Entry of R5 human immunodeficiency virus type 1 (HIV-1) into target cells requires sequential interactions of the viral envelope glycoprotein gp120 with the receptor CD4 and the coreceptor CCR5. We investigated replication of 45 R5 viral clones derived from the HIV-1JR-FLan library carrying 0–10 random amino acid substitutions in the gp120 V3 loop. It was found that 6.7% (3/45) of the viruses revealed ≥10-fold replication suppression in PM1/CCR5 cells expressing high levels of CCR5 compared with PM1 cells expressing low levels of CCR5. In HIV-1V3L#08 suppression of replication was not associated with entry events and viral production but with a marked decrease in infectivity of nascent progeny virus. HIV-1V3L#08 generated from infected PM1/CCR5 cells, was 98% immunoprecipitated by anti-CCR5 monoclonal antibody T21/8, whereas the other infectious viruses were only partially precipitated, suggesting that incorporation of larger amounts of CCR5 into the virions caused impairment of viral infectivity in HIV-1V3L#08. The results demonstrate the implications of an alternative influence of CCR5 on HIV-1 replication.

Entry of R5 human immunodeficiency virus type 1 (HIV-1) into a target cell requires cooperative interactions of the viral envelope protein gp120 with the receptor CD4 and the coreceptor CCR5 (or CXCR4 for X4 HIV-1) (1–3). These interactions depend on the concentration and distribution of receptor and coreceptor molecules on the cell surface (4–7). Cells with a large amount of CD4 only require trace amounts of CCR5 for maximal susceptibility to infection by R5 HIV-1, whereas cells low in CD4 require larger amounts of CCR5 for maximal infection (6, 8). Sequential binding of the viral surface glycoprotein gp120 to CD4 and CCR5 initiates R5 HIV-1 infection; CD4 attachment induces a conformational change in gp120 that exposes a CCR5 binding domain (9–11). The coreceptor-binding site located in the bridging sheet and the V3 loop of gp120 also play a crucial role in interacting with the N-terminal domains of the CCR5 (12–16). Finally, direct interaction between CCR5 and the V3 loop (35–37 amino acid residues) in gp120 induces structural rearrangements of a fusion peptide of gp41, allowing fusion of viral and cellular membranes (14, 15, 17–19).

Envelope viruses are known to down-modulate the receptor expression on infected cells to prevent reinfec tion (20, 21). Post-entry, HIV infection leads to a rapid and potent down-modulation of CD4 molecules expressed at the cell surface. Three viral gene products, Nef, Env, and Vpu, are involved in trafficking and catabolism of down-modulating CD4. Nef enhances CD4 internalization and directs the receptor to lysosomes for degradation (22–27), whereas Env and Vpu interfere with the transport of newly synthesized CD4 to the cell surface (28, 29). Without strict CD4 down-modulation, CD4 induces trapping and aggregation of nascent progeny virions at the cell surface by the high affinity of gp120 for CD4 (30) and a dramatic reduction in the infectivity of released virions by recruitment and sequestration of gp120 molecules away from budding sites or recruitment of nonfunctional gp120-CD4 complexes at the virion surface (31, 32).

On the other hand, strict down-modulation of coreceptor CCR5 is not observed in HIV-infected cells, although CCR5 expression on the cell surface is partially reduced by Nef (33). Partial down-regulation of CCR5 and strict down-regulation of CD4 prevent the superinfection of cells in which viral replication is already progressing. CCR5 binding domains, V3 loop, and the bridging sheet domain of gp120 are not exposed until conformational changes in gp120 are induced by interaction with CD4 on the cell surface; therefore, CCR5 would not trap nascent progeny virions at the cell surface. This may be one reason why CCR5 does not need to be strictly down-regulated after viral entry. However, a presence of an unknown inhibitory effect of CCR5 on R5 HIV-1 replication is possible. This paper addresses whether the level of CCR5 in CD4+ T-cell lines influences R5 HIV-1 replication, including late stages of the viral lifecycle. To evaluate the effect of V3 loop on viral replication with respect to CCR5 expression, 45 replication-competent mutant viruses carrying multiple amino acid substitutions in the gp120 V3 loop were used which were derived from an R5 HIV-1 V3 loop library using HIV-1JR-FLan as background (34). The library contained a set of random combinations of 0–10 polymorphic amino acid substitutions observed in 31 R5 clinical isolates. Replication of the viruses in a CD4+ T-cell line PM1 expressing low levels of CCR5 and PM1/CCR5 cells expressing
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high levels of CCR5 was examined. It was found that the viruses revealed different replication phenotypes with respect to CCR5 expression. The present study focused on a viral clone, HIV-1JR-FLan, with a replication in PM1 cells comparable with wild type HIV-1JR-FLan, but with dramatically suppressed replication in PM1/CCR5 cells. This is the first report that suppression of replication by high expression of CCR5 is V3 loop-dependent and associated with late stages of viral replication.

EXPERIMENTAL PROCEDURES

Cells and Viruses—The human CD4+ T-cell line PM1 (35) was provided by the National Institutes of Health (NIH) AIDS Research and Preference Regent Program, Division of AIDS NIAID, NIH, and maintained in RPMI1640 (Invitrogen) supplemented with 10% heat-inactivated fetal calf serum (Vitromex). PM1/CCR5 cells were generated by standard retrovirus-mediated transduction of PM1 cells by coculture with PA317 clone #8 cells transfected with pG1TKneo-CCR5 (36) without cloning. MAGIC5 (37) and 293T cells were maintained in Dulbecco’s modified Eagle’s medium (ICN Biomedicals) supplemented with 10% heat-inactivated fetal calf serum.

pJR-FLan was created in our laboratory from pJR-FL (kindly provided by Dr. Y. Koyanagi, Kyoto University), incorporating AfIII and NheI sites into Env at 6395 and 6562 nucleotides, respectively. R5 viruses, carrying a set of random amino acid substitutions in the gp120 V3 loop, were derived from the HIV-1 V3 loop library (34). For construction of V3 loop mutant viruses, amino acid substitutions were introduced into the gp120 V3 loop, as described previously (34). For virus preparation, 293T cells (1×10⁶) were transfected with 10 µg of molecular clone DNA using the calcium phosphate Precipitation Mammalian Transfection System (Promega). The supernatant was collected at 24 h post-transfection and filtered through a 0.22-µm filter unit (Millipore) and stored at −80 °C until use.

HIV-1 single-cycle luciferase reporter viruses were produced by cotransfection of 293T cells with pNL-LucR−E– (38) and Env-expressing plasmids pCXN-EnvJR-FLan, pCXN-EnvJR-FLan-A69T, pCXN-EnvV3L#08, or pCXN-EnvV3L#08-A69T. Culture supernatant containing pseudoviruses at a final concentration of 8 ng/ml p24 was added to 1×10⁴ cells/well PM1 or PM1/CCR5 cells in a 48-well plate. After 2 h, the cells were washed twice with phosphate-buffered saline (PBS), and firefly luciferase activity was measured 48 h post-infection according to the manufacturer’s directions (Promega).

Viral Replication Assay—For determination of replication phenotype, 4×10⁴ of PM1 or PM1/CCR5 cells were infected with 8 ng of p24 Gag for 2 h. After washing twice with PBS, the infected cells were incubated at 37 °C in a 5% CO₂ atmosphere. On day 6 post-infection, p24 Gag in the supernatant was measured using a p24 Gag ELISA (Zeptometrix).

Flow Cytometry—Cell surface expression of CD4 and CCR5 was analyzed by flow cytometry. Cells were incubated in the staining solution (3% fetal calf serum plus 0.05% sodium azide in PBS) with the mouse monoclonal antibodies (mAbs) anti-human CD4 (SK3, BD Biosciences Pharmingen) or anti-human CCR5 (2D7, BD Biosciences Pharmingen) at 4 °C for 30 min. The cells were washed with PBS, and fluorescein isothiocyanate-conjugated goat anti-mouse IgG antibody was used for antibody-staining. Flow cytometry was performed with a FACSCalibur flow cytometer (BD Biosciences) and analyzed with BD Cell Quest Version 3.1 software (BD Biosciences Pharmingen).

Quantitative PCR—Virus (8 ng p24 Gag) was pretreated in culture fluids with 690 units of DNase I (Worthington Biochem) and added to PM1 or PM1/CCR5 cells (4×10⁵) for 2 h at 37 °C. Cells were then washed and incubated at 37 °C for 8 h. Total DNA was purified using the QiAamp DNA blood kit (Qiagen) and eluted in a total volume of 200 µl. Two µl of DNA was analyzed by real-time quantitative PCR. Late reverse transcription products were detected using primers amplifying the region between nucleotides 685 and 789 of the provirus: forward primer (5′-ACATCAACGAGCCATGCAAAAT-3′), reverse primer (5′-ATCTGGCTCTGTTCTATGAGG-3′), and probe (5′-FAM-CATCAAGGAACTGCGAATGGGATAGA-TAMRA-3′). Reactions were performed in triplicate in TaqMan Universal PCR master mix using 0.9 pmol of each primer/µl and 0.25 pmol of probe/µl. After 10 min at 95 °C, reactions were cycled for 15 s at 95 °C followed by 1 min at 60 °C for 40 repeats on an ABI Prism model 7700 thermal cycler (Applied Biosystems).

Virus Infectivity Assay—For infectivity assay, 5×10⁵ of MAGIC5 cells (37) were plated 1 day before infection into 48-well tissue culture plates. After absorption of virus for 2 h at 37 °C, cells were washed twice with PBS and further incubated at 37 °C in 5% CO₂. At 48 h post-infection, the cells were stained, and the number of blue foci in each well was counted (39).

Western Blot Analysis—Four days post-infection, viruses in the supernatant of HIV-1-infected cells were pelleted by centrifugation at 175,000×g for 60 min. Viral proteins (10 ng of p24 Gag) were separated by 4–20% SDS-PAGE, transferred to a polyvinylidene difluoride Immobilon P membrane (Millipore), and blocked with 5% milk in PBST (137 mM NaCl, 8.1 mM KCl, 0.17 mM Na₂HPO₄, 2.68 mM KH₂PO₄, and 0.05% Tween 20) for 8 h at room temperature. Immunodetection was performed with anti-gp120 antibody (Aalto Bio Reagents) and anti-gp41 antibody (2F5; National Institutes of Health AIDS Research and Preference Regent Program) followed by a secondary antibody conjugated to horseradish peroxidase (Sigma) and Chemi-Lumi One (Nacalai Tesque).

Virus Precipitation Assay—Virus immunoprecipitation assay was performed as described previously (40). Anti-HLA-DR (L243), anti-CCR5 (3A9), and anti-CXCR4 (12G5) mAbs were purchased from BD Biosciences Pharmingen. Anti-CCR5 (T21/8) mAb was purchased from BioLegend. A rat immunoglobulin G1 (IgG1) mAb against hepatitis C virus, Mo-8 (41), was used as a rat isotype-matched negative control. Virus (5 ng of p24 Gag) in PBS containing 3% bovine serum albumin was mixed with the mAb at a concentration of 10 µg/ml in a final volume of 100 µl and incubated for 12 h at 4 °C. Then, 10 µl of Pansorbin (Calbiochem), a suspension of heat-killed Staphylococcus aureus cells pretreated for 1 h with 3% bovine serum albumin, was added to the virus/mAb mixture. After incubation for 30 min at room temperature, captured viruses were
RESULTS

Replication Suppression of Viruses from the R5 HIV-1 V3 Loop Library in PM1/CCR5 Cells—An R5 HIV-1 V3 loop library was constructed carrying a set of random combinations of 0–10 amino acid substitutions in the V3 loop (34) (Fig. 1). Replication of 45 viruses randomly selected from the library was compared in PM1 and PM1/CCR5 cells (Table 1). PM1/CCR5 cells, generated by standard retrovirus transduction of PM1 cells with a CCR5 expression lentivirus, regularly expressed high level of CCR5 and with similar levels of CD4 compared with PM1 cells (Fig. 2). A total of 36% of the viruses (16/45) failed to replicate in both cell types (ratio 1.9). Viruses were classified into the following three groups; 1) those that grew 10-fold more in PM1/CCR5 than in PM1 cells (ratio ≥ 10), designated R5\(^+\) phenotype, 2) those that revealed comparative replication kinetics in PM1 and PM1/CCR5 cells (0.1 < ratio < 10), designated R5\(^+\)H, and 3) viruses where replication was drastically suppressed in PM1/CCR5 cells (ratio ≤ 0.1), designated R5\(^-\) phenotype. Six of 45 viruses (13%), including HIV-1\(_{V3L=10}^\) (V3 loop 0 amino acid substitutions), HIV-1\(_{V3L=16}^\) (V3 loop 6 amino acid substitutions), HIV-1\(_{V3L=21}^\) (V3 loop 1 amino acid substitution), and HIV-1\(_{V3L=29}^\) (V3 loop 9 amino acid substitutions) were classified as R5\(^+\) phenotype, whereas 3 of the 45 (6.7%) viral clones, HIV-1\(_{V3L=20}^\), HIV-1\(_{V3L=21}^\), and HIV-1\(_{V3L=29}^\) were R5\(^-\) phenotype. These results indicated that the R5 HIV-1 V3 loop library contained unique replication phenotypes with respect to expression levels of CCR5 in the CD4\(^+\) T-cell line. Note that these viruses carried amino acid substitutions in the V3 loop alone, indicating that the variety of replication phenotypes was dependent on their V3 loop structure.

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Attention was then focused on the R5\(^+\) phenotype, HIV-1\(_{V3L=0}^\) to ascertain why high expression of CCR5 had a suppressive effect on viral replication. Replication of HIV-1\(_{V3L=0}^\) was markedly suppressed in PM1/CCR5 cells, whereas the virus showed similar replication kinetics to HIV-1\(_{JR-FLan}^\) with 8 ng of p24 Gag in PM1 cells (Fig. 3, A and B). On day 6 post-infection, p24 Gag in the supernatant was 33-fold lower in PM1/CCR5 than PM1 cells. However, no replication suppression of HIV-1\(_{V3L=0}^\) was observed in peripheral blood mononuclear cells or macrophages derived from three different donors compared with HIV-1\(_{JR-FLan}^\) (data not shown). HIV-1\(_{V3L=0}^\) contained 8 amino acid substitutions: Ile\(^{208}\) to Val, His\(^{209}\) to Thr, Ile\(^{209}\) to Met, Phe\(^{215}\) to Leu, Thr\(^{317}\) to Ala, Glu\(^{320}\) to Asp, Ile\(^{321}\) to Val, and Asp\(^{324}\) to Asn in gp120 V3 loop alone (Fig. 3C).

A revertant of HIV-1\(_{V3L=0}^\) designated HIV-1\(_{V3L=0-A69T}^\) was isolated that restored replication ability in PM1/CCR5 cells with an additional substitution Ala\(^{69}\) to Thr in the C1 region of gp120 (Fig. 3C). HIV-1\(_{JR-FLan-A69T}^\) and HIV-1\(_{V3L=0-A69T}^\) showed similar replication kinetics to HIV-1\(_{JR-FLan}^\) in PM1 cells (Fig. 3A). However, HIV-1\(_{JR-FLan-A69T}^\) and HIV-1\(_{V3L=0-A69T}^\) showed a slightly higher replication profile in PM1/CCR5 cells on days 4 and 5 compared with HIV-1\(_{JR-FLan}^\) (Fig. 3B).

It is possible that suppression of HIV-1\(_{V3L=0}^\) replication in PM1/CCR5 cells was due to high susceptibility of the virus to the chemokine(s) produced by the cells. To exclude this possibility, susceptibility of HIV-1\(_{V3L=0}^\), HIV-1\(_{V3L=0-A69T}^\), HIV-1\(_{JR-FLan}^\), and HIV-1\(_{JR-FLan-A69T}^\) to a \(\beta\)-chemokine, RANTES (regulated on activation normal T cell expressed and secreted), was measured, but no differences were detected (data not shown).

Entry Efficiency of HIV-1\(_{V3L=0}^\) into PM1/CCR5 Cells—To investigate whether the entry efficiency of HIV-1\(_{V3L=0}^\) decreased, real-time PCR was utilized to analyze cellular accumulation of the late reverse transcriptase product, gag, synthesized shortly after virus entry into the cells (Fig. 4A). There was no decrease in gag DNA synthesis of HIV-1\(_{V3L=0}^\) in PM1/CCR5 compared with PM1 cells. Rather, the DNA copies of HIV-1\(_{V3L=0}^\) were 3.3-fold higher in PM1/CCR5 than in PM1 cells. Higher expression of CCR5 could promote more efficient viral entry when CD4 is not significantly expressed, consistent with previous reports for other viruses (1.9–2.5-fold) (6, 8). Moreover, there was no clear difference in DNA synthesis among the four viruses in PM1/CCR5 cells.

In addition, the virus pseudotyped with the envelope proteins of HIV-1\(_{V3L=0}^\) revealed 2.0-fold more efficiency in PM1/CCR5 than in PM1 cells (Fig. 4B). Luciferase activity of other viruses in PM1/CCR5 cells also increased 1.7–2.2-fold compared with PM1 cells. Measured luciferase activity in cells infected with pseudotyped viruses serves as an indirect estimation of viral entry, integration, and transcriptional activity. The results demonstrate that suppression of HIV-1\(_{V3L=0}^\) replication in PM1/CCR5 cells is not associated with early stages of the viral lifecycle.

Production of Virus from PM1/CCR5 Cells Infected with HIV-1\(_{V3L=0}^\)—A comparison was made of the number of virions generated from PM1/CCR5 and PM1 cells in the presence of a reverse transcriptase inhibitor (AZT) and a CCR5 inhibitor.
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TABLE 1
Replication of viruses from the HIV-1 V3 loop library in PM1 and PM1/CCR5 cells

| Viral clone | V3 sequence | p24 Gag antigen (ng/ml)a | Ratiob |
|-------------|-------------|--------------------------|--------|
| HIV-1JR-Flan | CTRPNNNTRKSIHIGPGRAFYTTGEIIGDIRQAHC | 140 | 270 | 1.9 |
| #01 | ...........RG.PM............HV | 18 | 50 | 2.8 |
| #02 | ...........L.L.A.D.N........ | 17 | 54 | 3.2 |
| #03 | ...........RG.PL.C.A.AV | <1.0 | <1.0 | – |
| #04 | ...........RGVYL.FV | <1.0 | <1.0 | – |
| #05 | ...........V.R.NL.V.N | 70 | 49 | 0.7 |
| #06 | ...........R.VS.L.A.V.N | 46 | 17 | 0.4 |
| #07 | ...........RG.PM.W.A.H | 23 | 20 | 0.9 |
| #08 | ...........VTM.L.A.DV.N | 83 | 8.0 | 0.1 |
| #09 | ...........GVNL.L.A.DV | 51 | 20 | 0.4 |
| #10 | ...........S.A.HV.N | 21 | 220 | 10 |
| #11 | ...........RGV.L.FV | <1.0 | <1.0 | – |
| #12 | ...........V.N.A.PI.N | <1.0 | <1.0 | – |
| #13 | ...........R.VNL.W.Q | 4.0 | 5.0 | 1.3 |
| #14 | ...........RGVPL.A | <1.0 | <1.0 | – |
| #15 | ...........G.L.C.H.H | <1.0 | <1.0 | – |
| #16 | ...........RG.YM.L.QV | 6.0 | 160 | 27 |
| #17 | ...........RGVPL.A.DV.N | 3.0 | 10 | 3.3 |
| #18 | ...........YL.L.P.N | <1.0 | <1.0 | – |
| #19 | ...........GVLT.W.A | 38 | 29 | 0.8 |
| #20 | ...........RGVYM.C.A.N | <1.0 | <1.0 | – |
| #21 | ...........VNL.C.Q | 7.0 | 140 | 20 |
| #22 | ...........VPM.A.VAV.N | 5.0 | 53 | 11 |
| #23 | ...........VNL.L.D | 120 | 14 | 0.1 |
| #24 | ...........R.VPL.W.A.N | 3.0 | 3.0 | 1.0 |
| #25 | ...........R.VM.V.N | 70 | 6.0 | 0.1 |
| #26 | ...........GVTL.L.V | <1.0 | <1.0 | – |
| #27 | ...........GVNL.L.V | 13 | 8.0 | 0.6 |
| #28 | ...........G.PL.L.A.HV | 40 | 47 | 1.2 |
| #29 | ...........RG.PM.FV.N | 7.0 | 100 | 27 |
| #30 | ...........SM.W.A.P | 2.0 | 2.0 | 1.0 |
| #31 | ...........L.C.H.N | <1.0 | <1.0 | – |
| #32 | ...........RG.L.L.N | 3.0 | 3.0 | 1.0 |
| #33 | ...........T.W.FV.N | 3.0 | 5.0 | 1.7 |
| #34 | ...........GVYM.L.S.D | 7.0 | 170 | 24 |
| #35 | ...........RGV.L.W.A.H | 2.0 | 2.0 | 1.0 |
| #36 | ...........RG | 3.0 | 3.0 | 1.0 |
| #37 | ...........TM.L.A.P | <1.0 | <1.0 | – |
| #38 | ...........G.Y.L.D | 3.0 | 23 | 7.7 |
| #39 | ...........Y.P | 5.0 | 37 | 7.4 |
| #40 | ...........RG.TL.W.A | <1.0 | <1.0 | – |
| #41 | ...........G.M.C.FV.N | <1.0 | <1.0 | – |
| #42 | ...........RG.L.A.FV.N | <1.0 | <1.0 | – |
| #43 | ...........L.A.FV | 60 | 24 | 0.4 |
| #44 | ...........VP.C.A.A | <1.0 | <1.0 | – |
| #45 | ...........RGET.L.AV.N | <1.0 | <1.0 | – |

a PM1 or PM1/CCR5 cells (4 x 10⁴) were infected with each virus (8 ng of p24 Gag). On day 6 the extent of viral replication was measured by p24 Gag ELISA. Results represent the average of three independent experiments.
b Ratio, the concentration of p24 Gag in the supernatant of PM1/CCR5 cells was divided by that of PM1 cells.

(TAK-779) to block secondary infection by nascent progeny virus. The concentrations of AZT and TAK-779 used were 4 and 1 μM, respectively, 78- and 32-fold higher than the respective IC₅₀ levels (the concentration required to inhibit 50% of the blue foci formation in MAGIC5 cells) (data not shown). Note that on day 2 post-infection, the number of viruses generated from
HIV-1V3L#08-infected PM1/CCR5 cells was 5.7-fold higher than PM1-infected cells (Fig. 5). HIV-1JR-FLan, HIV-1JR-FLan-A69T, HIV-1V3L#08, and HIV-1V3L#08-A69T showed similar results. Production of these viruses generated from PM1/CCR5 cells was 3.8–8.7-fold higher than from PM1 cells, demonstrating that V3 loop structure of HIV-1V3L#08 did not affect viral assembly and budding. Increased virus production from PM1/CCR5 cells was

FIGURE 2. Expression of CD4 and CCR5 in PM1 and PM1/CCR5 cells. The cells were stained with mAb directed against CD4 (SK3) or CCR5 (2D7) and analyzed by flow cytometry. A, CD4 in PM1 cells. B, CCR5 in PM1 cells. C, CD4 in PM1/CCR5 cells; D, CCR5 in PM1/CCR5 cells. The shaded histograms indicate background staining with secondary antibody alone. The open histograms represent staining with indicated primary antibody.

FIGURE 3. Suppression of HIV-1V3L#08 replication in PM1/CCR5 cells. Replication kinetics of HIV-1JR-FLan, HIV-1JR-FLan-A69T, HIV-1V3L#08, and HIV-1V3L#08-A69T in PM1 cells (A) and in PM1/CCR5 cells (B). Cells (4 × 10⁵) were infected with 8 ng of p24 Gag. Viral replication was monitored by measuring p24 Gag production. C, amino acid substitutions in the gp120 V3 loop and C1 of HIV-1JR-FLan, HIV-1V3L#08, and HIV-1V3L#08-A69T. The analysis was repeated three times; error bars represent the S.D. of three replicates. *, p < 0.01; **, p < 0.001. Statistical significant differences were calculated by t test versus HIV-1JR-FLan.

FIGURE 4. Entry of HIV-1JR-FLan, HIV-1JR-FLan-A69T, HIV-1V3L#08, and HIV-1V3L#08-A69T into PM1 or PM1/CCR5 cells. A, PM1 and PM1/CCR5 cells were infected with 30 ng of p24 HIV-1 for 2 h. Eight hours after infection, synthesized proviral HIV-1 DNA was determined by TaqMan real-time PCR. B, PM1 or PM1/CCR5 cells were infected for 2 h with viruses pseudotyped with the envelope of HIV-1JR-FLan, HIV-1JR-FLan-A69T, HIV-1V3L#08, or HIV-1V3L#08-A69T (8 ng of p24 Gag). At 48 h post-infection, luciferase activity in cell lysate was determined. The analysis was repeated three times; error bars represent S.D. of three replicates. *, p < 0.01; **, p < 0.001. Statistical significant differences were calculated by t test versus HIV-1JR-FLan.

HIV-1V3L#08-infected PM1/CCR5 cells was 5.7-fold higher than PM1-infected cells (Fig. 5). HIV-1JR-FLan, HIV-1JR-FLan-A69T, HIV-1V3L#08, and HIV-1V3L#08-A69T showed similar results. Production of these viruses generated from PM1/CCR5 cells was 3.8–8.7-fold higher than from PM1 cells, demonstrating that V3 loop structure of HIV-1V3L#08 did not affect viral assembly and budding. Increased virus production from PM1/CCR5 cells was
consistent with the higher entry efficiency, as shown in Fig. 4, although the increased levels were not equal. This excluded the possibility that replication suppression of HIV-1V3L#08 in PM1/CCR5 cells was due to decreased virus production.

HIV-1V3L#08 Infectivity Generated from PM1/CCR5 Cells—To further investigate replication suppression of HIV-1V3L#08 in PM1/CCR5 cells, infectivity of viruses generated from 293T, PM1, and PM1/CCR5 cells was determined using MAGIC5 cells containing a /H9252-galactosidase gene driven by the HIV long terminal repeat (37) (Fig. 6). Infectivity from 293T cells, used for experimental preparation of viruses, was similar among the four viruses. However, infectivity of HIV-1V3L#08 generated from PM1/CCR5 cells showed a 20-fold decrease compared with HIV-1JR-FLan whereas infectivity of viruses, including HIV-1V3L#08 from PM1 cells, revealed similar infectivity. Note that HIV-1V3L#08-A69T, with an additional substitution of Ala69 to Thr plus the eight amino acid substitutions of HIV-1V3L#08, reacquired a potent infection ability from PM1/CCR5 cells. The decrease in infectivity in progeny HIV-1V3L#08 from PM1/CCR5 cells could result in impaired replication of HIV-1V3L#08 in PM1/CCR5 cells, as shown in Fig. 3B.

CCR5 Incorporation into HIV-1V3L#08 Virions—To elucidate the significance of CCR5 levels on decreased infectivity in HIV-1V3L#08, we examined whether high expression of CCR5 inhibits incorporation of Env proteins gp120 and gp41 into virus particles. Amounts of gp120 and gp41 on the virus envelopes were compared (Fig. 7). There was no decrease in gp120 and gp41 in HIV-1V3L#08 generated from PM1/CCR5 cells, indicating that high levels of CCR5 has no effect on the incorporation efficiency of gp120 and gp41 into the virions. A slight increase in gp120 and gp41 incorporation into virions was observed in HIV-1JR-FLan-A69T and HIV-1V3L#08-A69T compared to HIV-1JR-FLan and HIV-1V3L#08 generated from 293T, PM1, and PM1/CCR5 cells.

Another putative mechanism could be that CCR5 is incorporated into virus particles and specifically impaired the intrinsic function of HIV-1V3L#08 in PM1/CCR5 cells. To examine this possibility, the virions were precipitated using anti-CCR5 mAbs (Fig. 8). It has been reported that HLA-DR is incorporated into the viral envelope from the infected cell membrane (42, 43). We confirmed that an anti-HLA-DR mAb L243 could precipitate
virions generated from PM1 or PM1/CCR5 cells, whereas anti-E2 protein mAb of hepatitis C virus Mo-8, anti-CD4 mAb SK3, or anti-CXCR4 12G5 could not. mAb 2D7 (44), which could recognize the second extracellular loop of CCR5 on the surface of PM1 or PM1/CCR5 cells via flow cytometry (Fig. 2), failed to precipitate viral particles (Fig. 8, A and B). Similarly, 3A9 (45), an anti-CCR5 mAb recognizing the N terminus via flow cytometry (data not shown), could not precipitate viruses either. Surprisingly, another anti-CCR5 mAb (T21/8), also recognizing the N terminus, partially precipitated viruses generated from PM1 (Fig. 8A) and PM1/CCR5 cells (Fig. 8B) but not from 293T cells (data not shown). Note that HIV-1V3L#08 generated from PM1/CCR5 cells was almost completely precipitated (98%) by mAb T21/8 (Fig. 8B), whereas HIV-1V3L#08 generated from PM1 cells and the other three viruses from both cell types were partially precipitated (69–88%) by mAb T21/8 (Fig. 8A). The results indicate that sufficient levels of CCR5 were incorporated into HIV-1V3L#08 particles to be precipitated by T21/8. The larger amount of incorporated CCR5 reduced viral infectivity in HIV-1V3L#08, although the recruitment of CCR5 into virions and the molecular mechanism of decreased infectivity in HIV-1V3L#08 are unclear. For comparison of the amount of CCR5 at the surface of the virions, we purified virions by precipitation using anti-gp120 antibody 2G12 from exosomes contaminated in the virion by Western blot analysis. However, we could not detect CCR5 possibly because of low affinity of anti-CCR5 antibodies (data not shown).

Amino Acid Substitutions in the V3 Loop Responsible for R5L Phenotype—In 45 viral clones, three viruses revealed an R5L phenotype (Table 1). The amino acid substitutions Ile307 to Val and Ile309 to Met were common to all three viruses, suggesting that these two substitutions could be responsible for the R5L phenotype. To assess the contribution of the eight substitutions in the HIV-1V3L#08 V3 loop to the R5L phenotype, eight V3 loop mutant viruses containing one substitution each were prepared, and their replication phenotypes were determined (Table 2). Replication of HIV-1I307V and HIV-1I309M was suppressed in PM1/CCR5 cells, whereas these viruses could replicate in PM1 cells (>60 ng/ml p24 Gag), consistent with the common substitutions obtained in three R5L viruses described above (Table 1).

To ascertain the importance of these substitutions, V3 mutant viruses containing random combinations of between two and six of the eight substitutions from HIV-1V3L#08 were prepared. Most of the recombinant viruses preferentially replicate in PM1 cells (ratio ≤ 0.5) (Table 3). Four of 12 viruses revealed a R5L phenotype (ratio ≤ 0.1) and contained I307V and/or I309M. Viruses containing I307V always showed a low p24 Gag ratio (ratio ≤ 0.3), whereas ratios in I309M-containing viruses were not as low (Fig. 9), e.g. 1.4 for HIV-1V3L#130 (Table 3). In addition, F315L frequently conferred a low p24 Gag ratio combined with other substitutions, although F315L alone could not confer R5L phenotype. The results show that the R5L phenotype cannot be conferred by just one or two substitutions; a combination of several substitutions, including key substitutions, is essential. The key amino acid residues at 307 and 309 were directly at the N-terminal of the GPGR sequence in the V3 loop crown, suggesting that the amino acid substitutions adjoining the V3 crown may be important in regulating replication kinetics in cells expressing high levels of CCR5.

**DISCUSSION**

We investigated the replication ability of 45 viral clones from the R5 HIV-1 V3 loop library and found that the library contained different replication phenotypes with respect to the expression level of CCR5 (Table 1). The report focused on one of the viruses, HIV-1V3L#08, that was suppressed 33-fold in PM1/CCR5 cells expressing high levels of CCR5 compared with parental PM1 cells (Fig. 3B). This suppression of viral growth was not associated with early events in the lifecycle but with a decrease in viral infectivity in nascent progeny virus from cells expressing high levels of CCR5. MAGIC5 (37) and NP2/CD4/hiCCR5 cells expressed CD4 at levels similar to and CCR5 at levels compatible to or higher than that of PM1/CCR5 cells;
however, HIV-1V3L#08 showed no CCR5-dependent replication suppression compared with MAGI/CCR5, NP2/CD4/lowCCR5 expressing a similar level of CD4, and a lower level of CCR5 (data not shown). The results suggest that distribution or localization of CCR5 on the surface in PM1 cell line may be distinct from these adherent cells.

Depending on the surface of the host cell, HIV-1 incorporates cell-derived molecules into its envelope (46, 47). HLA-DR, β2-microglobulin, ICAM-1 (intracellular adhesion molecule 1), and other cellular surface proteins were incorporated into a budding virion (48, 49), whereas CD4, CXCR4, and CCR5 were not detectable (50). Using the anti-CCR5 mAb, T21/8, we found CCR5 incorporated into a budding virion (Fig. 8). In contrast, two anti-CCR5 mAbs, 2D7 and 3A9, failed to capture virions from PM1 and PM1/CCR5 cells despite the fact that 2D7 (Fig. 2) and 3A9 (data not shown) could recognize CCR5 at the cell surface, indicating that the epitopes of 2D7 and 3A9 were lost by conformation change or concealed from the antibodies by interaction with other molecule(s) on a virus particle. The epitope of 2D7 is mapped to the second extracellular loop including Lys171-Glu172 by alanine mutagenesis scan (44). The tertiary structure of CCR5 including Ser7 and IYD11 in the N terminus and His88 and Trp94 in the first extracellular loop are estimated to contribute to the binding of 3A9 by phage displayed peptide libraries (45), whereas T21/8 epitope has not been mapped but should be located in the N terminus of 22 amino acid residues from Met1 to Lys22; T21/8 was raised against a peptide corresponding to residues 1–22 of CCR5 according to the manufacturer. For viral entry, binding of coreceptors to HIV-1 gp120 is mediated by the V3 loop and the coreceptor-binding site located in the bridging sheet of gp120 (12, 14–16). The N terminus and the first and second extracellular loops of CCR5 are thought to interact with gp120 (51, 52). Loss of 2D7 and 3A9 binding with CCR5 at the viral envelope indicate that conformation of CCR5 at the viral envelope is distinct from that of CCR5 at the host cell surface.

The following scenario could explain the decreased infectivity in HIV-1V3L#08 from PM1/CCR5 cells. In HIV-1V3L#08, HIV-1JR-FLan, and HIV-1JR-FLan-A69T generated from PM1 and PM1/CCR5 cells, cellular CCR5 is incorporated into the viral envelope but does not impair viral infectivity due to the low level of CCR5. A similar amount of CCR5 incorporated into HIV-1V3L#08 from PM1 cells is not sufficient to impair viral

**TABLE 3**

| Viral clone | V3 loop sequence | p24 Gag (ng/ml) | PM1 | PM1/CCR5 | Ratio |
|-------------|------------------|----------------|-----|----------|-------|
| HIV-1JR-FLan | CTRPNNTRKHIGPGAYTTEIGDIRQAH | 140 | 270 | 1.9 |
| HIV-1V3L#08 | .............VTM.L.A..DV...N..... | 83 | 8.0 | 0.1 |
| HIV-1V3L#102 | .............T..L........N..... | 200 | 72 | 0.4 |
| HIV-1V3L#103 | .............TM........DV..... | 150 | <1.0 | <0.1 |
| HIV-1V3L#104 | .............VT........L.A..V...N..... | 210 | 84 | 0.4 |
| HIV-1V3L#117 | .............VT........L.A..V...N..... | 110 | <1.0 | <0.1 |
| HIV-1V3L#121 | .............TM........DV..... | 120 | <1.0 | <0.1 |
| HIV-1V3L#124 | .............T..L........A.....DV...N..... | 150 | 40 | 0.3 |
| HIV-1V3L#125 | .............T..L........A.....DV...N..... | 14 | 170 | 11 |
| HIV-1V3L#127 | .............TM........L.A..A.....N..... | 200 | <1.0 | <0.1 |
| HIV-1V3L#128 | .............TM........L.A..A.....N..... | 160 | 30 | 0.2 |
| HIV-1V3L#130 | .............TM........L.A..A.....N..... | 130 | 180 | 1.4 |
| HIV-1V3L#132 | .............TM........L.A..A.....N..... | 160 | 70 | 0.4 |
| HIV-1V3L#133 | .............TM........L.A..A.....N..... | 120 | 20 | 0.2 |

* PM1 or PM1/CCR5 cells (4 × 10⁵) were infected with each virus (8 ng of p24 Gag). On day 6 the extent of viral replication was measured by p24 Gag antigen ELISA. Results represent the average of three independent experiments.

* Ratio, the concentration of p24 Gag in the supernatant of PM1/CCR5 cells was divided by that of PM1 cells.

**FIGURE 9. Effect of each amino acid substitution on preferential replication of V3 mutant viruses.** Ratios of p24 Gag in seven mutant viruses in Table 2 and 12 mutant viruses in Table 3 were plotted. Closed circle, mutant virus containing one substitution in the V3 loop (Table 2). Open circle, virus containing a random combination of multiple substitutions in the HIV-1V3L#08 V3 loop (Table 3).
infectivity either. Larger amounts of CCR5 are recruited by HIV-1V3L#08 from the PM1/CCR5 cell surface into the virions, and the CCR5 levels are above the threshold needed for suppression of viral replication. HIV-1V3L#08 from PM1/CCR5 cells was almost completely precipitated by T21/8, indicating that most of the virus particles contained sufficient levels of CCR5 for capture (Fig. 8B), although immunoprecipitation efficiency of virions does not proportionally reflect the amount of CCR5. Otherwise incorporated CCR5 may specifically impair infectivity of CCR5 cells is supposed to be higher than in CCR5-expressing human donors being reported (53, 54). CCR5 expression levels in peripheral blood mononuclear cells from PM1/CCR5 isolates, suggesting that HIV-1 has evolved to overcome problems in viral replication caused by the high expression levels of CCR5 in target cells in vivo. The results demonstrate the significant implications of an alternative influence of CCR5 on R5 HIV-1 replication.

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REFERENCES
1. Berger, E. A., Doms, R. W., Fenyo, E. M., Korber, B. T., Littman, D. R., Moore, J. P., Sattentau, Q. J., Schuitemaker, H., Sodroski, J., and Weiss, R. A. (1998) Nature 391, 240
2. Doms, R. W., and Peiper, S. C. (1997) Virology 235, 179–190
3. Moore, J. P., Trkola, A., and Dragic, T. (1997) Curr. Opin. Immunol. 9, 551–562
4. Lin, Y. L., Mettling, C., Portales, P., Reyes, J., Clot, J., and Corbeau, P. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 15590–15595
5. Peters, P. J., Bhattacharya, J., Hibbitts, S., Dittmar, M. T., Simmons, G., Bell, I., Simmonds, P., and Clapham, P. R. (2004) J. Virol. 78, 6915–6926
6. Platt, E. J., Wehrly, K., Kuhmann, S. E., Chesebro, B., and Kabat, D. (1998) J. Virol. 72, 2855–2864
7. Reyes, J., Baillat, V., Portales, P., Clot, J., and Corbeau, P. (2003) J. Acquired Immune Defic. Syndr. 34, 114–116
8. Walter, B. L., Wehrly, K., Swamstrom, R., Platt, E., Kabat, D., and Chesebro, B. (2005) J. Virol. 79, 4828–4837
9. Kwong, P. D., Wyatt, R., Robinson, J., Sweet, R. W., Sodroski, J., and Hendrickson, W. A. (1998) Nature 393, 648–659
10. Trkola, A., Dragic, T., Arthos, J., Binley, J. M., Olson, W. C., Allaway, G. P., Cheng-Mayer, C., Robinson, J., Maddon, P. J., and Moore, J. P. (1996) Nature 384, 184–187
11. Wyatt, R., Kwong, P. D., Desjardins, E., Sweet, R. W., Robinson, J., Hendrickson, W. A., and Sodroski, J. G. (1998) Nature 393, 705–711
12. Reeves, J. D., Gallo, S. A., Ahmad, N., Miamidian, J. L., Harvey, P. E., Sharron, M., Pohlmann, S., Sfakianos, J. N., Derdeyn, C. A., Blumenthal, R., Hunter, E., and Doms, R. W. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 16249–16254
13. Reeves, J. D., Miamidian, J. L., Biscone, M. J., Lee, F. H., Ahmad, N., Picerney, T. C., and Doms, R. W. (2004) J. Virol. 78, 5476–5485
14. Rizzuto, C. D., Wyatt, R., Hernandez-Ramos, N., Sodroski, J., Hendrickson, W. A., and Sodroski, J. I. (1998) Science 280, 1949–1953
15. Rizzuto, C., and Sodroski, J. I. (2000) AIDS Res. Hum. Retroviruses 16, 741–749
16. Suphaphiphat, P., Thitithanonant, A., Parks-Uccaralertkun, S., Essex, M., and Lee, T. H. (2003) J. Virol. 77, 3832–3837
17. Wu, L., Gerard, N. P., Wyatt, R., Choe, H., Polaron, C., Ruffing, N., Bordet, A., Cardoso, A. A., Desjardins, E., Newman, W., Gerard, C., and Sodroski, J. (1996) Nature 384, 179–183
18. Wyatt, R. D., and Sodroski, J. (1998) Science 280, 1884–1888
19. Zhang, W., Canziani, G., Plugariu, C., Wyatt, R., Sodroski, J., Sweet, R., Kwong, P., Hendrickson, W., and Chaiwen, I. (1999) Biochemistry 38, 9405–9416
20. Delwart, E. L., and Panganiban, A. T. (1989) J. Virol. 63, 273–280
21. Stock, F. T., and Rubin, H. (1966) Virology 29, 628–641
22. Aiken, C., Konner, J., Landau, N. R., Lenburg, M. E., and Trono, D. (1994) Cell 76, 853–864
23. Bresnahan, P. A., Yonemoto, W., Ferrell, S., Williams-Herman, D., Geleziunas, R., and Greene, W. C. (1998) Curr. Biol. 8, 1235–1238
24. Craig, H. M., Pandori, M. W., and Guattelli, J. C. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 11229–11234
25. Greenberg, M., DeTulio, L., Rapoport, I., Skowronski, J., and Kirchenhaus, T. (1998) Curr. Biol. 8, 1239–1242
26. Mangasarian, A., Foti, M., Aiken, C., Chin, D., Carpenter, J. L., and Trono, D. (1997) Immunity 6, 67–77
27. Piquet, V., Chen, Y. L., Mangasarian, A., Foti, M., Carpenter, J. L., and Trono, D. (1998) EMBO J. 17, 2472–2481
28. Geleziunas, R., Bour, S., and Wainberg, M. A. (1994) FASEB J. 8, 593–600
29. Willey, R. L., Maldarelli, F., Martin, M. A., and Strebel, K. (1992) J. Virol. 66, 7193–7200
30. Palese, P., Tobi, K., Ueda, M., and Comans, R. W. (1974) Virology 61, 397–410
31. Lanna, J., Mangasarian, A., and Trono, D. (1999) Curr. Biol. 9, 622–631
32. Levesque, K., Zhao, Y. S., and Cohen, E. A. (2003) J. Biol. Chem. 278, 28346–28353
33. Michel, N., Allespach, I., Venzke, S., Fackler, O. T., and Keppeler, O. T. (2005) Curr. Biol. 15, 714–723
34. Yusa, K., Maeda, Y., Fujiioka, A., Monde, K., and Harada, S. (2005) J. Biol. Chem. 280, 30083–30090
35. Luco, P., Cocchi, F., Balotta, C., Markham, P. D., Louie, A., Farci, P., Palese, R., Gao, R. C., and Reitz, M. S., Jr. (1995) J. Virol. 69, 3712–3720
36. Maeda, Y., Foda, M., Matsushita, S., and Harada, S. (2000) J. Virol. 74, 1787–1793
37. Hachiya, A., Aizaawa-Matsuoka, S., Tanaka, M., Takahashi, Y., Ida, S., Gataanaga, H., Hiraibayashi, Y., Kojima, A., Tatsumi, M., and Oka, S. (2001) Antimicrob. Agents Chemother. 45, 495–501
38. Mariani, R., Rutter, G., Harris, M. E., Hope, T. J., Krausslich, H. G., and
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Landau, N. R. (2000) J. Virol. 74, 3859–3870
39. Kimpton, J., and Emerman, M. (1992) J. Virol. 66, 2232–2239
40. Esser, M. T., Graham, D. R., Coren, L. V., Trubey, C. M., Bess, J. W., Jr., Arthur, L. O., Ott, D. E., and Lifson, J. D. (2001) J. Virol. 75, 6173–6182
41. Inudoh, M., Kato, N., and Tanaka, Y. (1998) Microbiol. Immunol. 42, 875–877
42. Cantin, R., Fortin, J. F., Lamontagne, G., and Tremblay, M. (1997) Blood 90, 1091–1100
43. Cantin, R., Fortin, J. F., Lamontagne, G., and Tremblay, M. (1997) J. Virol. 71, 1922–1930
44. Lee, B., Sharron, M., Blanpain, C., Doranz, B. J., Vakili, J., Setoh, P., Berg, E., Liu, G., Guy, H. R., Durell, S. R., Parmentier, M., Chang, C. N., Price, K., Tsang, M., and Doms, R. W. (1999) J. Biol. Chem. 274, 9617–9626
45. O’Connor, K. H., Konigs, C., Rowley, M. J., Irving, J. A., Wijeyewickrema, L. C., Pustowka, A., Dietrich, U., and Mackay, I. R. (2005) J. Immunol. Methods 299, 21–35
46. Ott, D. E. (2002) Rev. Med. Virol. 12, 359–374
47. Tremblay, M. J., Fortin, J. F., and Cantin, R. (1998) Immunol. Today 19, 346–351
48. Hoxie, J. A., Fitzharris, T. P., Youngbar, P. R., Matthews, D. M., Rackowski, J. L., and Radka, S. F. (1987) Hum. Immunol. 18, 39–52
49. Ott, D. E. (1997) Rev. Med. Virol. 7, 167–180
50. Lallos, L. B., Laal, S., Hoxie, J. A., Zolla-Pazner, S., and Bandres, J. C. (1999) AIDS Res. Hum. Retroviruses 15, 895–897
51. Dragic, T., Trkola, A., Lin, S. W., Nagashima, K. A., Kajumo, F., Zhao, L., Olson, W. C., Wu, L., Mackay, C. R., Allaway, G. P., Sakmar, T. P., Moore, J. P., and Maddon, P. J. (1998) J. Virol. 72, 279–285
52. Farzan, M., Choe, H., Vaca, L., Martin, K., Sun, Y., Desjardins, E., Ruffing, N., Wu, L., Wyatt, R., Gerard, N., Gerard, C., and Sodroski, J. (1998) J. Virol. 72, 1160–1164
53. Moore, J. P. (1997) Science 276, 51–52
54. Wu, L., Paxton, W. A., Kassam, N., Ruffing, N., Rottman, J. B., Sullivan, N., Choe, H., Sodroski, J., Newman, W., Koup, R. A., and Mackay, C. R. (1997) J. Exp. Med. 185, 1681–1691