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REVIEW ARTICLE

An overview of the determinants of CCR5 and CXCR4 co-receptor function

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HIV-1 entry into target cells

Virus entry into target cells is the key first step of virus replication and is mediated by interactions between viral envelope glycoproteins and plasma membrane receptors. The human immunodeficiency virus type 1 (HIV-1) envelope glycoproteins gp120 and gp41 are non-covalently associated and form trimers on the surface of the virus particle (Moore *et al.*, 1993). HIV-1 entry into target cells is initiated by the consecutive interaction of the soluble unit gp120 with CD4 and a co-receptor, whereas virus–cell membrane fusion is mediated by the transmembrane unit gp41 (Moore *et al.*, 1993).

Gp120 is composed of five constant domains (C1–C5) and five variable loops (V1–V5), which are organized into an inner and an outer domain, connected by a four-stranded anti-parallel β-sheet, called the bridging sheet. This domain includes the V1/V2 stem and two strands derived from C4 (Kwong *et al.*, 1998; Wyatt *et al.*, 1998; Wyatt & Sodroski, 1998). The first extracellular domain of the CD4 receptor (Bour *et al.*, 1995; Wu *et al.*, 1997*a*) associates with a highly conserved groove at the interface of the inner and outer domains and the bridging sheet of gp120 (Kwong *et al.*, 1998). Gp120–CD4 complex formation generates a large bonding energy that drives reordering of the gp120 core structure (Kwong *et al.*, 1998; Myszka *et al.*, 2000; Wyatt & Sodroski, 1998). Changes in the orientation of the V1/V2 and V3 loops, as well as the bridging sheet, cooperatively create/expose a co-receptor-binding site on gp120 (Kwong *et al.*, 1998; Rizzuto *et al.*, 1998; Wyatt & Sodroski, 1998). The predicted co-receptor-binding surface on gp120 has a hydrophobic core surrounded by a positively charged periphery and is composed of both conserved and variable residues (Kwong *et al.*, 1998; Rizzuto *et al.*, 1998). The gp120/co-receptor interaction drives additional conformational changes within the gp120/gp41 trimer that trigger the insertion of the gp41 fusion peptide into the plasma membrane (Chan *et al.*, 1997; Weissenhorn *et al.*, 1997).

HIV-1 co-receptors

A number of CC- and CXC-chemokine receptors, belonging to the seven transmembrane G protein-coupled receptor family, have been shown to act as HIV-1 co-receptors *in vitro* (Zhang *et al.*, 1998; Zhang & Moore, 1999). However, numerous studies have shown that CCR5 and CXCR4 are the major HIV-1 co-receptors *in vivo* (Zhang *et al.*, 2000; Zhang & Moore, 1999). In particular, the resistance to infection by HIV-1 of $\Delta 32/\Delta 32$ individuals, who lack CCR5 entirely, and the protective effect of $\Delta 32$ heterozygosity on early disease progression demonstrate that this co-receptor is responsible for virus transmission and early stage HIV-1 replication (Bennetts *et al.*, 1997; de Roda Husman *et al.*, 1997; Garred *et al.*, 1997; Huang *et al.*, 1996; Meyer *et al.*, 1997; Michael *et al.*, 1997).

The selective use of the CCR5 and/or CXCR4 co-receptors to a large extent explains the cellular tropism of different HIV-1 isolates (Berger *et al.*, 1998; Doms & Moore, 1997; Fenyo *et al.*, 1997). CCR5 is the principal co-receptor for HIV-1 variants that are sexually transmitted and persist within the majority of infected individuals (R5 isolates). The appearance of variants that use CXCR4 or both co-receptors (X4 and R5X4 isolates) signals accelerated $CD4^+$ T-cell loss and disease progression (Connor *et al.*, 1997; Simmons *et al.*, 1996). The phenotypic switch from R5 to X4 viruses *in vivo* typically occurs only after several years of infection. This is surprisingly slow given that changing only a few residues in gp120 can be sufficient to convert an R5 virus into an R5X4 virus *in vitro* and that such changes must be occurring continuously *in vivo* given the error rate of reverse transcription (Chan *et al.*, 1999; Chavda *et al.*, 1994; Chesebro *et al.*, 1992; Cho *et al.*, 1998; Cocchi *et al.*, 1996; Harrowe & Cheng-Mayer, 1995; Hwang *et al.*, 1991; Ivanoff *et al.*, 1991; Kwong *et al.*, 2000; Menzo *et al.*, 1998; Moore & Nara, 1991; Morris *et al.*, 1994; Page *et al.*, 1992; Shioda *et al.*, 1992; Trkola *et al.*, 1996; Verrier *et al.*, 1999; Wang *et al.*, 1999 *a*; Wu *et al.*, 1996). These observations imply that the transition to CXCR4 usage is specifically suppressed *in vivo* (Michael & Moore, 1999). It should be noted here that co-receptor usage and switching has been analysed most extensively for clade B isolates, which predominate in North America and Western Europe (Bazan *et al.*, 1998; Berger, 1997). Non-clade B viruses now cause the vast majority of new HIV-1 infections worldwide and should therefore be the major focus of vaccine efforts and drug development efforts. Compared to clade B isolates, however, these viruses are

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understudied and their immunogenic and biological properties remain largely unknown.

Determinants of CCR5 co-receptor function

Early attempts to identify the determinants of CCR5 co-receptor function relied on the use of chimera comprising segments of CCR5 and related chemokine receptors such as murine CCR5 or CCR2b, which do not mediate HIV-1 fusion and entry (Alkhatib *et al.*, 1997*a*; Atchison *et al.*, 1996; Bieniasz *et al.*, 1997; Doranz *et al.*, 1997*b*; Picard *et al.*, 1997; Rucker *et al.*, 1996; Wang *et al.*, 1999*b*). Results from these studies are difficult to interpret and reconcile because of differences in chimeric constructs, HIV-1 isolates and detection systems that were used to test the role of the CCR5 extracellular domains in co-receptor function. The general conclusion that can be drawn from these studies is that multiple CCR5 domains directly or indirectly contribute to its co-receptor activity, and that the CCR5 amino-terminal domain (Nt) plays a privileged role in virus fusion and entry. When the CCR5 Nt is grafted onto other chemokine receptors, such as CCR1 or CCR2b, it enables them to mediate virus fusion and entry (Atchison *et al.*, 1996; Bieniasz *et al.*, 1997; Doranz *et al.*, 1997*b*; Picard *et al.*, 1997; Rucker *et al.*, 1996). In contrast, no CCR5 extracellular loop alone can confer HIV-1 co-receptor activity upon another chemokine receptor. [The one exception is extracellular loop 1 (ECL1) of human CCR5 placed into murine CCR5 (Atchison *et al.*, 1996; Picard *et al.*, 1997).] Substituting the CCR5 Nt with the Nt of a related chemokine receptor such as CCR2b or murine CCR5, however, is not accompanied by a significant loss of CCR5 co-receptor function (Atchison *et al.*, 1996; Bieniasz *et al.*, 1997; Doranz *et al.*, 1997*b*; Picard *et al.*, 1997; Rucker *et al.*, 1996). Finally, it should be noted that deletion of the CCR5 carboxy-terminal domain or the Asp-Arg-Tyr sequence in the second intracellular loop has no effect on virus fusion and entry *in vitro*. Therefore, CCR5-mediated intracellular signalling and endocytosis are not required for its co-receptor activity in cell lines but may play a role *in vivo* (Alkhatib *et al.*, 1997*a*; Atchison *et al.*, 1996; Doranz *et al.*, 1997*b*).

Point mutagenesis studies have provided a clearer picture of the determinants of CCR5 co-receptor function. It has been shown that the negatively charged and tyrosine residues in the CCR5 Nt (Asp-2, Tyr-3, Tyr-10, Asp-11, Tyr-14, Tyr-15 and Glu-18) are important for CD4-induced gp120–CCR5 binding and virus entry (Blanpain *et al.*, 1999; Doranz *et al.*, 1997*b*; Dragic *et al.*, 1998; Farzan *et al.*, 1998; Rabut *et al.*, 1998). Other Nt residues that were found to be involved in co-receptor function include Ser-6, Ser-7, Ile-9, Asn-13, Gln-21 and Lys-22 (Blanpain *et al.*, 1999; Farzan *et al.*, 1998; Rabut *et al.*, 1998). Residues in the CCR5 extracellular loops that were found to influence co-receptor function include Gln-93 in ECL1 (Kuhmann *et al.*, 1997); Gly-163 in the transmembrane helix 4 (TM4)/ECL2 junction (Siciliano *et al.*, 1999); Tyr-184, Ser-185 and Arg-197 in ECL2 (Doranz *et al.*, 1997*b*; Ross *et al.*, 1998); Asp-276 and Gln-280 in ECL3 (Doranz *et al.*, 1997*b*; Farzan *et al.*, 1998). It should be noted that Gly-163 was only studied in the context of a Gly → Arg substitution; hence the introduction of a guanidinium rather than the loss of a hydrogen atom may be responsible for the lack of co-receptor function of this CCR5 mutant (Siciliano *et al.*, 1999). Furthermore, residues 184 and 185, like residues 197 and 276, have to be substituted together in order to compromise virus fusion and entry (Doranz *et al.*, 1997*b*; Ross *et al.*, 1998). Despite minor discrepancies between the different point mutagenesis studies, all lend support to the finding that a cluster of residues located in the CCR5 Nt is required for HIV-1 fusion and entry by participating in gp120 binding to CCR5.

Many reports have speculated on the role of CCR5 post-translational modifications in co-receptor function; the CCR5 Nt undergoes both *O*-glycosylation and tyrosine sulfation (Farzan *et al.*, 1999). It is presently not known whether *O*-glycosylation plays a role in co-receptor function, but this possibility is suggested by the preponderance of amino-terminal serines that are important for virus entry. Moreover, *N*-linked oligosaccharides critically affect CXCR4 co-receptor function (Chabot *et al.*, 2000). Inhibition of cellular sulfation pathways, including tyrosine sulfation, greatly decreases gp120 binding to CCR5 as well as the entry of R5 and R5X4 HIV-1 strains into target cells (Farzan *et al.*, 1999). Two groups recently demonstrated that CCR5 Nt-based peptides containing sulfotyrosines, but not tyrosines or phosphotyrosines, specifically bind soluble gp120–CD4 complexes (Cormier *et al.*, 2000; Farzan *et al.*, 2000). Soluble envelope glycoproteins from both R5 and R5X4 strains bind to CCR5 Nt sulfopeptides, but soluble envelope glycoproteins from an X4 strain do not (Cormier *et al.*, 2000). The CCR5 Nt therefore specifically interacts only with gp120 proteins from isolates that use this co-receptor. Recently, CCR5 Nt sulfopeptides were shown to bind to conserved residues in the C4/V3 stem region of gp120 (Cormier *et al.*, 2001). Residues in the V3 crown, however, were shown to be important for gp120 binding to cell surface CCR5 but not to the Nt sulfopeptides (Cormier *et al.*, 2001). Furthermore, the affinity of soluble gp120–CD4 for CCR5 Nt sulfopeptides is about 10–100-fold lower than for the native, membrane-associated co-receptor (Cormier *et al.*, 2000; Trkola *et al.*, 1996; Wu *et al.*, 1996).These observations lend support to the idea that there is a second gp120-binding site on CCR5 that consolidates the association between the co-receptor and the envelope glycoprotein, lowering the K_d into the nanomolar range (Doranz *et al.*, 1997*b*; Dragic *et al.*, 2000; Rucker *et al.*, 1996).

The characterization of inhibitors of CCR5-mediated HIV-1 entry has provided further insight into the structure–function relationships of this co-receptor. Agents that target CCR5 co-receptor function belong to one of four categories of molecules: monoclonal antibodies (MAbs), chemokines and their derivatives, peptides and small molecules (< 1 kDa).

Anti-CCR5 MAbs whose epitopes include residues in the Nt strongly inhibit gp120 binding to CCR5 but only moderately inhibit HIV-1 fusion and entry, whereas MAbs whose epitopes include residues in ECL2 potently inhibit HIV-1 fusion and entry but only moderately inhibit gp120 binding (Lee *et al.*, 1999; Olson *et al.*, 1999; Wu *et al.*, 1997*b*). Anti-ECL2 MAbs, therefore, must act through a secondary mechanism in order to disrupt CCR5 co-receptor function more efficiently than anti-Nt MAbs. Possibly, these MAbs inhibit important post-gp120-binding steps, such as conformational changes in CCR5 or its oligomerization (Kuhmann *et al.*, 2000). Chemokines and their derivatives inhibit HIV-1 fusion and entry both by blocking gp120 binding to CCR5 and by decreasing co-receptor availability on the cell surface (Alkhatib *et al.*, 1997*b*; Trkola *et al.*, 1998).

Peptides based on the TM helices of CCR5 inhibit HIV-1 replication and chemokine-induced signalling (Tarasova *et al.*, 1999), presumably by disrupting helix–helix interactions, which may influence CCR5 conformation and/or oligomerization (Kuhmann *et al.*, 2000). The disruption of TM helix–helix interactions might also explain the inhibitory effect of TAK-779 on HIV-1 fusion and entry. TAK-779, a small-molecule CCR5 antagonist, inhibits gp120 binding to CCR5 by inserting into a pocket formed by TM helices 1, 2, 3 and 7 (Baba *et al.*, 1999; Dragic *et al.*, 2000). Whatever the mechanism of inhibition by these different agents may be, the data thus far suggest that CCR5 co-receptor function is not limited to gp120 binding to the co-receptor Nt. Other regions of this co-receptor have functions that are necessary for the successful completion of virus fusion and entry either by providing a secondary gp120-binding site on CCR5, and/or by mediating conformational changes or the oligomerization of CCR5. Possibly, CCR5 interactions with CD4 or other cell surface molecules also contribute to its co-receptor function (Golding *et al.*, 1999; Lapham *et al.*, 1996).

Determinants of CXCR4 co-receptor function

The determinants of CXCR4 co-receptor function were studied using chimera of CXCR4 and related chemokine receptors such as CXCR2 or rat and murine CXCR4, which do not mediate virus fusion and entry (Brelot *et al.*, 1997; Doranz *et al.*, 1999; Lu *et al.*, 1997; Reeves *et al.*, 1998; Willett *et al.*, 1998). No single domain of CXCR4 can confer co-receptor activity upon CXCR2 (Doranz *et al.*, 1999; Lu *et al.*, 1997). Replacing the CXCR4 Nt by that of CXCR2 does not abolish co-receptor activity, but replacing the CXCR4 ECL2 by that of CXCR2 generates a non-functional chimeric co-receptor (Doranz *et al.*, 1999; Lu *et al.*, 1997). Substitution of the CXCR4 Nt by that of CCR5 generates a hybrid co-receptor capable of mediating fusion of both R5 and X4 isolates, albeit inefficiently (Doranz *et al.*, 1997*b*). Sequence differences between human and murine CXCR4 ECL2s are responsible for murine CXCR4 lack of co-receptor activity (Parolin *et al.*, 1998). Using a rat/human CXCR4 chimera, it was shown that the HIV-1_{NDK} isolate requires both the Nt and ECL2 for efficient fusion and entry, whereas HIV-1_{LAI} only requires the presence of the CXCR4 ECL2 (Brelot *et al.*, 1997). HIV-2_{ROD} also requires both the CXCR4 Nt and ECL2 for fusion and entry (Reeves *et al.*, 1998; Willett *et al.*, 1998). The general conclusion from all of these studies is that the CXCR4 Nt and ECL2 are essential for its co-receptor activity, but are not used equally by all HIV-1 and HIV-2 isolates.

Studies of CXCR4 point mutants did not delineate more precisely a region of CXCR4 that plays a pivotal role in virus fusion and entry. Several mutations enable CXCR4 to mediate weak fusion and entry of R5 strains, including conversion of Asp-187 to a neutral residue (Chabot & Broder, 2000; Wang *et al.*, 1998), alanine substitutions of Arg-30 and Asp-193 (Chabot *et al.*, 1999), and removal of an *N*-linked glycosylation site in the CXCR4 Nt (Chabot *et al.*, 2000). Charged residues Asp-193, Arg-183 and Arg-188 in ECL2 were shown to differently affect the entry of various HIV-1 strains; for example, only HIV-1_{NDK} was sensitive to substitutions of Asp-193, whereas all other test strains were sensitive to changes in Arg-183 and Arg-188 (Brelot *et al.*, 1999). In a recent report, Glu-15 and Glu-32 in the Nt, Asp-97 in ECL1 and Arg-188 in ECL2 were found to be involved in CXCR4-mediated entry of X4 and R5X4 isolates (Chabot *et al.*, 1999). Another study found that multiple substitutions of Tyr-7, Asp-10, Tyr-12, Asp-20, Tyr-21, Asp-22, Ser-23 and Glu-26 in the Nt and Asp-182, Tyr-184, Asp-187, Tyr-190 and Asp-193 in ECL2 influence HIV-1 entry, albeit in an isolate-dependent manner (Kajumo *et al.*, 2000). No clear patterns of CXCR4 amino acid usage by X4 and R5X4 isolates, taken as phenotypic groups, were observed (Kajumo *et al.*, 2000). A study by Brelot *et al.* (2000) confirms the role of Tyr-7, -12 and -21 in the Nt, as well as Asp-193 in ECL2 and Asp-262 in ECL3, in CXCR4 co-receptor function. Presumably, Tyr, Asp and Glu residues important for CXCR4-mediated virus entry are implicated in gp120 binding. It should be noted here that the CXCR4 Nt is sulfated, presumably due to the presence of sulfotyrosines. Inhibition of cellular sulfation pathways, including tyrosine sulfation, blocks CXCR4-mediated HIV-1 entry (unpublished results from the author's laboratory). It is therefore probable that, like gp120 binding to CCR5, gp120 binding to CXCR4 requires the presence of sulfotyrosines.

The use of MAbs, chemokines and their derivatives, peptides and small molecules (< 1 kDa) that inhibit CXCR4-mediated HIV-1 entry has provided further insight into the structure–function relationships of this co-receptor. Little is known, however, about the mechanisms of action of these agents. Lack of a practical and reproducible binding assay has made it difficult to determine whether inhibitors of CXCR4-mediated entry block gp120 binding to the co-receptor. Also, surprisingly few anti-CXCR4 MAbs have been generated and only one has been extensively characterized. MAb 12G5 recognizes an epitope in ECL2 and inhibits HIV-1 fusion and

entry both in an isolate- and a cell type-specific manner (McKnight *et al.*, 1997; Strizki *et al.*, 1997). Differences in gp120 affinities for CXCR4 and post-translational modifications of CXCR4 in different cell types could account for these discrepancies. Other anti-CXCR4 MAbs, whose epitopes remain to be determined, also variably inhibit the entry of the HIV-1_{NL-43} isolate (Hori *et al.*, 1998).

The CXCR4 ligand SDF-1α and its derivatives inhibit HIV-1 fusion and entry by decreasing co-receptor availability on the cell surface and by inhibiting gp120 binding to CXCR4 (Amara *et al.*, 1997; Bleul *et al.*, 1996; Doranz *et al.*, 1999; Bandres *et al.*, 1998). Peptides consisting of the 16 amino-terminal residues of SDF-1α are sufficient to inhibit HIV-1 entry (Heveker *et al.*, 1998). Positively charged peptides, such as T22, presumably inhibit gp120–CXCR4 binding by associating with the negatively charged surface of CXCR4 (Arakaki *et al.*, 1999; Doranz *et al.*, 1997*a*; Murakami *et al.*, 1997, 1999; O'Brien *et al.*, 1996; Tamamura *et al.*, 1998*a*, *b*; Xu *et al.*, 1999). Peptides derived from CXCR4 TM helices inhibit HIV-1 entry, presumably by disrupting CXCR4 conformation and/or oligomerization (Tarasova *et al.*, 1999).

Small molecules such as distamycin analogues and bicyclams potently inhibit CXCR4 co-receptor function (Este *et al.*, 1999; Howard *et al.*, 1998*a*, *b*; Schols *et al.*, 1997*a*, *b*). The antiviral activity of the AMD3100 bicyclam was shown to depend on residues in ECL2 and TM4 of CXCR4 (Donzella *et al.*, 1998; Labrosse *et al.*, 1998). A recent study identified Asp-171 in TM4 and Asp-262 in TM6 as being essential for inhibition of SDF-1α binding and HIV-1 antiviral activity (Gerlach *et al.*, 2001). Upon binding to these residues AMD3100 spans the main ligand-binding cavity of CXCR4 and probably constrains the receptor in an inactive conformation. Surprisingly, an AMD3100-resistant X4 isolate continues to use CXCR4 as a co-receptor (Schols *et al.*, 1997*a*, 1998). Furthermore, replication of this isolate can no longer be inhibited by SDF-1α, but continues to be sensitive to T22, suggesting that AMD3100 and SDF-1α, but not T22, inhibit entry by convergent mechanisms. Resistance to AMD3100 and SDF-1α is associated with the accumulation of mutations in both constant and variable domains of gp120 (Schols *et al.*, 1997*a*, 1998). These changes probably allow the virus to exploit a different docking site on CXCR4. Alternatively, the resistant isolates may recognize an altered conformation of the original binding site.

Conclusions

The evidence accumulated to date indicates that there are similarities and differences in the way envelope glycoproteins from R5 and X4 HIV-1 isolates interact with their respective co-receptors. Similarities between CCR5 and CXCR4 gp120-binding sites are further underscored by the ability of R5X4 isolates to interact with both co-receptors. Negatively charged and tyrosine residues dispersed throughout the extracellular domain of CXCR4 are involved in co-receptor function, but each X4 HIV-1 isolate uses a slightly different subset of amino acids in order to gain entry into target cells. In contrast to X4 isolates, all R5 isolates characterized to date interact with the same cluster of negatively charged and sulfotyrosine residues in the CCR5 Nt. Furthermore, the CCR5 Nt specifically associates with residues in the C4/V3 stem region of gp120. Since the majority of these gp120 residues are conserved between R5 and X4 isolates, subtle differences in amino acid sequence and/or conformation of the C4/V3 stem region of gp120 probably determine co-receptor specificity and may account for the ability of a few residue changes in gp120 to induce a switch in co-receptor usage.

It is notable, however, that all chemokine receptors described to date have Tyr-Asp-Glu-rich regions in their extracellular domains, yet most do not mediate HIV-1 entry, and some do so only poorly. It also seems that the Nts of most if not all chemokine receptors contain sulfotyrosines. Hence, the unique features that make CCR5 and CXCR4 efficient HIV-1 co-receptors remain to be identified. Perhaps it is the way that the different Tyr-Asp-Glu motifs are exhibited on the surfaces of these receptors, or their ability to interact with CD4, or each other, or other molecules on the cell surface, that ultimately renders them efficient mediators of virus entry.

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