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Chemokine Receptors in HIV-1 and SIV Infection

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Seven transmembrane segment (7TMS) receptors for chemokines and related molecules have been demonstrated to be essential, in addition to CD4, for HIV and SIV infection. The β-chemokine receptor CCR5 is the primary, perhaps sole, coreceptor for HIV-1 during the early and chronic phases of infection, and supports infection by most primary HIV-1 and many SIV isolates. Late-stage primary and laboratory-adapted HIV-1, HIV-2, and SIV isolates can use other 7TMS receptors. CXCR4 appears especially important in late-stage HIV infection; several related receptors can also be used. The specificity of SIV viruses is similar. Commonalities among these receptors, combined with analyses of mutated molecules, indicate that discrete, conformationally-dependent sites on the chemokine receptors determine their association with the third variable and conserved regions of viral envelope glycoproteins. These studies are useful for elucidating the mechanism and molecular determinants of HIV-1 entry, and of inhibitors to that entry.

Key words : Chemokine, Receptor, HIV, SIV, CCR5, Transmembrane segment, CXCR4

Chemokines, their receptors and HIV coreceptor functions

While all well-characterized isolates of HIV-1 are dependent on CD4 as the primary viral receptor, different HIV-1 viruses can infect different subsets of CD4-expressing cells (Dalgleish *et al.*, 1984; Klatzmann *et al.*, 1984; Maddon *et al.*, 1986; Koenig *et al.*, 1986; Pope *et al.*, 1994; Broder and Berger, 1995; Weissman *et al.*, 1995). Macrophage-tropic (M-tropic) HIV-1 isolates, which lack the ability to infect most laboratory cell lines, require the presence of the chemokine receptor CCR5 to effect fusion with the target cells (Ashorn *et al.*, 1990; Choe *et al.*, 1996). These viruses, which infect primary macrophages and T cells, predominate during the early and chronic phases of HIV-1 infection. As HIV-1 infection progresses, viruses emerge which gain the ability to infect laboratory T cell lines as a consequence of their ability to use the α-chemokine receptor CXCR4 in addition to CCR5 as their coreceptor (Åsjö *et al.*, 1986; Schuitemaker *et al.*, 1992; Choe *et al.*, 1996; Feng *et al.*, 1996). These isolates are referred to as primary T cell-line tropic or dual-tropic viruses, reflecting their ability to infect laboratory cell lines, as well as primary T lymphocytes and macrophages. Dual-tropic viruses serve as precursors to viruses that have been extensively passaged on T cell lines. These viruses, dubbed laboratory-adapted, or T-tropic viruses, use CXCR4, but not CCR5, as a coreceptor (Choe *et al.*, 1996; Feng *et al.*, 1996). As a consequence they cannot infect macrophages but still infect primary T cells and T cell lines.

Both CCR5 and CXCR4 are G-protein coupled, 7TMS receptors (Premack and Schall, 1996). CCR5 is present on monocytes, some macrophages, microglia and CD4-positive T cells as well as CD8-positive T cells; the level of CCR5 on T cells is inducible with interleukin 2 (Loetscher *et al.*, 1996). Thus, activated T cells (or T cells in an infectious milieu) have up-regulated HIV coreceptor expression. The ligands for CCR5 include MIP-1α, MIP-1β and RANTES, factors which inhibit HIV-1 replication of M-tropic virus on peripheral blood mononuclear cells (PMBC) (Cocchi *et al.*, 1995). CXCR4 is a ubiquitous α-chemokine receptor present on virtually all hematopoietic cells, as well as many epithelial and mesenchymal cells. The known ligand for CXCR4, SDF-1α, inhibits the replication of T-tropic viruses on PBMC (Bleul *et al.*, 1996a; Oberlin *et al.*, 1996).

As indicated in Table I, a number of other coreceptors including CCR3 (expressed on eosinophils, basophils, mast cells, brain microglia, and some TH-2 T lymphocytes), CCR2b, CCR8, and the orphan receptor APJ have been identified by our group and others, for both M-tropic and dual-tropic viral isolates; HIV-2 and SIV envelopes may use yet other coreceptors in

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Table I. Human coreceptors for the primate immunodeficiency viruses grouped according to efficiency of viral entry on CF 2Th cells in the presence of CD4

High
CCR5 (dual- and M-tropic)
CXCR4 (dual- and T-tropic)
gpr15 (siv)
str133 (siv)
Moderate
CCR3 (M-, dual-, and T-tropic)
apj (dual-, and T-tropic)
gpr1 (siv)
Low but detectable
CCR8 (M-, dual- and T-tropic)
CCR9 (T-tropic)
v28 (dual- and T-tropic)
gpr15 (M-tropic)
str133 (M-, dual-, T-tropic)
CCR2b (dual- and T-tropic)

Those receptors listed as 'high' support entry levels at or above entry of the M-tropic virus ADA on CCR5 for the viral envelope which uses it most efficiently. Receptors listed as 'moderate' support entry at 10~30% of the entry of ADA on CCR5. Receptors listed as low support entry lower than 10% of entr of ADA on CCR5. Type of virus using receptor is indicated in pa-

addition to CCR5, including STRL33/Bonzo, BOB/GPR-15, APJ and GPR-1. The physiological relevence of these other receptors remains to be demonstrated, although evidence exists for an important role for CCR3 for HIV-1 infection in the central nervous system (He *et al.*, 1997).

Thus, the overwhelming number of viral isolates from recently infected individuals utilize CCR5 as their obligate coreceptor, even though CXCR4 and other potential coreceptors are also present on candidate target cells. Exactly why viral use of CCR5 predominates during most of HIV-1 infection, and what are the immune determinants for the emergence of dual- and multi-tropic viruses, are important issues that remain to be explored.

Lessons from coreceptor chimeras

Several groups have attempted to define the molecular determinants of coreceptor function by assessing HIV-1 infection and syncytium formation using receptor chimeras (Atchison *et al.*, 1996; Rucker *et al.*, 1996; Bieniasz *et al.*, 1997; Doranz *et al.*, 1997; Farzan *et al.*, 1997). In general, the results are complex. Following the initial report by Rucker, Samson and colleagues, several groups have exploited sequence similarities between CCR2b and CCR5 (human but not mouse), between CXCR4 and CXCR1, and between CCR3 and CCR1. The following conclusions are generally agreed upon by these studies.

First, the amino-terminal region plays a critical role in tropism for M-tropic strains, perhaps an even more dramatic role for dual-tropic strains, and likely no role for T-tropic or laboratory-adapted strains. Second, different viral Env proteins within a tropic group behave similarly but not identically and may exhibit slight overlap with respect to their CCR5 binding sites. Third, the second extracellular loop of the receptor is a dominant recognition site for T-tropic and laboratory adapted strains. Thus, work based on the generation of chimeric receptors supports the hypothesis that the gp120 binding site on the chemokine receptor is a discontinuous surface with contributions from several of the extracellular domains of the latter proteins.

There are several caveats in interpreting data gathered with this approach. A major concern stems from topological constraints in the chemokine receptors. While commonly represented in a two-dimensional serpentine arrangement, physical studies have revealed that the first and second extracellular loops as well as the amino-terminal peptide and the third extracellular loop are stabilized by disulfide bonds, and that multiple interactions are likely to exist between the amino-terminal and third loop as well as extracellular loops 1 and 2. Since each chimera is a multiply substituted structure (ie, the sum of a number of point mutations), loss of function due to substitution of a particular region of the receptors could be overinterpreted. For example, deletion of 4 or 8 residues from the amino-terminus of CCR5 had no effect on a fusion assay using the JRFL or 89.6 envelope glycoproteins. Thus, one would conclude from these data that residues 1~8 of CCR5 are not necessary for support of viral entry. However, when the same deletions were evaluated in a CCR2b molecule containing the amino-terminus of CCR5, dramatic decreases were seen in the entry of 89.6 Env with deletion of residues 2~5, and entry of JR-FL was ablated following deletion of residues 4~8. Thus, the extracellular regions of the chemokine receptors are probably not independent domains.

A second caveat with the chimeric receptor approach relates to the nature of the constructs. In many instances, transmembrane helices are also included as part of the chimeric construct. As has been well documented for other chemoattractant receptors such as the C5a receptor (Kolakowski *et al.*, 1995), the transmembrane segments are integrally coupled to the binding affinity and presumably the conformation of the extracellular regions. An example of this type of complexity was observed with CCR5/CCR2b chimeras reported by Atchison *et al.* (1996), and Farzan *et al.* (1997). One chimera was produced that contained only the extracellular amino-terminal 32 amino acids of CCR2b fused to the CCR5 backbone (Atchison *et al.*, 1996). This receptor exhibited wild-type entry for the Bal M-

tropic virus. A second construct was reported in which the sequence consisting of the amino-terminal domain, first transmembrane segment and first intracellular loop of CCR2b was fused to CCR5 via a common MscI site. This construct was well expressed, had gain of function for MCP-1 binding as well as retention of binding for MIP-1α and RANTES, but failed to function as an HIV-1 coreceptor for the ADA and YU-2 M-tropic viruses. Thus, while the amino-terminus of CCR2b can apparently substitute for CCR5, inclusion of the anchoring transmembrane segment 1 and first intracellular loop negate this retained function. Since it is unlikely that any part of the CD4/Env complex associates with these latter regions, we suggest that the three dimensional arrangement of the extracellular regions is influenced by the transmembrane and intracellular domains. Thus, chimeric receptors are a valuable first approximation to defining binding sites but any conclusions must be further substantiated using independent biochemical analyses.

A final caution with respect to recombinant viral entry assays relates to the quantitation itself. Many factors potentially affect virus entry, including the level of coreceptor expression, potential quaternary structure of the surface receptors, variation in CD4 levels, and host cell post-translational modification of receptors. Even when these factors are controlled, it is important that the entry assay is performed in a linear range of the assay.

Scanning mutagenesis of CCR5

An alternative approach to defining regions of envelope glycoprotein and chemokine receptor interaction is afforded by alanine-scanning mutagenesis. The most common approach is to substitute alanine for charged amino acid residues, because of the importance of electrostatic protein-protein interactions. However, we reasoned that the interaction of gp120 with CCR5 might involve hydrophobic interactions, as the binding site on gp120 appears to become exposed only after CD4 binding. We were particularly interested in the role of tyrosyl residues in CCR5, because aromatic residues have previously been demonstrated to be important for a number of hydrophobic protein-protein contacts, including the gp120/CD4 interaction (Arthos *et al.*, 1989). Dramatic alterations were observed on the ability of M-tropic YU2 and dual-tropic 89.6 envelopes to infect cells expressing CCR5 with mutation of residues Tyr^{10}, Asp^{11}, Tyr^{14}, Tyr^{15}, Glu^{18}, Gln^{21} and Lys^{22}. A greater sensitivity of dual-tropic 89.6 to Glu^{18} is notable because this residue is present as glutamate in both CCR5 and CXCR4. The $SIV_{mac}239$ envelope glycoprotein also showed sensitivity to changes in Tyr^{10}, Asp^{11} and Tyr^{14}, but not Tyr^{15} or Glu^{18}. These differing dependencies play a role in the different ability of other coreceptors, like GPR-1, GPR15 and STRL33 to support $SIV_{mac}239$ entry, but not efficient HIV-1 entry, as described below.

Examination of point mutants in the extracellular loops revealed a less clear picture, with none of the mutants significantly effecting YU2 entry, while certain positions in the first and second extracellular loops had partial effects on the dual-tropic 89.6 viral entry. The possibility that the first and second extracellular loops contribute to dual-tropic (as well as T-tropic) viral entry is consistant with the chimeric receptor studies. $Gln^{280}\rightarrow$ Ala in the third extracellular loop significantly decreased YU2 and 89.6 entry. As this residue is predicted to be at the membrane interface with transmembrane segment 7, it is possible that this mutation grossly affects the conformation of extracellular loop 3 and, by extension, through the putative disulfide bond, the amino-terminal region as well.

The N-terminal motif YDINYY and additional SIV and HIV-1 coreceptors

The alanine-scanning mutagenesis studies described above indicate that a tyrosine- and aspartic acid-enriched sequence in the amino-terminus of CCR5 (the YDINYY motif) plays a central role in coreceptor function. Further, these data suggest that additional coreceptors for HIV-1, HIV-2 and SIV might be identified through analysis of the genomic database for sequences containing a similar amino-terminal motif. A collection of publicly available G-protein receptor sequences was compiled, with special attention given to known motifs associated with chemokine receptors. These include the DRYLAIV signature sequence following transmembrane segment 3, conserved cysteine residues in the amino-terminal region and third extracellular loop, and acidic properties to the amino-terminal peptide (Fig. 1). In addition, some receptors that lacked one or more of these characteristics, but contained regions similar to the YDINYY region of CCR5, were also included for analysis. We have identified several additional coreceptors using this approach including two receptors for $SIV_{mac}239$, and two for several dual- and T-tropic HIV-1 viruses. All of these receptors have an identifiable region that is similar to the YDINYY region in CCR5 for the occurrance of tyrosines and acidic amino acids.

$SIV_{mac}239$ can use CCR5 as a coreceptor. However, previous studies have indicated that a novel coreceptor for SIV may exist on CEMx174 cells and U87 glioma cells (Farzan *et al.*, in press; Clapham *et al.*, 1991). Neither of these cell lines expresses CCR5, but both can support replication of $SIV_{mac}239$. Furthermore, $SIV_{mac}239$, but not M-tropic HIV-1, can replicate in PBMC from individuals lacking functional CCR5 protein (Clapham *et al.*, 1991). We therefore screened for

Receptors for primary dual- or T-tropic HIV-1 viruses

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ccr5            MDYQVSSPIYDINYYTSEPC
cxcr4             MEGISIYTSDNYTEEMG
apj             MEEGGDFDNYYGADNQSECEY
ccr3      MTTSLDTVETFGTTSYYDDVGLLCEK
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Receptors for SIVmac239

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ccr5 (rhesus)   MDYQVSSPIYDIDYYTSEPC
ccr5            MDYQVSSPIYDINYYTSEPC
gpr15            MDPEETSVYLDYYYATSPN
gpr1     MEDLEETLFEEFENYSYDLDYYSLESD
strl33             MAEHDYHEDYGFSSFN
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Fig. 1. Alignment of the N-terminii of receptors supporting high or moderate entry of $SIV_{mac}239$ or the primary dual or 5-topic viruses. Residues aligned with those demonstrated to be important for CCR5 entry of SIV-mac239 (top) or the dual tropic virus 89.6 (bottom) are shown in bold. Glycosylated asparagines are underlined. Primary T-tropic viruses do not enter cells expressing CCR5 but typically use the remaining receptors shown.

expression of candidate 7TMS receptors in CEMx174 and U87 cells and tested the ability of those present to support infection by $SIV_{mac}239$. Two receptors, GPR-1 and GPR-15, present respectively on U87 and CEMx174 cells, suppported infection of $SIV_{mac}239$ (Chen *et al.*, 1997). GPR-15 is present on T cells and also weakly supports the entry of a subset of M-tropic viruses. At the same time, Farber and Berger identified STRL33 as a coreceptor supporting efficient infection by SIV and marginal infection by HIV-1 (Farzan *et al.*, 1997b). Independently and in parallel, Littman and colleagues used an elegant expression cloning system to identify two SIV coreceptors, termed Bonzo and BOB, that were identical to STRL33 and GPR-15, respectively (Liao *et al.*, 1997). The ability of these coreceptors to support SIV and some HIV-1 infection underscores the importance of the tyrosine-rich amino-terminal motif for HIV-1 and SIV infection (Fig. 1). It is notable that STRL33 lacks residues analogous to Tyr^{15} and Glu^{18}; $SIV_{mac}239$ showed no sensitivity to changes at these positions in CCR5 that interfered with HIV-1 infection (Arthos *et al.*, 1989).

Our success using this approach encouraged us to test other 7TMS receptors with amino-terminal tyrosines and acidic amino acids arranged similarly to those in CCR5 (Weissman *et al.*, 1997). One such receptor, APJ, supports entry of the dual-tropic viruses 89.6, ELI and UG21. APJ, like GPR-1, GPR-15 and STRL33, lacks several of the hallmark motifs of the chemokine coreceptors. It shares amino-terminal homology with CCR5, but also has pronounced amino-terminal homology with CCR3 and CXCR4. APJ supports entry of viruses which also use CCR3 and CXCR4. The similarity of APJ to CCR5 and CXCR4 suggest that it could function as a bridge by which viruses can adapt to

Table II. CD4-dependence of gp120 binding to CCR5

Receptor+gp120 strain	K_d, nM +CD4	−CD4
Human CCR5+gp120 Yu2	5+	nd
Human CCR5+gp120 $SIV_{mac}239$	4.4+1.6	nd
Rhesus CCR5+gp120 Yu2		
Rhesus CCR5+gp120 $SIV_{mac}239$	8.2+1.1	14.4+3.9
Human CCR5(N^{13}→D)+gp120 $SIV_{mac}239$	4.5+3.7	3.0+1.2
Rhesus CCR5(D^{13}→N)+gp120 $SIV_{mac}239$	8.2+1.7	nd
Rhesus CCR5(T^{9}→D)+gp120 $SIV_{mac}239$	11.4+	4.0+

Binding affinity for each of the glycoproteins indicated was determined in the presence or absence of 100 nM soluble CD4 (Wu *et al.*, 1996; Horuk *et al.*).
nd: not detected.

CXCR4 from CCR5. We have also shown that the receptor identified in the Genbank database as CCR9, weakly supports the entry of at least on dual-tropic virus. Doms and colleagues have also reported that CCR8, as well as the orphan receptor V28, supports some HIV-1 infection (Deng *et al.*, 1997). The physiological relevance of these new receptors remains to be proven, howecer, their identification validates the importance of the tyrosine-rich amino-terminal motif as a key structural feature for coreceptor function (Table II). As described below, an independent line of evidence calls attention to this region as well.

CD4-independent interaction of SIV gp120 with rhesus CCR5

Rhesus CCR5 displays identical coreceptor activity towards HIV-1 M-tropic strains as does the human receptor, despite eight amino acid substitutions. Five of these are conservative changes in the transmembrane segments, one conservative Lys→Arg replacement in the second extracellular loop, and two non-conservative substitutions in the amino-terminal region (Thr ^{9}→Ile and Asp^{13}→Asn). To more fully characterize the interaction between gp120 and rhesus CCR5, we performed soluble glycoprotein binding assays with radiolabelled gp120 of YU2 and $SIV_{mac}239$. In each case nanomolar affinity binding was observed in the presence of soluble CD4. However, $SIV_{mac}239$ Env binding to rhesus CCR5 could be demonstrated in the absence of CD4 (Table II). In the case of YU2, binding of Env was CD4-dependent for both rhesus and human CCR5. Thus, a dramatic difference in the requirement for CD4 was observed with minimal changes between the two coreceptors. Given the important role of the YDINYY motif as described above, we were interested in the fact that the asparagine residue at position 15 in human CCR5 was non-conservatively replaced with aspartic acid in the rhesus homologue. To test the hypothesis that this position is critical for dictating the CD4-dependence

of CCR5 binding, the respective point mutants were made in both human and rhesus CCR5. Change of this single position on the CCR5 molecule conferred gain of function (CD4-independence) for human CCR 5 binding of $SIV_{mac}239$ gp120, while resulting in the acquisition of CD4 dependence for the rhesus CCR5/ $SIV_{mac}239$ gp120 interaction. CD4-independent SIV and HIV-2 entry has been previously reported (Endres *et al.*, 1996), but entry of $SIV_{mac}239$ pseudotyped virus in the absence of CD4 remains to be confirmed.

Further evidence for both the importance of the YDINYY region of the coreceptor and its differential use by M-tropic HIV-1 and SIV, or HIV-2 envelopes was obtained through analysis of the alanine scanning mutants. In contrast to the entry data obtained with YU 2 and 89.6 Env the entry of $SIV_{mac}239$ was enhanced greater than 2-fold by the mutants Tyr^{15}→Ala and Asp^{18} →Ala (Fig. 1). The introduction of the negatively charged aspartic acid at position 13, which confers CD4-independence to $SIV_{mac}239$ binding, may be correlated with differences in gp120 structure that change the precise epitopes contacting the amino-terminal sequence of CCR5.

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