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Utilization of Chemokine Receptors, Orphan Receptors, and Herpesvirus-Encoded Receptors by Diverse Human and Simian Immunodeficiency Viruses

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Human immunodeficiency virus type 1 (HIV-1) requires both CD4 and a coreceptor to infect cells. Macrophage-tropic (M-tropic) HIV-1 strains utilize the chemokine receptor CCR5 in conjunction with CD4 to infect cells, while T-cell-tropic (T-tropic) strains generally utilize CXCR4 as a coreceptor. Some viruses can use both CCR5 and CXCR4 for virus entry (i.e., are dual-tropic), while other chemokine receptors can be used by a subset of virus strains. Due to the genetic diversity of HIV-1, HIV-2, and simian immunodeficiency virus (SIV) and the potential for chemokine receptors other than CCR5 or CXCR4 to influence viral pathogenesis, we tested a panel of 28 HIV-1, HIV-2, and SIV envelope (Env) proteins for the ability to utilize chemokine receptors, orphan receptors, and herpesvirus-encoded chemokine receptor homologs by membrane fusion and virus infection assays. While all Env proteins used either CCR5 or CXCR4 or both, several also used CCR3. Use of CCR3 was strongly dependent on its surface expression levels, with a larger number of viral Env proteins being able to utilize this coreceptor at the higher levels of surface expression. ChemR1, an orphan receptor recently shown to bind the CC chemokine I309 (and therefore renamed CCR8), was expressed in monocyte and lymphocyte cell populations and functioned as a coreceptor for diverse HIV-1, HIV-2, and SIV Env proteins. Use of ChemR1/CCR8 by SIV strains was dependent in part on V3 loop sequences. The orphan receptor V28 supported Env-mediated cell-cell fusion by four T- or dual-tropic HIV-1 and HIV-2 strains. Three additional orphan receptors failed to function for any of the 28 Env proteins tested. Likewise, five of six seven-transmembrane-domain receptors encoded by herpesviruses did not support Env-mediated membrane fusion. However, the chemokine receptor US28, encoded by cytomegalovirus, did support inefficient infection by two HIV-1 strains. These findings indicate that additional chemokine receptors can function as HIV and SIV coreceptors and that surface expression levels can strongly influence coreceptor use.

The initial interaction of human immunodeficiency virus type 1 (HIV-1) with target cells is made via a high-affinity interaction between the virus envelope glycoprotein (Env) and CD4. However, this initial interaction is not sufficient to trigger the conformational changes in Env that lead to fusion between the virus envelope and target cell membrane (reviewed in reference 51). Recently, a number of cellular coreceptors which, in combination with CD4, permit virus entry have been described. These cellular coreceptors are members of the chemokine receptor family of seven-transmembrane G-protein-coupled receptors (GPCRs) that play an important role in the recruitment of leukocytes and monocytes to sites of inflammation. The coreceptor utilization of a particular HIV-1 strain is a primary determinant of virus tropism. Virus strains that can infect primary T cells and transformed T-cell lines (T-tropic strains) use the CXC chemokine receptor CXCR4 (30), while strains that can replicate in primary T cells and macrophages (M-tropic strains) typically use the CC chemokine receptor CCR5 (3, 17, 24, 26, 27). In addition, certain virus strains can also use the CC chemokine receptors CCR2b and CCR3 (17, 26). Expression of CD4 and the appropriate chemokine receptor in a variety of cell types renders them permissive for viral entry and Env-mediated cell-cell fusion.

The importance of CCR5 for initial HIV-1 infection has been well established. M-tropic viruses are preferentially transmitted both sexually and through intravenous drug use and are prevalent during the asymptomatic period of infection (21, 62, 66, 74). That CCR5 is critical for infection of a naive host is shown by a natural knockout of CCR5, Δccr5, in which a 32-bp frameshift deletion found with an allelic frequency of 10% in the Caucasian population prevents transport of the truncated molecule to the cell surface (45, 59, 64). Individuals homozygous for Δccr5 are highly resistant to virus infection, while heterozygotes have some degree of protection from virus infection and typically progress to disease more slowly than homozygous wild-type individuals (23, 37, 40, 45, 50, 64). The role of CXCR4 in viral pathogenesis seems to be primarily in the later stages of infection, as viruses that use this receptor are generally not identified until several years postinfection (21, 68–70). Furthermore, CXCR4 does not appear to be present at levels that can be used for infection of macrophages and is present at low levels in activated/memory T cells (relative to naive cells), the primary cellular targets of initial infection (11, 49, 54).

Although the ability of a virus to use CCR5 is important for

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initial infection, its utilization does not appear to be absolutely necessary, since several HIV-positive individuals homozygous for ccr5 have been reported (9, 55, 71). Furthermore, macrophages from ccr5 homozygotes can be readily infected by HIV-1 89.6 (59), a virus strain that can use CXCR4, CCR5, CCR3, and CCR2b for virus entry (26). Receptors other than CCR5 and CXCR4 may play a role in certain aspects of viral pathogenesis. For example, CCR3 is expressed in microglia, and infection of these cells is inhibited by the CCR3 ligand eotaxin implicating CCR3 in HIV neurotropism (35). Here we found that utilization of CCR3 by HIV-1 was strongly dependent on its surface expression levels, likely explaining past differences regarding which virus strains can use this coreceptor (17, 24, 26, 27).

Given the possibility that utilization of coreceptors other than CCR5 and CXCR4 by HIV influences viral pathogenesis, we tested the ability of chemokine receptors, related orphan receptors, and chemokine receptor homologs encoded by herpesviruses to support HIV-1, HIV-2, and simian immunodeficiency virus (SIV) Env-mediated membrane fusion and viral entry. While many of the receptors were found to be nonfunctional for any Env protein tested, ChemR1 (65), an orphan chemokine receptor recently shown to bind the CC chemokine I309 (and now designated CCR8) (38, 72), was utilized by a wide variety of HIV-1, HIV-2, and SIV Env constructs. Furthermore, ChemR1/CCR8 was expressed in both activated monocytes and peripheral blood lymphocytes and thus could potentially have a role for HIV infection in vivo. An additional orphan receptor, V28, previously shown to function as a coreceptor for HIV-2 ROD/B (61), was also found to support fusion by several viral Env proteins. Finally, a chemokine receptor encoded by cytomegalovirus (CMV), US28, supported inefficient virus infection for HIV-1 ADA and Ba-L. The use of ChemR1/CCR8 by diverse virus strains and its expression in both T cells and activated monocytes suggests that it may serve as a relevant HIV-1 coreceptor in vivo.

MATERIALS AND METHODS

Plasmids. Plasmids encoding HIV-1 Envs were obtained from the NIH AIDS Research and Reference Reagent Program, the WHO Network for HIV Isolation and Characterization, and Beatrice Hahn: p92RW020-5 (clade A), p92UG037-8 (clade A), p92BR020-4 (clade B), p91US005.11 (clade B), p93BR019.10 (clade BF recombinant), p92BR025-9 (clade C), p93ZR001.3 (clade D), p92UG024.2 (clade D), p92TH022-4 (clade EA recombinant), and p93BR029.2 (clade F) (31, 32). SIV/DeltaB670-Cl3 and SIVmac17E-Fr have been described previously (4, 5). HIV-2 ST and HIV-2 ST/24.2C were kindly provided by Beatrice Hahn (University of Alabama, Birmingham) (39, 43). Plasmids encoding the HIV-1 JR-FL, ADA, Ba-L, HXB2, and NL4-3 Envs for use in making luciferase virus were provided by John Moore (Aaron Diamond AIDS Research Center). The NL4-3 luciferase virus backbone (pNL-Luc-E^-R^-) was provided by Ned Landau (Aaron Diamond AIDS Research Center) (15, 20).

The coding region of ChemR1/CCR8 was cloned as a 5.3-kb fragment in pBluescript SK+ (65) and was transferred to pcDNA3 (Invitrogen) as a 1,116-bp *Hin*dIII-*Xba*I fragment. An oligonucleotide (AAGCTTGCCTTGATGG) was used in the cloning process to introduce a *Hin*dIII site 6 bp ahead of the ATG start codon and to improve the region surrounding the ATG to the Kozak consensus. The plasmids encoding the orphan receptors GPR9, GPR5, and GPR-9-6 in the expression vector pCDN (2) and the plasmid encoding the orphan receptor V28 in the expression vector pCR3.1 (Invitrogen) were a kind gift of Derk Bergsma (Smith-Kline Beecham). All constructs were expressed under control of the CMV promoter. Plasmid pcDNA3-CCR3p, encoding CCR3 under the control of the CMV promoter in pcDNA3, was provided by Stephen Peiper (University of Louisville). The plasmid encoding US28 under the control of the CMV promoter was provided by P. E. Kolattukudy (Ohio State University) (42). The plasmid encoding the human herpesvirus 8 (HHV8) GPCR (open reading frame 74) under the control of CMV in pcDNAI/amp was provided by D. Ganem (University of California, San Francisco). Clones encoding US27 and UL33 were transferred into pcDNA1 (Invitrogen) by using standard techniques (48). Plasmids encoding the HHV6 GPCRs U51 and U12 under the control of the CMV promoter in pcDNA3 were provided by U. A. Gompels (University of London) (34). All other plasmids have been described by our lab previously (7, 26).

Cells and viruses. HeLa and QT6 cells were cultured as previously described (7, 26). QT6 cells stably expressing CD4 (QT6.T4.6) were made by transfecting QT6 cells with the CD4-expressing plasmid pT4 and selection with medium containing 0.6 mg of G418 (Geneticin; Gibco BRL) per ml. Vaccinia viruses encoding HIV-1 Envs included vSC60 (BH8), vCB39 (ADA), vCB28 (JR-FL), vCB32 (SF162), vCB36 (RF), vBD3 (89.6), vCB43 (Ba-L), vCB51 (BK132, a T-tropic primary clade B virus), and vCB53 (CM243, an M-tropic primary clade E virus) (12). Recombinant vaccinia viruses encoding HIV-2 and SIV Envs included vSC50 (HIV-$2_{SBL6669}$) (13), v194 (SIVmac251), vCB74 (SIVmac239), vCB75 (SIVmac316), and vCB76 (SIVmac316mut) (28). We also used the recombinant virus vTF1.1, encoding the T7 RNA polymerase (2).

Gene reporter fusion assay. To quantitate cell-cell fusion events, we used a luciferase-based gene reporter assay (26, 53). Target QT6 cells were transfected with human CD4, T7-luciferase, and coreceptor constructs as indicated in the text and figure legends. Proteins were generally introduced into target QT6 cells in 24-well plates by transfection of 2 μg of each plasmid by calcium phosphate precipitation. The transfectant was removed after 4 h, the medium was replaced, and the cells were incubated at 37°C overnight. T7 RNA polymerase and Env proteins were introduced into effector HeLa cells by recombinant vaccinia viruses. Vaccinia virus-encoded proteins were produced by infecting cells at a multiplicity of infection of 10 for 1.5 to 4 h at 37°C. Effector cells were then trypsinized, washed with phosphate-buffered saline (PBS), resuspended in medium, and incubated at 32°C overnight in the presence of rifampin. After overnight incubation at 32°C, cells were washed with PBS and added to the target cells at 37°C in the presence of cytosine arabinoside and rifampin. For experiments with plasmid-encoded Envs, HeLa effector cells were infected for 30 to 45 min with vaccinia virus expressing T7 polymerase (vTF1.1) and then transfected with the Env constructs. The following morning, cells were lifted by using 0.5 mM EDTA in PBS and treated in the same way as the suspension effector cells. After 8 h, 2×10^5 to 3×10^5 cells were lysed in 150 μl of 0.5% Triton X-100 in PBS and assayed for luciferase activity by using commercially available reagents (Promega). Luciferase activity was quantitated with a Wallac 1450 Microbeta luminometer detector within the linear range of the detector. Results are either presented as relative light units or as a signal-to-noise ratio comparing the signal with a particular coreceptor to the signal with CD4 and pcDNA3 (no coreceptor), as indicated in the figure legends. Coreceptor usage by a particular Env protein was evaluated in at least three independent experiments.

Infection with luciferase virus. Viral stocks were prepared as previously described by transfecting 293T cells with plasmids encoding the envelopes indicated and the NL4-3 luciferase virus backbone (pNL-Luc-E^-R^-) (15, 20). The resulting supernatant was sterile filtered and stored at −80°C. For infection, U87-MG cells were prepared in 24-well plates and transfected with pT4 and the desired coreceptor. Medium was changed after 4 h, and cells were allowed to express overnight. Cells were infected the next day with 100 μl of viral supernatant in the presence of 4 μg of Polybrene per ml in a total volume of 500 μl. An additional 0.5 ml of medium was added 1 day prior to harvest of cells. Cells were lysed at 4 days postinfection by resuspension in 150 μl of 0.5% Nonidet P-40–PBS, and 50 μl of the resulting lysate was assayed for luciferase activity.

Flow cytometry. To evaluate surface levels of CCR3, we performed flow cytometry on a variety of CCR3 transfectants by using the CCR3-specific monoclonal antibody 7B11, from Charles Mackay (35, 36). 7B11 was obtained through the NIH AIDS Research and Reference Reagent Program. QT6 cells near confluency in a six-well plate were transfected with 10 μg of plasmid per well of pcDNA3-CXCR4, pcDNA3-CCR3, or pcDNA3-CCR3p by using calcium phosphate precipitation. The transfectant was removed after 4 h, the medium was replaced, and the cells were incubated at 37°C overnight. Where indicated, medium was supplemented with 10 mM sodium butyrate to boost expression levels. After 16 to 18 h, cells were lifted by using 5 mM EDTA in PBS and washed once with PBS. Cells were then resuspended in PBS-FCS (100 μl of PBS supplemented with 2% fetal calf serum [FCS]) in the presence of either 7B11 or 12G5, a negative-control monoclonal antibody specific for the chemokine receptor CXCR4 (29), and incubated for 30 min at 4°C. 12G5 was the kind gift of Jim Hoxie. Both 12G5 and 7B11 are of isotype immunoglobulin G2a (IgG2a). Primary antibodies were used at a final concentration of 0.5 to 5 μg/ml. Cells were then washed twice in PBS-FCS and then resuspended in 100 μl of PBS-FCS in the presence of a 1:100 dilution of fluorescein isothiocyanate-conjugated, affinity-purified $F(ab')_2$ donkey anti-mouse IgG (Jackson ImmunoResearch Laboratories). Cells were incubated for 30 min at 4°C, washed twice with PBS-FCS, and resuspended in 1 ml of PBS-FCS. Cells were analyzed by fluorescence-activated cell sorting (FACS) on a Becton-Dickinson FACScan. Live cells were gated and analyzed by using CellQuest v3.1f. Relative surface expression was determined as the mean fluorescence intensity with respect to cells transfected with CXCR4 and stained with 7B11.

Leukocyte preparations. Peripheral blood mononuclear cells (PBMC) were isolated from buffy coats of healthy donors by density gradient centrifugation on Lymphoprep (Nycomed, Oslo, Norway). $CD4^+$ T cells were prepared as follows. T lymphocytes were obtained by Lymphokwik-T (One Lambda, Los Angeles, Calif.) treatment of the PBMC. The cells were incubated first with anti-CD56, anti-CD19, anti-CD14, anti-DR, and anti-CD8 monoclonal antibodies and subsequently with immunomagnetic particles coated with goat anti-mouse IgG (Im-

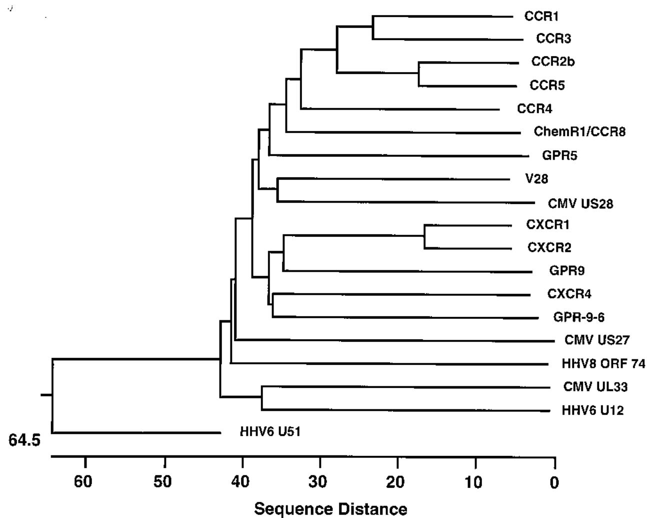

FIG. 1. Structure similarity diagram of human chemokine receptors. A protein sequence cladogram was generated by using the Clustal sequence alignment routine of the program MegAlign for Macintosh (DNASTAR, Madison, Wis.). Accession numbers for the sequences are (from top to bottom) L10918, U51241, U03905, X91492, X85740, Y08456, L36149, U20350, P32952, M68932, M73969, U32674, X71635, U45982, P09703, U75698, P16849, X83413, and P52382. A number of these receptors have been independently cloned by several groups, although only a single accession number is given per receptor. ORF, open reading frame.

munotech, Marseilles, France). Finally, CD4$^+$ cells were recovered after pelleting immunoreactive cells with a magnet. CD8$^+$ T cells were purified by a similar procedure, except that anti-CD8 in the first incubation was replaced by an anti-CD4 monoclonal antibody. These T-cell preparations contained more than 98% CD4$^+$ or CD8$^+$ T cells as evaluated by flow cytometry. Monocytes were isolated by double clumping of the PBMC previously depleted of T cells by rosetting. This cell population was more than 90% pure, as confirmed by flow cytometry and May-Grünwald-Giemsa staining. Monocytes (2×10^6 cell/ml) in primary culture were incubated in RPMI culture medium plus 10% FCS in the presence of 100 ng of bacterial lipopolysaccharide per ml for various times before RNA preparation. All reagents used for cell isolation contained less than 10 pg of endotoxin per ml as determined by the *Limulus* assay (LAL, QCL-1000; Whittaker Bioproducts, Walkersville, Md.).

Reverse transcription-PCR (RT-PCR). Total RNA was isolated by phenol-chloroform extractions. Total RNA samples (1 μg) were treated with 5 U of RNase-free DNase I (Gibco BRL) in the presence of 40 U of RNasin (Promega) per μl for 30 min at 37°C. Reverse transcription was performed by using a SuperScript preamplification system kit (Gibco BRL). PCRs were performed on cDNA samples, using as forward primers 5′-TCCATGCCGTGTATGCCC-3′, 5′-GGGTCAGAAGGATTCCTATG-3′, and 5′-CCAAATGGGAGAGTGGTTAC-3′ and as reverse primers 5′-CCACGTTGAATGGGACCC-3′, 5′-GGTCTCAAACATGATCTGGG-3′, and 5′-TCAGCGATGTTATCTTGCAG-3′ for ChemR1, β-actin, and lysozyme transcripts, respectively. PCR conditions were as follows: 93°C for 2 min 30; 93°C for 1 min; 60, 58, or 55°C (for the three transcripts, respectively) for 2 min; and 72°C for 3 min (35 cycles). Amplified products (363-bp product for ChemR1, 245-bp product for β-actin, and 175-bp product for lysozyme) were electrophoresed on 2% agarose gel stained with ethidium bromide. β-Actin primers were used as positive controls for PCR in cDNA preparations, and the expected 245-bp product was detected for all cellular preparations.

RESULTS

Use of CCR3 as a fusion coreceptor. Although CCR5 and CXCR4 are the primary HIV-1 receptors in vivo, additional chemokine receptors, including CCR3 and CCR2b, have been implicated in both viral infection and pathogenesis (17, 22, 26, 67). Given the broad spectrum of chemokine receptors used by some HIV-1 strains, we extended our search for additional coreceptors to other potential members of the chemokine receptor family. To do this, we obtained a number of orphan receptors with significant homology to the chemokine receptor family, as well as chemokine receptors or chemokine receptor homologs encoded by herpesviruses (Fig. 1), and tested them for the ability to support fusion with a panel of HIV-1, HIV-2, and SIV Env proteins. To ensure that the Env proteins were functional, we tested their ability to mediate cell-cell fusion with cells expressing CD4 and either CCR1, CCR2, CCR3, CCR4, CCR5, CXCR1, CXCR2, or CXCR4 (Table 1). Recombinant vaccinia viruses and plasmids containing the *env* gene under control of the T7 promoter were used to express Env in effector cells. These effector cells were also infected with vTF1.1, a recombinant vaccinia virus that expresses T7 polymerase. The effector cells were mixed with quail QT6 target cells expressing CD4, the indicated chemokine receptor under control of the CMV promoter, and luciferase under control of the T7 promoter. In this assay, cell-cell fusion results in cytoplasmic mixing with subsequent expression of luciferase, providing a readily quantifiable marker for membrane fusion (26, 53).

As we have shown previously, all SIV Env proteins tested used CCR5, but not CXCR4, as a fusion coreceptor (28). Three HIV-2 Env proteins, including that of HIV-2 ST, an isolate previously shown to be unable to induce syncytia with transformed T-cell lines (43), also used CCR5 as a fusion coreceptor. A cytopathic variant of HIV-2 ST derived by serial passaging on SupT1 cells, termed HIV-2 ST/24.2C, also used CCR5 but not any other receptor tested (39). Therefore, the ability of HIV-2ST/24.2C to form syncytia with SupT1 cells is likely due to the presence of an as yet unrecognized coreceptor (Table 1). The T-tropic HIV-2 strain SBL6669 efficiently used both CCR5 and CXCR4 as fusion coreceptors, and its Env can be considered a dual-tropic HIV-2 Env protein.

All HIV-1 Env proteins used CCR5 or CXCR4 or both. As we have shown previously (26), HIV-1 89.6 was also able to utilize CCR2b and CCR3 as fusion coreceptors. HIV-1 ADA could also use CCR3, though not as efficiently as HIV-1 89.6. However, studies have differed in terms of what envelopes use CCR3. In particular, JR-FL, which was originally considered to use CCR5 only (24, 26), was later shown to use CCR3 (22, 35). This discrepancy was rationalized by the known inefficient expression levels of CCR3 and by the finding that CCR3 expression levels can be limiting for coreceptor activity (17). To resolve this issue directly, we expressed CCR3 by using two plasmids that differ in their 5′ untranslated regions in the presence or absence of sodium butyrate, which increases the transcriptional activity of the CMV promoter (56). We used a monoclonal antibody to CCR3 (7B11) (35, 36) to measure surface expression levels of CCR3 by FACS and found that expression levels varied approximately 15-fold (mean channel fluorescence [MCF] values are given above bars in Fig. 2). When tested in the cell-cell fusion assay, the HIV-1 JR-FL Env protein was able to mediate progressively greater levels of cell-cell fusion as CCR3 expression levels increased, while the SIVmac251 Env protein did not (Fig. 2). We found that HIV-1 BH8, Ba-L, and BK132 could also fuse with cells expressing higher levels of CCR3, while HIV-1 RF did not (Table 1). Thus, CCR3 serves as a functional coreceptor for a larger panel of T-, M-, and dual-tropic viruses when expressed at higher levels. Whether the utilization of CCR3 expressed at such high levels is relevant for infection in vivo is not known and will obviously be dependent on CCR3 expression levels in cells that also express CD4.

ChemR1/CCR8 supports membrane fusion by diverse virus strains. To determine if newly identified chemokine receptors could function as coreceptors for HIV-1, we initially focused on a recently cloned novel protein related to chemokine receptors, ChemR1 (Fig. 1) (65). Recently, ChemR1 has been shown to bind the CC chemokine I309 (38, 72). As a conse-

TABLE 1. Usage of CC and CXC chemokine receptors as fusion cofactors[a]

Source of Env	Tropism	CCR1	CCR2b	CCR3	CCR4	CCR5	CXCR1	CXCR2	CXCR4
SIVmac251	T	−	−	− (−)	−	+++	−	−	−
SIVmac239	T	−	−	− (−)	−	+++	−	−	−
SIVmac316	M	−	−	− (−)	−	+++	−	−	−
SIVmac316mut	T	−	−	− (−)	−	+++	−	−	−
SIV/DeltaB670-Cl3	T	−	−	−	−	++	−	−	−
SIVmac17E-Fr	M	−	−	−	−	+++	−	−	−
HIV-2 SBL6669	T	−	−	−	−	+++	−	−	+++
HIV-2 ST	NA	−	−	−	−	++	−	−	−
HIV-2 ST/24.2C	NA	−	−	−	−	++	−	−	−
HIV-1 BH8	T	−	−	− (+++)	−	−	−	−	+++
HIV-1 89.6	D	−	+	++ (+++)	−	+++	−	−	+++
HIV-1 JR-FL	M	−	−	− (+++)	−	+++	−	−	−
HIV-1 ADA	M	−	−	+/− (+++)	−	+++	−	−	+/−
HIV-1 Ba-L	M	−	−	− (+++)	−	++	−	−	−
HIV-1 CM243	NSI	−	−	−	−	+	−	−	−
HIV-1 RF	T	−	−	− (−)	−	+++	−	−	+++
HIV-1 SF162	M	−	−	−	−	+++	−	−	−
HIV-1 BK132	NA	−	−	− (+++)	−	+/−	−	−	+++
HIV-1 92RW020-5	NA	−	−	−	−	++	−	−	−
HIV-1 92TH022-4	NSI	−	−	−	−	−	−	−	+
HIV-1 92UG037-8	NA	−	−	−	−	++	−	−	−
HIV-1 92BR025-9	NSI	−	−	−	−	+	−	−	−
HIV-1 92BR020-4	NSI	−	−	−	−	+++	−	−	−
HIV-1 93ZR001.3	NA	−	−	−	−	−	−	−	++
HIV-1 91US005.11	NSI	−	−	−	−	++	−	−	−
HIV-1 93BR029.2	NA	−	−	−	−	+	−	−	−
HIV-1 93BR019.10	NA	−	−	−	−	+	−	−	−
HIV-1 92UG024.2	SI	−	−	−	−	+/−	−	−	+++

[a] HeLa cells expressing the indicated HIV-1 Env proteins and T7 polymerase were mixed with QT6 cells expressing CD4, luciferase under control of the T7 promoter, and the indicated coreceptor. Cell-cell fusion was assessed 8 to 10 h later by lysis of cells followed by quantitation of luciferase activity in relative light units. Ability to use a given receptor is presented as the ratio of the signal given by the receptor versus the signal with CD4 alone: 0 to 5; −, 5 to 10, +/−; 10 to 20, +; 20 to 50, ++; >50, +++. In the case of CCR3, results shown are at the lowest level of surface expression, while results in parentheses are at the highest level of expression (CCR3p, a high-expression CCR3 plasmid, plus sodium butyrate). All results are the average of at least three experiments with the given coreceptor. The phenotypic tropism of each strain as obtained from the literature, is indicated. Tropism for a given strain is defined by one of two ways. One system (T, M, or D) defines a strain according to its ability to infect T-cell lines (T), macrophages (M), or both (D). The other system (SI or NSI) defines a strain according to its ability (SI) or inability (NSI) to form syncytia in an MT-2 assay system. NA means that a strain's biological phenotype either is unavailable or cannot be easily defined by using either system.

quence, ChemR1 has now been renamed CCR8 (38). ChemR1/CCR8 has 47% amino acid identity with CCR4, 39 to 44% with CCR1, CCR2b, CCR3, and CCR5, and 34% with CXCR4. Its gene is colocalized with other CC chemokine receptors to the 3p21.3-p24 region of the human genome and is expressed in Jurkat and MOLT-4 cells but not in the pre-B lymphoblastic JM-1 and lymphoblastic Raji cell lines (65). ChemR1/CCR8 is also expressed in PBMC and in unstimulated $CD4^+$ and $CD8^+$ T cells but not in unstimulated peripheral blood monocytes (65). Stimulation of monocytes with bacterial lipopolysaccharide made it possible to detect ChemR1/CCR8 transcripts by RT-PCR after 4 h and up to 24 h following the stimulation (Fig. 3). Transcripts encoding ly-

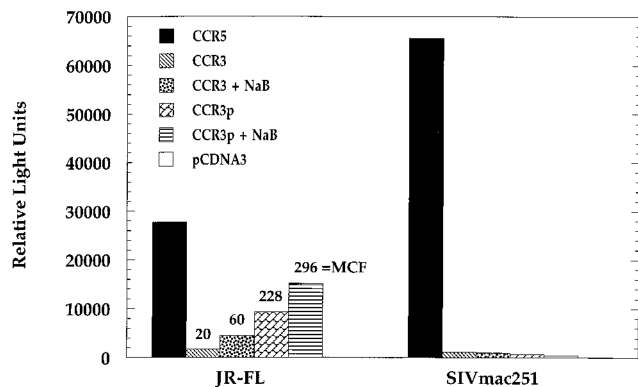

FIG. 2. Use of CCR3 as a coreceptor at different expression levels. CCR3 was expressed in quail QT6 cells under control of the CMV promoter, using two plasmids that differ in their 5′ untranslated regions (CCR3 and CCR3p). Expression was performed in the presence or absence of sodium butyrate (NaB). Cell-cell fusion with either the JR-FL or SIVmac251 Env protein was assessed in the luciferase-based gene reporter fusion assay and is shown in relative light units. CCR3 surface expression was assessed by using a monoclonal antibody to CCR3, 7B11, and FACS analysis and is reported as MCF above the columns. The MCF for QT6 cells transfected with CXCR4 and stained with 7B11 was 8.

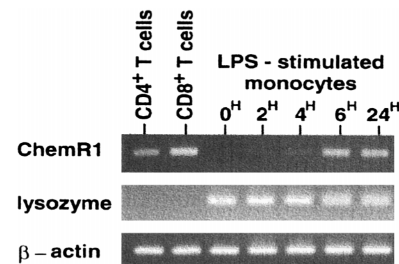

FIG. 3. Distribution of ChemR1/CCR8 transcripts. RT-PCR was performed on cDNA samples from $CD4^+$ and $CD8^+$ T cells and monocytes obtained from PBMC. ChemR1/CCR8-specific primers amplified the 363-bp product in the unstimulated $CD4^+$ and $CD8^+$ T cells and in monocytes stimulated with bacterial liposaccharide for various times (0 to 24 h). Lysozyme primers amplified the 175-bp product in the monocyte preparations. β-Actin primers were used as a positive control for PCR and RNA preparations, and the expected 245-bp product was detected for all cell preparations.

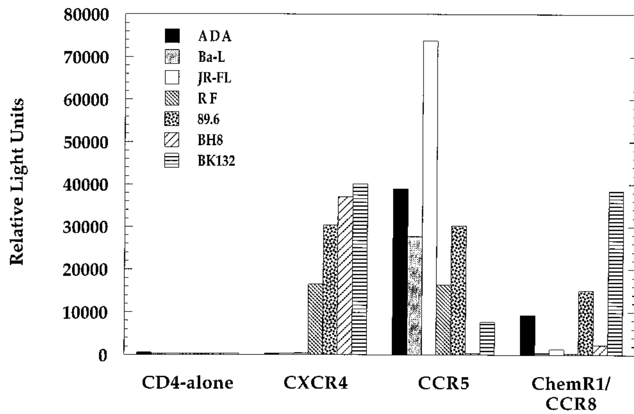

FIG. 4. ChemR1/CCR8 functions as a coreceptor for cell-cell fusion. HeLa cells expressing the indicated HIV-1 Env proteins and T7 polymerase were mixed with QT6 cells expressing CD4, luciferase under control of the T7 promoter, and the indicated coreceptor. Cell-cell fusion was assessed 8 to 10 h later by lysis of cells followed by quantitation of luciferase activity in relative light units. The results shown are from a typical experiment. The results from three to six independent experiments were used to compile the summary data presented in Table 1.

sozyme, a monocyte-specific marker, were found only in monocytes, not in the various T-cell populations. Thus, ChemR1/CCR8 transcripts are present in two primary targets of HIV-1 infection in vivo.

To examine the ability of ChemR1/CCR8 to function as a coreceptor for HIV and SIV, we tested the recombinant protein in the cell-cell fusion assay using a variety of M-tropic, dual-tropic, and T-tropic HIV-1, HIV-2, and SIV Env proteins. As shown in Fig. 4, expression of ChemR1/CCR8 in QT6 cells allowed fusion with HeLa cells expressing the M-tropic ADA Env, though approximately fourfold less efficiently than fusion obtained with CCR5. More efficient membrane fusion was observed with the dual-tropic 89.6 Env, while the primary T-tropic BK132 Env fused with cells expressing ChemR1/CCR8 nearly as efficiently as with cells expressing CXCR4. When a larger panel of HIV-1, HIV-2, and SIV Env proteins were examined for the ability to utilize ChemR1/CCR8, a substantial number, including those of two M-tropic SIV strains, two HIV-2 strains, and several primary HIV-1 strains, were found to elicit membrane fusion with cells expressing CD4 and ChemR1/CCR8 (Table 2). Thus, usage of ChemR1/CCR8 by HIV-1 does not seem to reflect any classical pattern of HIV tropism; a subset of both T-tropic and M-tropic strains can utilize the receptor.

ChemR1/CCR8 supports pseudovirus infection. Although Env-mediated cell-cell fusion assays generally reflect the ability of a virus to enter cells, important differences between cell-cell fusion and virus infection assays may occur. Therefore, we examined the ability of ChemR1/CCR8 to support infection by luciferase reporter viruses (15, 20). The luciferase reporter virus is a single-round infection vector with a nonfunctional *env* gene and a luciferase gene inserted into *nef*. This allows for single-round quantitation of virus infection following complementation with the desired Env protein. As shown in Fig. 5, HIV-1 ADA was able to infect cells expressing CD4 and ChemR1/CCR8, while the T-tropic strain NL4-3 did not. While infection efficiency was approximately 10-fold lower than that seen with CCR5, it was 100-fold higher than that observed with CD4 alone. This result demonstrates that ChemR1/CCR8 is fully functional as a virus coreceptor, supporting both Env-mediated cell-cell fusion and virus infection.

Utilization of orphan receptors by diverse virus strains. To more fully understand the range of chemokine receptors that

TABLE 2. Usage of ChemR1/CCR8, herpesvirus-encoded receptors, and orphan receptors as fusion cofactors[a]

Source of Env	Tropism	HHV8	ChemR1/CCR8	GPR9	GPR5	GPR-9-6	V28
SIVmac251	T	−	−	−	−	−	−
SIVmac239	T	−	−	−	−	−	−
SIVmac316	M	−	+++	−	−	−	−
SIVmac316mut	T	−	−	−	−	−	−
SIV/DeltaB670-Cl3	T	−	−	−	−	−	−
SIVmac17E-Fr	M	−	+	−	−	−	−
HIV-2 SBL6669	T	−	+++	−	−	−	++
HIV-2 ST	NA	−	−	−	−	−	−
HIV-2 ST/24.2C	NA	−	+/−	−	−	−	−
HIV-1 BH8	T	−	−	−	−	−	−
HIV-1 89.6	D	−	+++	−	−	−	+
HIV-1 JR-FL	M	−	−	−	−	−	−
HIV-1 ADA	M	−	+++	−	−	−	−
HIV-1 Ba-L	M	−	−	−	−	−	−
HIV-1 CM243	NSI	−	−	−	−	−	−
HIV-1 RF	T	−	−	−	−	−	−
HIV-1 SF162	M	−	−	−	−	−	−
HIV-1 BK132	NA	−	++	−	−	−	−
HIV-1 92RW020-5	NA	−	−	−	−	−	−
HIV-1 92TH022-4	NSI	−	−	−	−	−	−
HIV-1 92UG037-8	NA	−	++	−	−	−	−
HIV-1 92BR025-9	NSI	−	+	−	−	−	−
HIV-1 92BR020-4	NSI	−	−	−	−	−	−
HIV-1 93ZR001.3	NA	−	+/−	−	−	−	++
HIV-1 91US005.11	NSI	−	+/−	−	−	−	−
HIV-1 93BR029.2	NA	−	−	−	−	−	−
HIV-1 93BR019.10	NA	−	−	−	−	−	−
HIV-1 92UG024.2	SI	−	++	−	−	−	+

[a] See the footnote to Table 1 for experimental details.

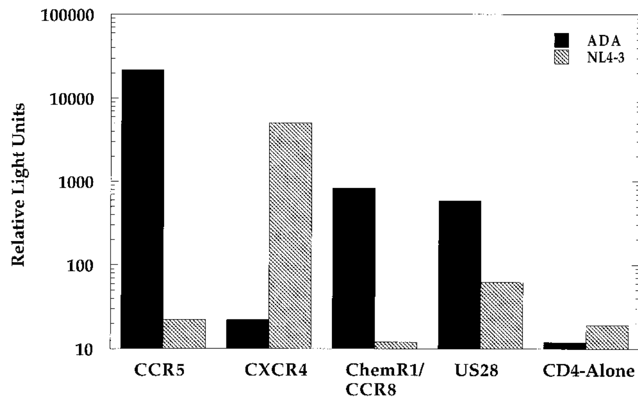

FIG. 5. ChemR1/CCR8 and US28 support virus infection. U87-MG cells expressing CD4 and the indicated coreceptor were infected with luciferase reporter viruses bearing either the HIV-1 ADA or HIV-1 NL4-3 Env protein. Luciferase activity was measured 4 days after infection.

can be used as fusion coreceptors, we assessed the abilities of four orphan receptors to support cell-cell fusion by the panel of HIV-1, HIV-2, and SIV Env proteins in the cell-cell fusion assay. GPR5, GPR9, and GPR-9-6 were nonfunctional as coreceptors for the Envs tested (Table 2), though the possibility that they can be used if expressed at higher levels cannot be ruled out. However, V28 was used by one HIV-2 and several HIV-1 isolates, though not as efficiently as either CCR5 or CXCR4. V28 is most highly expressed in neural and lymphoid tissues (60) and has recently been shown to serve as a coreceptor for HIV-2 (61). Both HIV-2 ROD (61) and the four virus strains found to utilize V28 in this study also use CXCR4 as a coreceptor. Thus, for the virus strains examined to date, V28 appears to be used by a subset of T- and dual-tropic Env proteins. Though the ligand for V28 is unknown, the receptor appears to be a member of the CC chemokine receptor family, though only distantly related to CCR5.

Chemokine receptor homologs encoded by herpesviruses. Several herpesviruses have been shown to encode for chemokine receptor-like proteins. US28, encoded by CMV, is a promiscuous chemokine receptor, binding a number of CC and CXC chemokines (33, 42). We found that expression of US28 in conjunction with CD4 rendered cells permissive for low level of membrane fusion with the ADA Env protein (Table 2). In addition, US28 supported virus infection by both ADA (Fig. 5) and Ba-L (not shown), although at relatively low levels. The extremely inefficient level of infection with Ba-L may explain why fusion was not observed between cells expressing the Ba-L Env and cells expressing CD4 and US28. No other viral Env proteins examined could utilize US28 as a fusion coreceptor under the conditions used. Using a smaller subset of Env proteins, two additional CMV-encoded seven-transmembrane-domain receptors, UL33 and US27 (14, 48), failed to function as a coreceptor for any virus strain tested (data not shown). Likewise, the chemokine receptor-like molecule GPCR encoded by HHV8 (6) and two GPCRs encoded by HHV6, U12 and U51 (34), failed to serve as a coreceptor (data not shown), at least at the expression levels used here.

DISCUSSION

CCR5 and CXCR4 are the primary coreceptors for M-tropic and T-tropic HIV-1 strains, respectively (3, 17, 24, 26, 27, 30). The critical role for CCR5 in viral transmission is shown by the remarkable resistance of individuals who lack CCR5 to HIV infection (23, 40, 45, 50, 64). However, several HIV-positive individuals who lack CCR5 have been reported (9, 55, 71), indicating that other coreceptors can sometimes be used to establish an infection. While the coreceptors used by the viruses that infected these individuals are not known, the ability of HIV-1 89.6 to readily infect macrophages from CCR5-negative individuals raises the possibility that receptors other than CXCR4 may be involved (59). Due to the genetic diversity of the primate lentiviruses, the large number of chemokine and orphan receptors that have been described, and the potential role for receptors other than CCR5 and CXCR4 in viral pathogenesis, we screened a panel of 28 viral Env proteins for the ability to use various chemokine receptors and chemokine-receptor like molecules.

Consistent with previous work, all HIV-1, HIV-2, and SIV Env proteins used CCR5, CXCR4, or both as coreceptors. HIV-2 ST, an isolate previously shown to exhibit a non-fusion-inducing phenotype with transformed T-cell lines (43), was found to use only CCR5 among those receptors tested. Since CCR5 is generally not expressed on T-cell lines, the nonfusogenic phenotype of HIV-2 ST reflects the fact that cells lacking CCR5 were used as targets. However, a fusogenic variant of HIV-2 ST, termed HIV-2 ST/24.2C, was also CCR5 restricted (39). Since HIV-2 ST/24.2C was derived by passaging on SupT1 cells, the ability of HIV-2 ST/24.2C to form syncytia on this cell line is likely due to the presence of a coreceptor not represented in our panel (Table 1). Likewise, the SIV strains tested all use CCR5 but not CXCR4. Therefore, the ability of many strains of SIV to replicate in CCR5-negative cell lines, such as CEMx174, must also be due to the presence of one or more additional coreceptors (16, 28, 47).

In addition to CCR5 and CXCR4, a more limited number of viruses used CCR3 as a coreceptor. A number of primary virus strains, including several neurotropic virus isolates (17, 26, 35, 67), have been shown to utilize CCR3 as a coreceptor for either cell-cell fusion or virus infection. CCR3 and CCR5 are both expressed in microglia, and infection of these cells is inhibited by eotaxin (which binds CCR3) and by RANTES (which binds CCR5 and CCR3), raising the possibility that utilization of CCR3 correlates with neurotropism (35). We found that the ability of a given virus isolate to use CCR3 as a coreceptor was strongly dependent on its cell surface expression. At low surface levels, HIV-1 89.6 and ADA used CCR3 as a coreceptor, while higher levels of CCR3 expression permitted fusion mediated by a larger number of M-, dual-, and T-tropic Env proteins. However, not all viruses used CCR3 when expressed at high levels, indicating a degree of Env specificity in the use of this coreceptor. Thus, like ChemR1/CCR8 and the orphan receptor STRL33 (44), CCR3 supports fusion by viruses with different tropisms.

The relationship between CCR3 expression levels and its use as an HIV-1 coreceptor raise an important point. Generally, the ability of a given virus strain to use a coreceptor for either cell-cell fusion or virus infection is determined by expressing the desired coreceptor along with CD4 in a cell that is otherwise nonpermissive for virus entry. Often, it is not known if coreceptor expression levels under these conditions reflect the levels seen in primary cells that might serve as targets for HIV infection. Minimally, the ability of a larger number of virus strains to use CCR3 when it is expressed at high levels demonstrates that this receptor, which has 53 and 32% amino acid identity with CCR5 and CXCR4, respectively, possesses the structural information required for Env-mediated membrane fusion. However, we do not yet know if the higher levels of surface expression used in our assay are less than, comparable to, or exceed the levels in relevant target cells such as micro-

glia. This underscores a general problem also encountered in studying orphan receptors. A receptor that is scored as negative in fusion or infection assays may in fact support membrane fusion if expressed at high enough levels, while a receptor that is scored as positive may function as a coreceptor only when expressed at unnaturally high levels. To evaluate the significance of coreceptor activity observed in a transient expression system, the number and types of viruses that can use the coreceptor, whether it is expressed in relevant target cells in vivo, and the levels at which it is expressed must be considered. The development of specific antibodies to chemokine and orphan receptors will make it possible to study coreceptor distribution in vivo and to determine whether surface expression levels achieved in transient expression systems is comparable to that observed on the surface of primary cells. The identification of orphan receptor ligands will also be important for evaluating the potential relevance of a coreceptor identified by in vitro assays. Entry of HIV into a variety of target cells is inhibited by ligands to coreceptors such as CCR3, CCR5, and CXCR4 (10, 17, 18, 54, 57). Thus, antibodies and ligands to newly identified coreceptors can be used to study their role in the infection of primary cells such as macrophages.

Among the orphan receptors tested, ChemR1/CCR8 was notable for the number of viruses that could use it for Env-mediated membrane fusion, the efficiency with which it supported fusion for some viral Env proteins, and the fact that it is expressed in relevant target cells for virus infection. The primary T-tropic strain BK132, for example, fused with cells expressing ChemR1/CCR8 as efficiently as with cells expressing CXCR4. Furthermore, ChemR1/CCR8 is expressed in $CD4^+$ T cells (65) and stimulated monocytes (Fig. 2), raising the possibility that it can function as a coreceptor for a subset of HIV-1, HIV-2, and SIV strains in vivo. However, ChemR1/CCR8 transcripts have not been detected in fully differentiated macrophages; ADA, which efficiently uses ChemR1/CCR8, cannot infect macrophages that are homozygous for Δccr5 (73a).

Use of ChemR1/CCR8 by SIV strains was dependent in part on V3 loop sequences. SIVmac316 used ChemR1/CCR8 efficiently, while SIVmac316mut (which differs from SIVmac316 by two amino acids in the V3 loop region) did not (41, 52). Thus, lentivirus V3 loop sequences have been shown to help govern use of CXCR4, CCR5, and ChemR1/CCR8 (8, 17, 19, 28). With the recent identification of the CC chemokine I309 as a ligand for ChemR1/CCR8 and the finding that I309 is a potent inhibitor of virus infection mediated by ChemR1/CCR8 (38), it should be possible to determine if this receptor participates in virus entry in primary cells.

The orphan receptor V28 also functioned as a coreceptor for several virus strains. V28 is expressed at the highest levels in neural and lymphoid tissues (60) and can be used by HIV-2 ROD/B for virus infection (61). We found that expression of V28 in conjunction with CD4 supported membrane fusion by the T-tropic HIV-2 strain SBL6669 and by three HIV-1 Env proteins, including the dual-tropic 89.6 Env. Given that V28 is expressed in neural tissues, it will be important to test its ability to support infection by neurotropic virus strains and to determine whether it is expressed in cells commonly infected with HIV-1.

Several potential seven-transmembrane-domain receptors, including US27, US28, and UL33 from CMV (14, 48), UL12 and UL51 from HHV6 (34), ECRF3 of herpesvirus saimiri (1), and a constitutively active receptor encoded by HHV8 (6), are encoded by herpesviruses. Of these proteins, US28 and ECRF3 have been shown to bind chemokines (1, 33, 42). We tested all of these proteins except ECRF3 for the ability to support membrane fusion mediated by a large panel of HIV-1, HIV-2, and SIV Env proteins. We found that US28 supported inefficient cell-cell fusion by the HIV-1 ADA Env protein and inefficient infection by HIV-1 ADA, and Ba-L Env proteins. This is in contrast to a recent study which found that US28 supported infection by a number of virus strains, including ADA, as efficiently as CCR5 (58). It is likely that differences in US28 expression levels account for this discrepancy, and it is not clear if the ability of US28 to support virus entry at high expression levels is relevant in vivo. To determine this, levels of US28 expression on CMV-infected cells must be measured and whether CMV infection renders nonpermissive cells susceptible to infection with HIV must be determined. The inefficient use of US28 by only 2 of 28 Env proteins tested here, coupled with the fact that US28 is expressed during the lytic cycle of virus infection in vitro (73), suggests that US28 may not serve as a relevant coreceptor in vivo.

The dual-tropic HIV-1 strain 89.6 is notable for its ability to use at least six chemokine receptors as fusion cofactors with varying efficiency. Sequence alignment of these receptors reveals only two absolutely conserved residues in the extracellular domains, suggesting that the 89.6 Env protein utilizes complex structural motifs that are conserved between a wide variety of chemokine receptors, interacts with somewhat different regions of these receptors, or perhaps acts by a combination of both. Indeed, 89.6 appears to be more dependent on the amino-terminal domain of CCR5 than are M-tropic strains, and it can utilize the first and second extracellular loops of CXCR4 (25, 46, 63). Thus, the ability of 89.6 to efficiently use the two major HIV-1 coreceptors is at least partially the result of differential usage of the receptor ectodomains. Additional structure-function studies with these and other coreceptors may reveal the means by which 89.6 and certain other Env proteins (like that from HIV-2 SBL6669) can use multiple coreceptors for Env-mediated membrane fusion.

The identification of chemokine receptors as necessary factors for HIV infection largely explains viral tropism at the level of entry into the cell. While CCR5 and CXCR4 are the major HIV-1 coreceptors, the possibility exists that other chemokine receptors or related molecules influence viral pathogenesis under some circumstances. The diversity of virus strains and the fact that HIV can be found in numerous tissues in vivo raise the possibility that the ability of a given virus strain to infect certain target cells may be dependent on its ability to use receptors other than CCR5 or CXCR4. A link between neurotropism and the ability to use CCR3 has already been proposed (35). The ability of multiple virus strains to use ChemR1/CCR8 and STRL33 as coreceptors, coupled with the expression of these receptors in CD4-positive cells in vivo, makes these molecules candidates for influencing viral pathogenesis. These and other chemokine receptors may also influence viral entry indirectly, by modulating the in vivo levels of both chemokines (by binding) and of other chemokine receptors (by potential heterologous down-regulation). In addition, new therapeutic strategies aiming to block interaction of HIV with CCR5 by using chemokines, small-molecule inhibitors, or antibodies will have to consider the possibility of viral escape from inhibition by shifting its coreceptor use from CCR5 to ChemR1/CCR8 or other coreceptors. Finally, this study shows a direct relationship between the levels of CCR3 expression and the ability of CCR3 to support Env-mediated membrane fusion. Clearly, the identification of chemokine receptors that can support virus entry in vitro is only the first step in understanding the complex requirements at the cell surface in vivo necessary to support virus infection.

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