Multiple Extracellular Elements of CCR5 and HIV-1 Entry: Dissociation from Response to Chemokines

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The human β-chemokine receptor CCR5 is an important cofactor for entry of human immunodeficiency virus–type 1 (HIV-1). The murine form of CCR5, despite its 82 percent identity to the human form, was not functional as an HIV-1 coreceptor. HIV-1 entry function could be reconstituted by fusion of various individual elements derived from the extracellular region of human CCR5 onto murine CCR5. Analysis of chimeras containing elements from human CCR5 and human CCR2B suggested that a complex structure rather than single contact sites is responsible for facilitation of viral entry. Further, certain chimeras lacking the domains necessary to signal in response to their natural chemokine ligands retained vigorous HIV-1 coreceptor activity.

The discovery of chemokines as modulators of the replication cycle of HIV-1 (*1*) and the subsequent identification of human chemokine receptors as essential cofactors in cell entry by HIV-1 (*2*) have provided a new perspective on the biology of this pathogenic human virus. Human CCR5 (*3*), a seven-transmembrane receptor for the chemokines MIP-1α, MIP-1β, and RANTES, confers susceptibility to infection by certain macrophage-tropic strains of HIV-1 in the presence of human CD4 (*4*, *5*). To investigate structural features of CCR5 that contribute to this function, we developed a transient transfection-infection system in which human CD4 is expressed in COS-7 cells in conjunction with a natural chemokine receptor or receptor variants. A synthetic epitope engineered into the NH_2-terminus of each chemokine receptor permitted rapid verification of surface expression by conventional immunoassay methods. Transfected cells were exposed to a macrophage-tropic strain of HIV-1, such as Ba-L (*6*), and cell entry was quantitated by measurement of intracellular expression of the viral capsid protein p24 by means of fluorescence-activated cell sorting (FACS). Expression of CD4 alone in COS-7 cells was insufficient to confer susceptibility to infection by Ba-L, because fewer than 0.7% of the CD4-positive cells exhibited detectable p24 immunoreactivity (Fig. 1). Consistent with recent reports, vigorous infection of cells by Ba-L was observed upon concurrent expression of CD4 and human CCR5, as represented by p24 antigen expression typically in 10 to 20% of CD4-positive cells (Figs. 1 and 2B). In studies with a recombinant virus containing a reporter gene, cell entry was accompanied by viral gene expression, and in other experiments, pretreatment with a reverse transcriptase inhibitor demonstrated the dependence of p24 expression on reverse transcription (*7*). The system thus represents a useful method for distinguishing between permissivity and nonpermissivity to infection by HIV-1.

Because the murine form of CCR5 (*8*) exhibits marked sequence similarity to the human form (82% amino acid identity) (Fig. 2A), its ability to serve as a coreceptor for HIV-1 was evaluated. Despite abundant expression on the cell surface (*9*), this molecule exhibited virtually no detectable capacity to support infection by the Ba-L virus in repeated experiments (Fig. 2B); a similar result was observed with a second macrophage-tropic virus (*7*). To identify the critical elements lacking in the nonfunctional murine form of CCR5, we prepared chimeric human/mouse CCR5 receptors and evaluated them for HIV-1 coreceptor function. Substitution of the NH_2-terminal extracellular segment of murine CCR5 by the corresponding human CCR5 segment substantially restored coreceptor function for Ba-L (HMMM, Fig. 2B). A chimera containing the NH_2-terminus of the murine receptor fused to the remaining components of the human receptor also exhibited robust coreceptor function (MHHH, Fig. 2B). The similar function of entirely complementary chimeras implies that elements within both the NH_2-terminus and distal portions of the receptor are contributory to HIV-1 coreceptor activity, whereas neither element alone is essential.

As observed with the complementary exchanges of the NH_2-terminus, selective replacement of extracellular loop 1 of murine CCR5 with the corresponding portion of human CCR5 also reconstituted vigorous coreceptor function (MHMM, Fig. 2B). At least some amount of coreceptor activity was also detected with a murine receptor containing either extracellular loop 2 or loops 2 and 3 from human CCR5 (MMHM and MMHH, Fig. 2B). Thus, rather than a single site of interaction between HIV-1 and the coreceptor, multiple elements distributed throughout the extracellular segments appear to contribute to viral entry.

Because the human/mouse chimeras involved exchanges between related receptors with very similar sequences, a second set of exchanges was prepared between two more distant receptors (71% identity, Fig. 3A) with disparate ligand specificities, human CCR5 (responsive to MIP-1α, MIP-1β, and RANTES) and human CCR2B (*10*) [responsive to MCP-1 and MCP-3 (*11*)]. Like murine CCR5, and consistent with earlier reports, human CCR2B (2222) exhibited

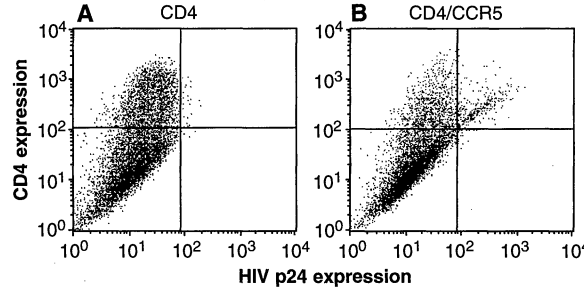

Fig. 1. Transient transfection-infection assay of CCR5-dependent cell entry by HIV-1 Ba-L. (**A**) Expression of CD4. (**B**) Concurrent expression of CD4 and CCR5. COS-7 cells transfected with the indicated expression plasmids were cultured with Ba-L and then assayed for CD4 and intracellular p24 expression by dual-color FACS (*20*). The appearance of many events in the upper right quadrant of (B) compared with few events in the upper right quadrant of (A) demonstrates the dependence of HIV infection on expression of both CD4 and CCR5. For subsequent comparative analysis with various chimeras, a window was drawn encompassing all CD4-positive cells, and the percentage of these cells that are positive for intracellular p24 (FITC+) was calculated. Typically, 10 to 20% of CD4-positive human CCR5-transfected samples are infected, representing at least a 25-fold increase over negative controls lacking CCR5.

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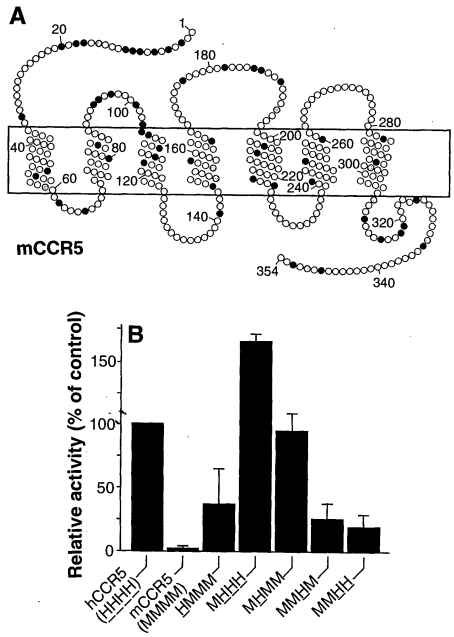

Fig. 2. HIV-1 coreceptor activity compared among chimeric receptors containing elements derived from human and murine forms of CCR5. (**A**) Primary sequence of murine CCR5 with designation of differences from human CCR5. Identical amino acids are indicated as open circles, residues with conservative substitutions as shaded circles, and residues with nonconservative substitutions as closed circles. (**B**) The coreceptor activity of each chimera (*9*) was calculated as a percentage of the activity of human CCR5 (hCCR5) in each of several transfection-infection assays. The activity of hCCR5 within each experiment was defined as 100%. Error bars represent the standard error of the mean (for each receptor variant, $n = 5$ to 7).

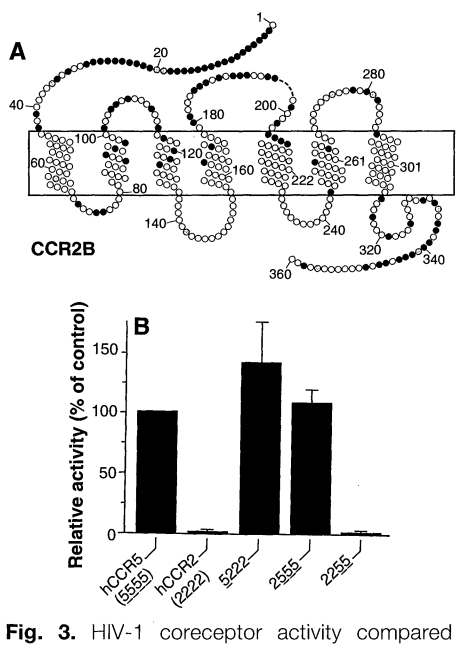

Fig. 3. HIV-1 coreceptor activity compared among chimeric receptors containing elements derived from human CCR5 and CCR2B. (**A**) Primary sequence of human CCR2B with designation of differences from human CCR5. Identical amino acids are indicated as open circles, residues with conservative substitutions as shaded circles, residues with nonconservative substitutions as closed circles, and residues within CCR5 lacking a corresponding residue within CCR2B as dashes. (**B**) Transfection-infection assay results with CCR5/CCR2B chimeras (*12*), determined by the methods described in Figs. 1 and 2.

no coreceptor activity for Ba-L in the transfection-infection system (Fig. 3B), despite abundant expression at the cell surface (*12*). As observed in the earlier chimeras, simple substitution of the NH_2-terminal segment of CCR2B with the corresponding segment from human CCR5 (5222) conferred robust susceptibility to HIV-1 cell entry (Fig. 3B). Again, a complementary chimera containing the NH_2-terminus of CCR2B and the remaining backbone of CCR5 (2555) was also fully functional as a coreceptor (Fig. 3B).

An additional fusion receptor (2255), representing the NH_2-terminal half of CCR2B (containing the extreme NH_2-terminal segment through extracellular loop 1) linked to the COOH-terminal half of human CCR5 (containing extracellular loops 2 and 3), repeatedly had no coreceptor function (2255, Fig. 3B), despite abundant expression on the cell surface (*12*). It was predicted that a chimera containing the NH_2-terminal half of human CCR2B and COOH-terminal half of human CCR5 (2255) would exhibit responses to diverse ligands, because the ligand-binding properties of these distinct receptors segregate largely to different regions of the receptor (*13*). Indeed, this receptor demonstrated substantial induction of phosphatidylinositol turnover in response to natural ligands for both CCR2B (MCP-1) and CCR5 (MIP-1β), whereas the parental receptors transmitted such signals exclusively in response to their native ligands (Fig. 4); the responses of 2255 to other ligands for CCR5 (MIP-1α and RANTES) were equally vigorous (*14*). The capacity of 2255 to mediate signal transduction responses to natural ligands did not correlate with HIV-1 coreceptor activity (Fig. 3B). This discordance was also observed in the converse situation, exemplified by the chimera described earlier containing the NH_2-terminus of CCR5 fused to the remainder of CCR2B (5222). Despite its vigorous function as a coreceptor for HIV-1 (Fig. 3), this chimeric molecule exhibited no detectable signaling response to cognate ligands for either parental receptor (Fig. 4). Taken together, these observations demonstrate that viral coreceptor activity is dissociable from ligand-dependent signaling responses. Whether or not the interaction of HIV-1 envelope proteins with CCR5 triggers receptor-dependent signal transduction remains to be determined. Nevertheless, this finding has potentially important pharmacologic implications, namely, that it may be feasible to develop therapeutic antagonists that disrupt receptor-virus interactions without eliciting the biologic consequences normally initiated by the binding of chemokines to CCR5.

Additional studies may help to determine whether or not other regions of chemokine receptors, such as transmembrane segments, also contribute to HIV-1 coreceptor activity. In the case of HIV-1 envelope protein (or proteins) as putative ligands for these receptors, the CCR5/CCR2 chimeras demonstrate that there is sufficient liberty within the overall interaction to accommodate substantial changes in one or another contributory segment of CCR5 without abolishing entirely coreceptor activity. Such relative lack of constraints may explain the reported ability of certain HIV-1 strains to engage multiple receptor types (*4*), because the interaction of a given envelope protein apparently can tolerate substantial variability in the chemokine receptor sequence. The murine form of CCR5, however, is incapable of mediating entry by the HIV-1 strains tested despite its substantial similarity to human CCR5. This fact provides one molecular basis for the absence of

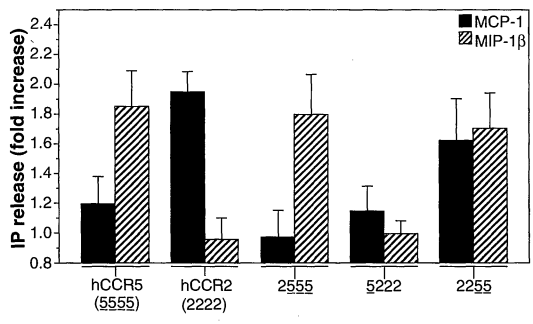

Fig. 4. Signaling responses of CCR5/CCR2 chimeras to chemokine ligands. COS-7 cells were transiently transfected with cDNAs encoding CCR5, CCR2, and the indicated CCR2/CCR5 chimeras (Fig. 3). Cells were loaded with myo-[^{3}H]inositol and incubated in the presence of 10 mM LiCl for 1 hour at 37°C in the presence of MCP-1 (100 nM) or MIP-1β (100 nM). Total [^{3}H]inositol phosphate was measured as described (*21*). Each data point was determined in triplicate, and the data shown are the means (± SEM) of three independent experiments. A value of 1.0 represents no phosphatidylinositol turnover.

infection of murine cells (*15*) and transgenic mice expressing human CD4 (*16*) and provides a rationale for transgenic approaches to developing animal models of HIV disease.

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9. We cloned cDNAs encoding human or murine CCR5 into the expression vector pcDNA3 (Invitrogen) after engineering the FLAG epitope into the NH_2-terminus as described (*13*). Expression of each construct was determined by FACS with an antibody to FLAG (anti-FLAG) (Boehringer Mannheim), and relative expression for each (see below) was calculated as the percentage of cells expressing human CCR5 on the cell surface normalized to the expression of hCCR5 (defined as 100%), with standard errors of the mean. The mean fluorescence intensity of the positive cells from any single sample never varied from the average by more than 30% in a single experiment. Therefore, neither the relative number of positive cells nor the absolute expression levels within transfected cells explains the differences in coreceptor activity. Chimeric receptors were prepared by the overlap polymerase chain reaction (PCR) method (*17*). hCCR5 (*HHHH*), human CCR5 (100% relative expression); mCCR5 (MMMM), murine CCR5 (126 ± 49%); *H*MMM, NH_2-terminus of human CCR5 [amino acids (aa) 1 to 32] fused to murine CCR5 (aa 35 to 354) (77 ± 22%); M*HHH*, NH_2-terminus of murine CCR5 (aa 1 to 34) fused to human CCR5 (aa 33 to 352) (73 ± 17%); M*H*MM, extracellular loop 1 and a portion of transmembrane domain 3 of human CCR5 (aa 86 to 118) replacing the corresponding segment of the murine receptor (aa 88 to 120) (37 ± 22%); MM*H*M, extracellular loop 2 and adjacent portions of human CCR5 (aa 134 to 210) replacing the corresponding region of the murine receptor (aa 136 to 212) (81 ± 30%); MM*HH*, NH_2-terminal half of mCCR5 (aa 1 to 162) fused to the COOH-terminal half of hCCR5 (aa 161 to 352) (80 ± 39%).
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12. We cloned cDNAs encoding human CCR2B or chimeras into the expression vector pCMV4 (*18*) after engineering the FLAG epitope into the NH_2-terminus as described (*13*). Expression of each construct (see below) was determined as described earlier. Chimeric receptors were prepared by the overlap PCR method (*17*). *5555*, human CCR5 (100% relative expression); 2222, human CCR2B (87 ± 2%); 5222, NH_2-terminus of CCR5 (aa 1 to 32) fused to CCR2B (aa 45 to 360) (27 ± 5%); *2555*, NH_2-terminus of CCR2B (aa 1 to 44) fused to CCR5 (aa 33 to 352) (108 ± 17%); 2255, CCR2B (aa 1 to 136) fused to CCR5 (aa 124 to 352) (119 ± 33%).
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20. COS-7 cells were transfected with 2 μg of plasmid DNA per well in a six-well plate as described (*19*). DNA samples consisted of appropriate combinations of 0.5 μg of a human CD4 expression plasmid [pCD4Neo (*19*)] or plain vector, and 1.5 μg of a chemokine receptor–expressing plasmid or plain vector. About 30 hours after addition of DNA, the medium in each well was replaced with 1.0 ml of medium containing HIV-1 Ba-L (~100 to 170 ng of p24 per sample; source: NIH AIDS Reagent Repository, passaged on primary human macrophages). About 10 hours later, an additional 1.0 ml of medium was added to each well. After 30 hours, the cells were recovered from the dish as described (*19*) and analyzed with a FacScan (Becton Dickinson). Staining for intracytoplasmic HIV-1 p24 was carried out with the Fix and Perm reagents (Caltag Laboratories), with a monoclonal antibody to p24 (Coulter Immunology) and goat anti-mouse fluorescein isothiocyanate (FITC)–conjugated secondary antibody (Becton Dickinson). Cells were further stained with phycoerythrin (PE)–conjugated anti-CD4 (Becton Dickinson). Appropriate controls indicated that the appearance of double-positive cells (FITC + PE) was dependent on cotransfection with both CD4 and human CCR5 expression plasmids and on the presence of HIV-1 Ba-L.
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Statistical Learning by 8-Month-Old Infants

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Learners rely on a combination of experience-independent and experience-dependent mechanisms to extract information from the environment. Language acquisition involves both types of mechanisms, but most theorists emphasize the relative importance of experience-independent mechanisms. The present study shows that a fundamental task of language acquisition, segmentation of words from fluent speech, can be accomplished by 8-month-old infants based solely on the statistical relationships between neighboring speech sounds. Moreover, this word segmentation was based on statistical learning from only 2 minutes of exposure, suggesting that infants have access to a powerful mechanism for the computation of statistical properties of the language input.

During early development, the speed and accuracy with which an organism extracts environmental information can be extremely important for its survival. Some species have evolved highly constrained neural mechanisms to ensure that environmental information is properly interpreted, even in the absence of experience with the environment (*1*). Other species are dependent on a period of interaction with the environment that clarifies the information to which attention should be directed and the consequences of behaviors guided by that information (*2*). Depending on the developmental status and the task facing a particular organism, both experience-independent and experience-dependent mechanisms may be involved in the extraction of information and the control of behavior.

In the domain of language acquisition, two facts have supported the interpretation that experience-independent mechanisms are both necessary and dominant. First, highly complex forms of language production develop extremely rapidly (*3*). Second, the language input available to the young child is both incomplete and sparsely represented compared to the child's eventual linguistic abilities (*4*). Thus, most theories of language acquisition have emphasized the critical role played by experience-independent internal structures over the role of experience-dependent factors (*5*).

It is undeniable that experience-dependent mechanisms are also required for the acquisition of language. Many aspects of a particular natural language must be acquired from listening experience. For example, acquiring the specific words and phonological structure of a language requires exposure to a significant corpus of language input. Moreover, long before infants begin to produce their native language, they acquire information about its sound properties (*6*). Nevertheless, given the daunting task of acquiring linguistic information from listening experience during early development, few theorists have entertained the hypothesis that learning plays a primary role in the acquisition of more complicated aspects of language, favoring instead experience-independent mechanisms (*7*). Young humans are generally viewed as poor learners, suggesting that innate factors are primarily responsible for the acquisition of language.

Here we investigate the nature of the

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