-based retrovirus vector infects only dividing cells (12) and, less efficiently, human T cells. Thus, the target cells for screening are limited. Recently, a human immunodeficiency virus (HIV)-based lentivirus vector was developed (12), and such vectors are beginning to be used in many applications (4, 7, 10, 11).

In this report, we describe the development of a lentiviral cDNA library expression system applicable for human T cells. The results showed significant utility of the system to clone genes through a high-throughput screening procedure. This system allowed us to identify genes that render cells resistant to HIV-induced cell death. Our lentivirus system is promising, as it can be applied to many library screening systems, and should accelerate the discovery of novel properties of the genes in many other cells including neurons and hematopoietic stem cells.

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MATERIALS AND METHODS

Cells. Human 293T were maintained in Dulbecco's modified Eagle medium containing 10% fetal calf serum, and MT-4 cells were maintained in RPMI 1640 containing 10% fetal calf serum.

Gateway-compatible lentiviral cDNA library system and HIV-1 challenge. A Gateway-compatible lentivirus vector DNA (pYK005C) was constructed through the insertion of a Gateway cloning system reading frame cassette (Invitrogen, Carlsbad, Calif.) into the EcoRI site of the multiple cloning sites (MCS) in the HIV-1-based vector DNA, pCSII-elongation factor 1α promoter (EF)-MCS-internal ribosome entry site (IRES)-humanized *Renilla* green fluorescent protein (hrGFP) (9). For the generation of the entry cDNA library, 10 ng (~1.5×10^9 copies) of the original cDNA library generated from human peripheral blood leukocytes (Invitrogen) was amplified by PCR with the following primers: 5′-GGGGACAAGTTTGTACAAAAAAGCAGGCT-3′ and 5′-GGGGACCACTTTGTACAAGAAAGCTGGGT-3′ (underlined nucleotides are the *att*B [B1 and B2] sequences in the forward and reverse primers, respectively). The cycling conditions were 94°C for 2 min, 94°C for 15 s, 55°C for 30 s, and 68°C for 5 min for 15 cycles and 68°C for 10 min. PCR products and pDONR201 DNA (Invitrogen) were incubated with BP Clonase enzyme mix (Invitrogen) for 16 h at 25°C by using the procedure recommended by the manufacturer, and the resulting recombinant molecules were transformed in DH5α. The transformants were selected with kanamycin (50 μg/ml), and the resultant entry cDNA library was prepared from pools of transformants. For the generation of the vector cDNA library, 300 ng of the entry cDNA library and 360 ng of pYK005C vector DNA, which is linearized by digestion with EcoRI, were incubated with LR Clonase enzyme mix (Invitrogen) for 19 h at 25°C. All resulting recombinant molecules were transformed in DH5α and selected on plates containing ampicillin (50 μg/ml). The resultant vector cDNA library was prepared from pools of transformants. For preparation of cDNA-expressing lentivirus vector, a vesicular stomatitis virus (VSV)-pseudotyped lentivirus vector was generated via calcium phosphate-mediated transfection of 293T cells as described before (9). Briefly, 1.2×10^7 cells were divided onto six TC dishes (100 × 20; Nunc, Roskilde, Denmark) 24 h before transfection. Seventeen micrograms of Vector cDNA library DNA, 12 μg of HIV Gag-Pol-expressing vector (pMDLg/pRRE), 5 μg of VSV-G protein-expressing vector (pMD-G), and 5 μg of HIV Rev-expressing vector (pRSV-Rev) per dish were cotransfected, then 48 h later, the culture supernatants were collected, and virus particles were concentrated 30-fold by centrifugation at 6,000 × *g* for 16 h. The concentrated viruses were titrated with MT-4 cells. For transduction of the cDNA library into T cells and HIV type 1 (HIV-1) challenge, 1.2×10^7 MT-4 cells were infected with 8×10^6 infectious doses of the

viral cDNA library. Three days later, the cells were challenged with HIV-1_{NL4-3} at a multiplicity of infection (MOI) of 0.05. For recovery of the cDNA sublibrary from surviving cells, MT-4 cells that survived HIV-1 challenge were collected and genomic DNA was extracted. The cDNAs from the surviving cells were amplified by PCR with primers that were used to amplify the original cDNA library as described above. This cDNA sublibrary was transferred to the pDONR201 vector by a BP reaction, and the resultant entry cDNA sublibrary was transferred to pYK005C lentivirus vector DNA by an LR reaction as described above. The viral cDNA sublibrary was prepared via transfection of 293T cells and used for the second round of screening.

Flow cytometric analysis. Two-color flow cytometric analysis was performed. Briefly, cells were stained with the optimal concentration of antibody for 30 min at 4°C and then washed. Phycoerythrin-conjugated anti-human CD4 and CD14 (eBioscience, San Diego, Calif.) and anti-mouse *H-2K*^{k} (Cedarlane, Ontario, Canada) were used. HIV-1 expression was examined with an anti-HIV-1 human serum followed by staining with biotin-conjugated anti-human IgG (Vector Laboratories, Burlingame, Calif.) and streptavidin-conjugated peridinin chlorophyll protein (BD Biosciences, San Jose, Calif.). The data were collected by FACScan (BD PharMingen, San Diego, Calif.) and analyzed with WinMDI software.

Sequence analysis. cDNA cloned into the pDONR201 vector was analyzed with the 5′-TCGCGTTAACGCTAGCATGGATCTC-3′ primer. The data were collected with the ABI 377 autosequencer. The sequence data were compared with the DNA database at the National Center for Biotechnology Information by using BLAST search.

Determination of individual cDNA length. The original cDNA library, the entry cDNA library, and the vector cDNA library were applied to *Escherichia coli* competent cells, and the cells were spread onto Luria-Bertani plates to develop bacterial colonies. cDNA fragments were amplified by PCR from these bacterial colonies containing each cDNA fragment. The PCR products were subjected to agarose gel electrophoresis and visualized with ethidium bromide. The migration distance of each cDNA fragment was compared with a DNA size marker. MT-4 cells transduced with the viral cDNA library were cloned by the limiting dilution method. cDNA fragments were amplified by PCR from the cloned cells. The length of each cDNA was determined as described above.

CD14 cDNA transduction and HIV-1 infection. A CD14 cDNA-expressing construct was made through the insertion of the Gateway cloning system reading frame cassette (Invitrogen) into the EcoRI site of the pIRES-hrGFP vector (Stratagene, San Diego, Calif.), and then a CD14 cDNA fragment was isolated from the library by an LR reaction. CD4$^+$CCR5$^+$ HeLa cells (6) were transfected by Lipofectamine 2000 (Invitrogen) with the CD14-expressing construct or empty vector (pIRES-hrGFP) as a control, and then 48 h later, the cells were infected with HIV-1_{NL4-3} at an MOI of 2. Cells were harvested 2, 12, 24, and 48 h after HIV-1 infection, and DNA was extracted as described before (20). For CD14 stable transduction, an *H-2K*^{k}-expressing lentivirus vector, which was constructed by replacing the mutant *Renilla reniformis* hrGFP sequence in the Gateway-compatible lentivirus vector DNA (pYK005C) with the *H-2K*^{k} sequence, was used. MT-4 or CD4$^+$ CCR5$^+$ HeLa cells were infected with either the CD14-expressing or control lentivirus vector at an MOI of 1, and then 2 days later, the cells were challenged with HIV-1_{NL4-3} at an MOI of 0.05. Cell killing activity was measured by trypan blue staining, and virus production in the culture supernatant was monitored by enzyme-linked immunosorbent assay (ZeptoMetrix Corp., Buffalo, N.Y.) for the HIV-1 $p24^{gag}$ antigen.

Real-time PCR assay. For the detection and quantification of individual forms of HIV-1 DNA, strong-stop (early reverse transcript), full-length/1-LTR circle (late reverse transcript), 2-LTR circle, and integrated forms, a real-time PCR assay was used as described previously (20). PCR was performed with an ABI PRISM 7700 sequence detection system (PE Applied Biosystems, Foster City, Calif.) and *Taq*Man universal PCR master mix (PE Applied Biosystems).

Statistical analysis. The Mann-Whitney U test was used to determine statistical significance, and P values of <0.05 were considered significant.

RESULTS

Transfer of a cDNA library from a cloning expression vector into a donor vector. Since some leukocytes would produce antiviral proteins, we started to isolate anti-HIV genes from a cDNA library generated from human peripheral blood leukocytes. Since a plasmid-based expression vector via transfection cannot be used for efficient and stable transduction into T cells, a lentiviral cDNA library-expressing system was used to introduce genes into human T cells. In this system, we used a recombination-cloning system referred to as Gateway (22). The Gateway system, which has been used to transfer individual genes (22), is based on the recombination system of the phage lambda that mediates integration and excision of the phage DNA into and from the *E. coli* genome, respectively. The integration involves recombination of the *att*P sites (P1 and P2) of the phage DNA within the *att*B sites (B1 and B2) located in the bacterial genome (BP reaction) and generates an integrated phage genome flanked by *att*L (L1 and L2) and *att*R (R1 and R2) sites. The next excision results in these *att*L and *att*R sites back to the *att*P and *att*B sites (LR reaction). First of all, the cDNA library fragments inserted between bacterial genome-derived B1 and B2 sites of the pCMV-SPORT6 cloning expression vector, referred to as the original cDNA library, were amplified by PCR. The PCR product was purified and incubated with a donor vector containing an insertion of the P1 and P2 sites, pDONR201, in the presence of BP clonase enzyme mix, which consists of a mixture of the phage protein integrase (Int) and the bacterial protein integration host factor. The BP clonase recombines the B1 and P1 sites as well as the B2 and P2 sites (BP reaction), and as a result, the cDNA library fragments were placed between derivatives of the L1 and L2 sites. The cDNA library-inserted pDONR201 was referred to as the entry cDNA library. Although a similar number of independent cDNA-carrying clones was obtained after this transfer, the mean size of the cDNA was clearly reduced from 1.75 ± 0.82 kb to 1.34 ± 0.66 kb (± standard deviations [SD]; $n = 60$; $P < 0.05$, Mann-Whitney U test), as shown in Table 1. Generally, the short DNA fragment tends to be more efficiently amplified during PCR. This property may account for the reduction of the cDNA size. However, omission of the PCR resulted in an obvious reduction of the number of independent cDNA-carrying clones by about 1/25. Thus, the PCR amplification before the BP reaction was indispensable.

TABLE 1. Quality of cDNA libraries

cDNA library	No. of primary clones	Mean insert size ± SD (kb)[a]
Original	1×10^7	1.75 ± 0.82
Entry	1.5×10^7	1.34 ± 0.66
Vector	8×10^7	1.26 ± 0.65
Viral	ND[b]	0.71 ± 0.54

[a] Mean insert size was determined by electrophoresis of PCR fragments from 60 bacterial colonies (original, entry, and vector libraries) or from 190 cDNA clones in viral cDNA library-infected cells.
[b] ND, not done.

Transfer of the entry cDNA library into a lentivirus vector. Next, the entry cDNA library was incubated with a lentivirus vector DNA that had derivatives of the R1 and R2 sites in the presence of the LR clonase enzyme mix that consists of a mixture of the phage protein excisionase (Xis), Int, and integration host factor. The LR clonase recombines the L1 and R1 sites as well as the L2 and R2 sites (LR reaction), and DNA fragments between L1 and L2 were placed between the B1 and B2 sites, respectively. Although *cis*-acting sequences derived from the lentivirus vector may interfere with wild-type HIV-1 replication, a part of the vector with a deletion of the U3 region (self-inactivating vectors) was not responsive to the interference (3). Therefore, a self-inactivating vector was used in this study. The cDNA library transferred into the lentivirus

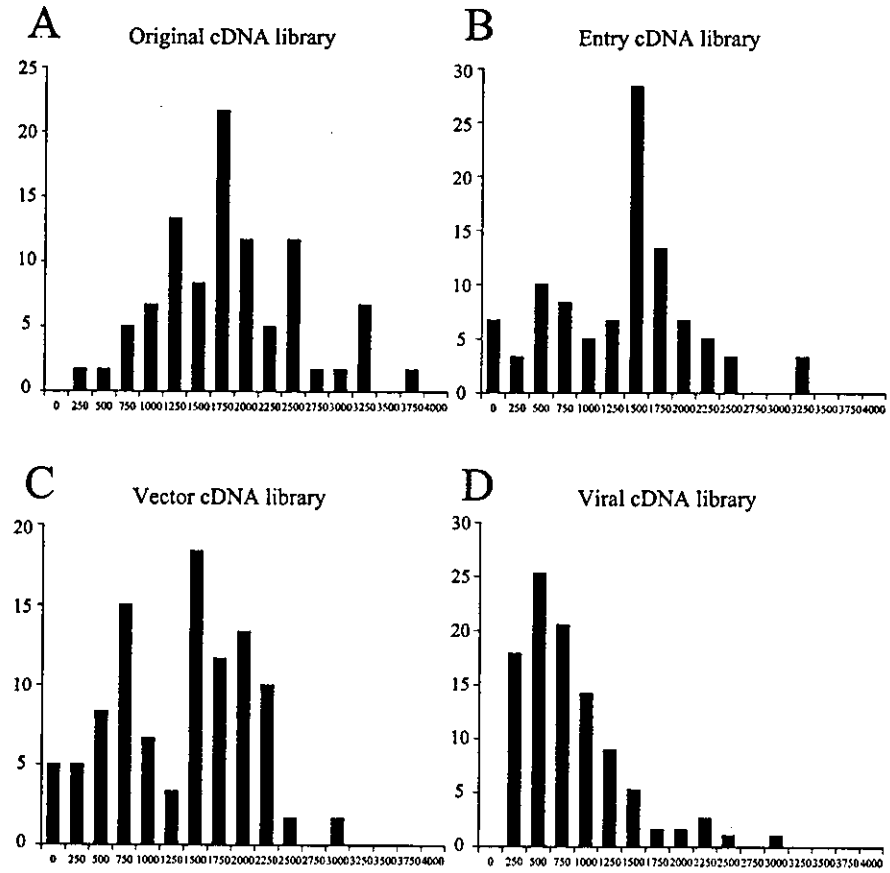


FIG. 1. Histogram analysis of lengths of individual cDNA fragments in each library. The lengths of cDNA fragments were determined as described in Materials and Methods and are plotted in 250-bp increments on the *x* axes. Percentages of individual clones are indicated on the *y* axes.

vector DNA was referred to as the vector cDNA library. Because this lentivirus vector expresses the cDNA library, under the control of elongation factor α promoter, along with GFP expression from a single bicistronic transcript, cDNA library-transduced cells are easily identified by flow cytometry or fluorescent microscopy. After this transfer, no reductions in the number or cDNA size of independent cDNA-carrying clones were observed (Table 1; Fig. 1), suggesting that the library inserted between the L1 and L2 sites would be transferred into another vector without significant loss of library complexity.

Generation of a cDNA library-expressing lentivirus vector. Next we prepared a cDNA library-expressing lentivirus vector, referred to as the viral cDNA library, via cotransfection of 293T cells with vector cDNA library DNA, a VSV-G protein expression DNA, an HIV Gag-Pol expression DNA, and an HIV Rev expression DNA. The infectious titer was approximately 4×10^6/ml, measured by using a human $CD4^+$-T-cell line, MT-4 cells. On the other hand, the infectious titer of the parental lentivirus vector with no cDNA inserted, CSII-EF-MCS-IRES-hrGFP (9), was 10 to 100 times higher than that of the viral cDNA library (data not shown). The average size of cDNA fragments in the transduced cells was 0.7 kb, which was shorter than that of cDNA fragments in the vector cDNA library, suggesting that the smaller cDNAs were enriched during lentivirus preparation and its infection into cells (Table 1; Fig. 1). To overcome this problem, size fractionation to enrich long cDNA fragments should be performed in future experiments. Nevertheless, some transduced cDNAs were more than 2,000 bp (Fig. 1), suggesting that this vector system can transduce more than 2,000-bp cDNA fragments.

Cloning of genes that prevent cells from HIV-1-induced cell death. Figure 2 shows an outline of the selection system used to isolate anti-HIV genes from the library used in this study. Twelve million MT-4 cells were infected with the viral cDNA library at an MOI of approximately 0.68. The total number of cDNA-transduced cells was estimated to be around 8×10^6, which was slightly smaller than the number of independent clones of the original cDNA library. Three days after cDNA transduction, the cells were challenged with HIV-1_{NL4-3} at an MOI of 0.05. About 30 days after HIV-1 challenge, when nontransduced culture cells had been completely killed, surviving cells, all of which were continuously growing, and GFP^+ cells were collected and cellular DNA was extracted. The cDNA fragments were recovered by PCR with B1 and B2 primers and transferred into pDONR201 vector DNA through the BP reaction. Then the cDNA sublibrary-expressing lentivirus was generated. After subsequent screening through transduction of the cDNA sublibrary in MT-4 cells and subsequent HIV-1 challenge, more than 25 independent cDNA clones were isolated, which were confirmed in further experiments to

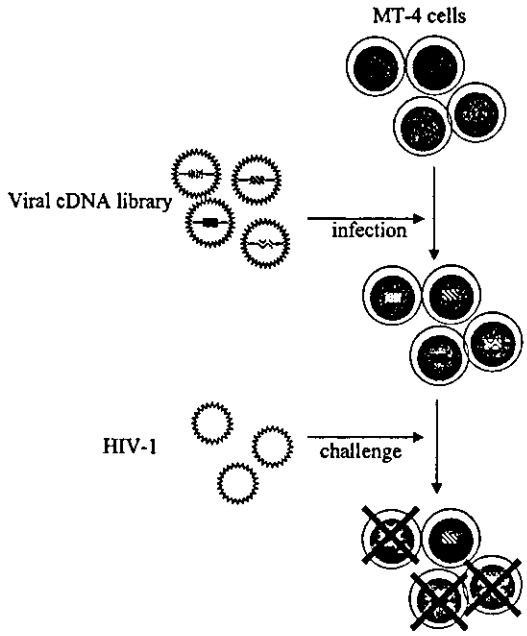


FIG. 2. Scheme for strategy used to select genes that arm cells with resistance to HIV-induced CPE. MT-4 cells were infected with the viral cDNA library and then challenged with HIV-1_{NL4-3}, which is highly cytopathic to MT-4 cells. If the introduced gene has anti-CPE, the cell will survive in the presence of HIV-1.

confer the cytopathic effect (CPE)-free phenotype in the transduced cells after HIV-1 challenge. Sequence analysis revealed that these clones contained full-length CD14 cDNA, and their sequence was identical to that of BC010507 in the GenBank database. Flow cytometric analysis showed that the anti-CD14 antibody reacted only with the cDNA clone-transduced $CD4^+$ cell population (Fig. 3A) identified by GFP expression (Fig. 3B and C).

To verify the effect of CD14 on HIV-1 infection, we independently prepared three cell lines: MT-4 cells transduced with a lentivirus vector that express CD14 along with GFP from a single bicistronic transcript, MT-4 cells transduced with an *H-2K*^{k}-expressing lentivirus vector, and nontransduced MT-4 cells. Flow cytometric analysis confirmed that all GFP-expressing cells simultaneously and persistently expressed CD14 on the cell surface (Fig. 4A). The three cell lines were mixed, and the cultures were challenged with wild-type HIV-1. Before HIV-1 infection, the mixed culture consisted of three cell populations: GFP^+ *H-2K*$^{k-}$, GFP^- *H-2K*$^{k+}$, and GFP^- *H-2K*$^{k-}$ cells (Fig. 4B). Only the GFP^+ *H-2K*$^{k-}$ population survived after HIV-1 infection (Fig. 4C). In contrast, the proportion of the three cell types was consistently maintained in HIV-1-uninfected cultures (Fig. 4D). Trypan blue staining confirmed that all dead cells were GFP^- and all GFP^+ cells were alive (Fig. 4E, F, and G). A subsequent flow cytometric analysis of cells stained by anti-HIV-1 human sera indicated that the CD14-transduced MT-4 cells also expressed HIV-1 antigen (Fig. 4H and I). When the CD14 gene was transduced into human $CD4^+$ $CCR5^+$ HeLa cells, these cells were also susceptible to HIV-1 infection but resistant to HIV-1-induced cell death (data not shown). To reveal the mechanism of how CD14 is blocking the HIV-1-induced cell death, the effect of CD14 for HIV-1 replication was examined. The surface expression of neither CD4 nor CXCR4 was altered in CD14-transduced MT-4 cells (data not shown). On the other hand, the HIV-1 replication in CD14-transduced cells determined by production of $p24^{gag}$ antigen in culture supernatant was significantly lower than that in control vector-transduced cells (Fig. 5A and B). To determine the level at which HIV-1 replication is inhibited by CD14, we used a real-time PCR assay to detect individual forms of viral cDNA at various times after HIV-1 infection. Since the preceding transduction with an HIV-1-based lentiviral vector will hamper the real-time PCR assay to measure the level of newly synthesized HIV-1 cDNA only originated from subsequent wild-type HIV-1 infection, a CD14-expressing plasmid DNA or control vector DNA was

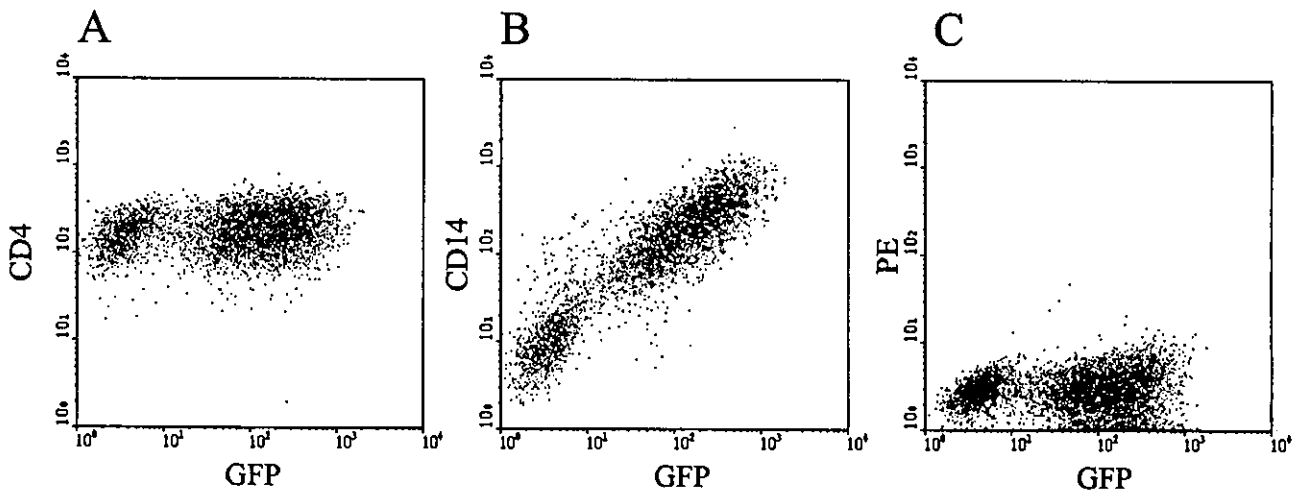
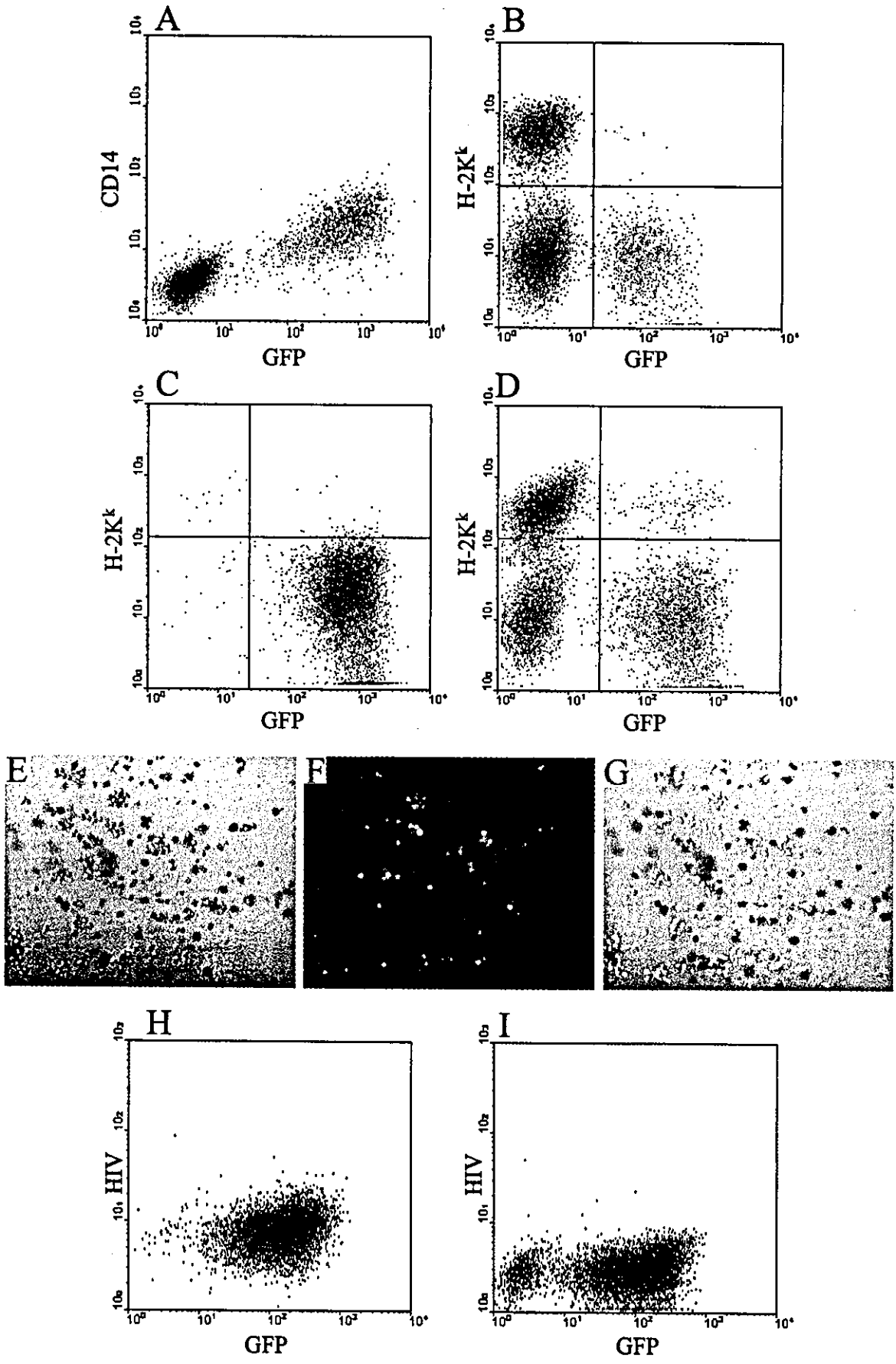


FIG. 3. Characterization of a cDNA clone that confers T-cell resistance to HIV-1-induced CPE. Cells transduced with the CD14-carrying vector isolated from this viral cDNA library were stained with anti-CD4 antibody (A), anti-CD14 antibody (B), or isotype-matched control antibody (C) and analyzed by flow cytometry. The results shown are data from one flow cytometry experiment, which is representative of three independent experiments. PE, phycoerythrin.


A
CD14
GFP
B
H-2K^k
GFP
C
H-2K^k
GFP
D
H-2K^k
GFP
E
F
G
H
HIV
GFP
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HIV
GFP

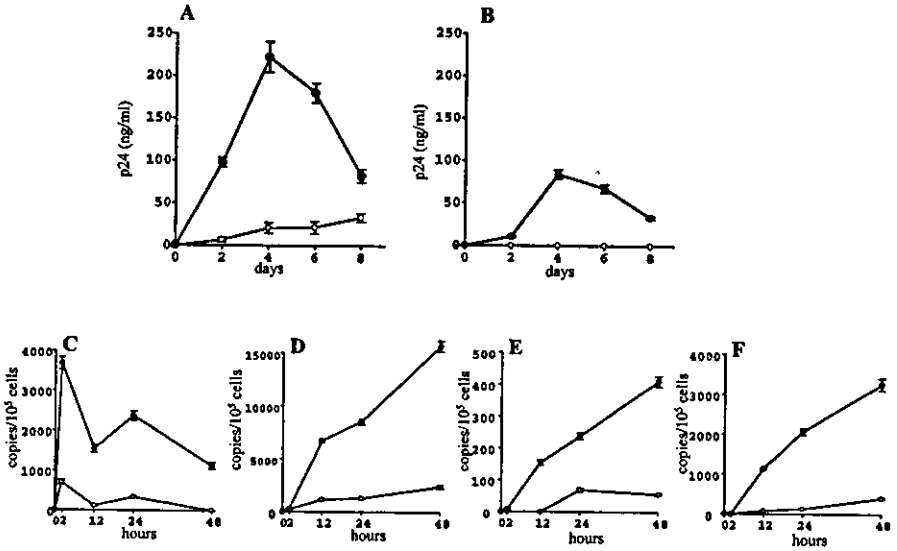

FIG. 5. Inhibition of HIV-1 replication in CD14-transduced cells. HIV-1 replication was evaluated by production of $p24^{gag}$ antigen in the culture supernatant of CD14- or empty vector-transduced MT-4 (A) or $CD4^+$ $CCR5^+$ HeLa cells (B) with a lentivirus vector expressing CD14 and *H-2K*^{k} or *H-2K*^{k} alone, respectively. To determine the level of HIV-1 entry efficiency, CD14- or empty vector-transfected $CD4^+$ $CCR5^+$ HeLa cells were challenged with DNase-treated $HIV\text{-}1_{NL4\text{-}3}$. Target cell DNA was isolated at the indicated time and used to detect early reverse transcripts (C), late reverse transcripts (D), 2-LTR circle (E), and the integrated form (F). Data are the means ± SD from duplicate experiments. The levels of the $p24^{gag}$ antigen or HIV-1 DNA in the CD14-transduced cultures were significantly lower than those of the empty vector-transduced cultures ($P < 0.05$, Mann-Whitney U test). Lines with open circles, CD14 vector; lines with filled circles, empty vector.

transfected into $CD4^+$ $CCR5^+$ HeLa cells. More than 70% of cells were confirmed to express the CD14 molecule on the cell surface determined by flow cytometry 2 days after transfection, and then the culture was infected with $HIV\text{-}1_{NL4\text{-}3}$ (X4 virus). The levels of early reverse transcripts, late reverse transcripts, 2-LTR circle, and the integrated form were significantly lower in the CD14-expressing culture than in control culture (Fig. 5C, D, E, and F). However, when $CD4^+$ $CCR5^+$ HeLa cells were infected with $HIV\text{-}1_{NL4\text{-}3}$ and then 2 days later transfected with the CD14-expressing DNA, the HIV-1 release, determined by production of the $p24^{gag}$ antigen in the culture supernatant, was similar in both the CD14-transfected and control cultures (data not shown). These data indicate that CD14 appears to partially inhibit the entry step in HIV-1 replication and provide the HIV CPE-free phenotype.

DISCUSSION

In this study, we established a lentivirus vector system to transduce a cDNA library into human T cells and successfully isolated an anti-CPE gene against HIV-1 infection. A wide variety of expression systems in mammalian cells have been developed, for example, plasmid-based, virus-based, and transposon-based systems. Among them, retrovirus-based and lentivirus-based expression systems can stably transduce genes into human T cells and are, furthermore, efficient enough to screen 10^7 genes. Recently, van Maanen et al. (21) reported the potential use of a lentivirus vector in an expression cloning system. However, the vast majority of cDNA libraries have not been yet constructed on lentivirus vectors. If a cDNA library is efficiently constructed on a lentivirus vector, this vector system will be strongly powerful to isolate genes and accelerate functional genomics. Hence, we used a site-specific recombination system, Gateway, for transferring a cDNA library from a transient expression vector to a lentivirus vector. The major advantage of this transfer system is that we can apply the system to many already established libraries. For instance, a cDNA library amplified in phage can be transferred to a mammalian expression vector including the lentivirus vector without significant loss of its complexity. Many reports have suggested that this technology allows for easy transfer of individual cDNA fragments, and this technology has become a powerful tool for a high-throughput screening system in functional genomics (8, 14, 18, 19). In this study, we used a premade cDNA library in a transient expression vector that cannot be efficiently intro-

FIG. 4. Resistance to HIV-1-induced cell death in CD14-transduced T cells. (A) Flow cytometric analysis of MT-4 cells infected with a CD14- and GFP-expressing lentivirus vector was performed. These cells (GFP^+ *H-2K*$^{k-}$) were mixed with cells infected with a lentivirus vector expressing *H-2K*^{k} alone (GFP^- *H-2K*$^{k+}$) and uninfected cells (GFP^- *H-2K*$^{k-}$) and then challenged with $HIV\text{-}1_{NL4\text{-}3}$. Flow cytometric analysis of the mixed culture is shown before HIV-1 challenge (B), 8 days after HIV-1 challenge (C), and 8 days after mock infection (D). Trypan blue staining (E) and fluorescent microscopic examination (F) were performed 3 days after HIV-1 challenge. A merged image of panels E and F is shown in panel G. Magnification, ×200. Flow cytometric analysis of the HIV-1-challenged culture 10 days after infection (H) or of an uninfected culture (I) was performed by staining with anti-HIV human serum. The results shown are data from one experiment, which is representative of three independent experiments.