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Topical Review

HIV Coreceptors

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Introduction

Viruses have evolved to recognize a variety of cell surface molecules and use them for delivering their genomes into cells. We will use the term virus receptors for those surface molecules in spite of the fact that they did not originally develop to serve as receptors for viruses, and in most cases do not transmit any signal to the interior of the cells after binding to the virus but virus components (for an overview of concepts and history *see* [33]). It appears that virtually any molecule that is exposed at the cell surface could serve as a virus receptor: members of the immunoglobulin supergene family, integrins, signaling receptors, sialic acids, heparan sulfate and other molecules (*see* [53, 106] for a list of identified animal virus receptors). The diversity of virus receptors is striking [106]; retroviruses, in particular, use quite diverse receptors possibly reflecting their ability for rapid genetic change [101]. Also there is no obvious relation between virus family and the receptor structure and function. Members of the same family can use different receptors, and different viruses can use the same receptor.

The lack of any obvious correlation between the viral attachment protein (VAP) and virus receptors suggests that the overall structure of any particular receptor does not play a significant role in virus evolution and that viruses, especially retroviruses, could rapidly evolve to accommodate new receptors [102]. The evolution process of some viruses, e.g., human immunodeficiency viruses (HIV), could involve the use of coreceptor molecules for gaining entry into the cell. Coreceptors should be distinguished from entry cofactors that may act at the late stages of the entry process. We will further use the term coreceptor molecules for those virus entry cofactors that cooperate with the primary receptor at the very initial stages of the entry process by forming complexes with the VAP. The use of coreceptors may help in increasing the rate of transition to new receptors or/and raise the efficiency of primary receptors in those host cells that are important for gaining advantages in replication.

It appears that in the evolution of HIV, the presently designated ‘‘coreceptors’’ may have initially served as receptors and the use of the ‘‘primary’’ receptor CD4 is a more recent adaptation, as was originally suggested by R. Weiss (reviewed in [33]). This possibility is supported by observations that one of the major human immunodeficiency virus type 1 (HIV-1) coreceptors, CXCR4, can mediate infection of CD4-negative cells, as was originally observed by Clapham and colleagues [26] by strains of human immunodeficiency virus type 2 (HIV-2) [44]. Isolates of simian immunodeficiency virus (SIV) can also infect CD4-negative cells by using the other major HIV-1 coreceptor, CCR5, as a receptor molecule [43, 64, 65] further indicating possible evolution pathways of coreceptor-receptor usage (*See also* Fig. 5 showing direct CD4-CCR5 interaction). However, because all clinically important HIV isolates are critically dependent on CD4, and in most cases could use either CXCR4 or CCR5 or other molecules in addition to CD4 to gain entry into cells, CD4 remains designated as the primary receptor for HIV.

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This article overviews the identification and characterization of the HIV coreceptors, what is their role in tropism and disease, and how the new knowledge could be used for prevention and treatment of diseases (*see* [8, 16, 32, 33, 35, 37, 69, 71] for background information and review of results obtained by the end of 1997). We discuss in more detail the coreceptor interactions with the HIV-1 envelope glycoprotein (Env) and CD4 leading to HIV-1 entry into cells and analyze data obtained by May 1998.

The Discovery of the HIV-1 Coreceptors

The principle cell types targeted by HIV-1 *in vivo* are helper T lymphocytes and cells of the monocyte-macrophage lineage via the CD4 receptor pathway, the primary high affinity receptor for HIV-1. While the presence of the CD4 molecule is clearly a major factor defining the tropism of HIV-1 for these target cells, it had long been recognized that human CD4 alone was not sufficient to confer a virus-susceptible phenotype to most nonhuman cell lines (and some human cell types) (reviewed in [33]). This suggested that an accessory factor(s) present in most human cells was needed for fusion, a notion that was supported by earlier observations that the block to HIV-mediated membrane fusion in nonhuman cells expressing human CD4 could be elevated by forming stable or transient hybrids with human cells.

In addition to this species restriction of HIV-1 Envelope glycoprotein (Env)-mediated fusion and infection, individual HIV-1 strains exhibit distinct tropisms for different types of CD4-expressing human cells. Macrophage (M)-tropic isolates infect primary macrophages and lymphocytes but not CD4-positive transformed cell lines and are typically not syncytia-inducing (NSI) in infected lymphoid targets, while T-cell line (T)-tropic isolates infect lymphocytes and CD4-positive transformed cells but not macrophages, and are often syncytia-inducing (SI). Isolates obtained from individuals immediately after infection are nearly always M-tropic/NSI, indicating an important role for these isolates in the transmission of HIV-1 infection. In fact, most isolates observed during the asymptomatic phase of infection are usually of the M-tropic/NSI type. In contrast, T-tropic/SI isolates often emerge later and have been linked to a more rapid progression of HIV-1 disease and accelerated immune destruction. The principle determinants of target cell tropism are located in the envelope gene (reviewed in [70]), and tropism appears linked to inherent differences in membrane fusion selectivity of the Env [15]. This led to the speculation that, like the species restriction in Env-mediated fusion, HIV-1 target cell tropism might reflect dependence of a particular isolate on fusion cofactors that are differentially expressed in specific cell types. It was later discovered that the principal cofactors/coreceptors required to overcome these obstacles to HIV-1 Env-mediated fusion and virus infection were in fact proteins, membranes of a superfamily of G protein coupled seven transmembrane domain receptors (reviewed in [17, 33]).

Soon after the discovery that four chemokine receptors can serve as HIV-1 coreceptors [2, 24, 29, 39, 40, 50], five new human molecules have been identified as entry cofactors — GPR1, GPR15, STRL33, V28 and CCR8, as well as one herpes virus chemokine receptor homologue, US28. Two human orphan seven-transmembrane domain receptors, GPR1 and GPR15, which are expressed in human alveolar macrophages, were shown to serve also as coreceptors for SIV isolates [47, 48]. The more efficient of these, GPR15, is also expressed in human CD4+ T lymphocytes and activated rhesus macaque peripheral blood mononuclear cells. Presumably, the simian homologues of these molecules would also be active in supporting SIV envelope-mediated membrane fusion although this has yet to be examined. The evidence suggesting that there were one or more as yet unknown coreceptors, in addition to CCR5, that mediate SIV infection was the observation that efficient SIV replication occurs in the human lymphoid cell line CEMx174, which does not express detectable amounts of CCR5 mRNA and does not support replication of CCR5-dependent M-tropic HIV-1 strains [22]. Several groups directed their efforts towards identifying the unknown coreceptors for SIV. D. Littman and his colleagues [30] isolated and cloned two human cDNAs each encoding an orphan seven transmembrane domain protein of the G-protein coupled receptor superfamily, related to the chemokine receptors, which were designated BOB and Bonzo. With the identification of the first HIV-1 coreceptor for Env-mediated membrane fusion, CXCR4 (then called fusin), these two orphan receptors represent the only additional immunodeficiency virus coreceptors that were functionally identified and cloned on the basis of their ability to confer a virus Env glycoprotein fusion permissive phenotype to an otherwise nonpermissive cell. Several research groups set out to examine previously cloned members of the seven-transmembrane domain G protein coupled receptor superfamily employing a variety of virus-cell infection and cell-cell fusion assay systems. GPR15 was in fact the same molecule as BOB, and STRL33 — the same as Bonzo. STRL33 is a novel human seven-transmembrane domain orphan receptor that is expressed in activated peripheral blood lymphocytes (PBLs) and T cell lines. It functions as an entry cofactor for Envs from M-tropic, T-tropic, and dual tropic strains of HIV-1 and SIV [3, 61]. The orphan receptor, V28, was also shown to function as HIV coreceptor [86].

Very recently several other chemokine and orphan receptors were found to function as HIV coreceptors.

The CC chemokine receptor CCR8 is an HIV-1 coreceptor [88]. It appears that CCR1 and CCR4 can be used by some HIV-2 isolates [66]. Two other chemokine receptors, CX3CR1 and CCR9, as well as one orphan receptor, APJ, were also very recently implicated in functioning as HIV coreceptors (*see* in [6]). Finally, with the finding that the human cytomegalovirus (CMV) encoded chemokine receptor homologue, US28, can also serve as an entry coreceptor for HIV [81], the number of coreceptors used by HIV and SIV is now 15 (CCR5, CXCR4, CCR3, CCR2b, STRL33, GPR1, GPR15, V28, CCR8, CCR9, CCR1, CCR4, CX3CR1, APJ and US28). This number does not include nonhuman analogues and is likely to grow.

The complex picture is further complicated by the existence of alternative primary receptors for HIV entry. The most noted of these is galactosyl ceramide (Gal-Cer) [52] which can mediate HIV-1 infection, albeit at low efficiency (reviewed in [33]). Gal-Cer is a monohexoside glycolipid inserted in the cellular plasma membranes by two aliphatic chains of their ceramide moieties. It contains one galactose residue in β-glycosidic linkage which protrudes outside the membrane and is the apparent binding site of gp120 and anti-Gal-Cer antibodies. These glycolipids were first proposed as alternative HIV receptors [52] based on inhibition of HIV infections by anti-Gal-Cer antibodies and binding of recombinant gp120 to them [52] as well as the association of greater infectivity with higher expression of those molecules [46]. However, it appears that Gal-Cer does not serve as a coreceptor for HIV because anti-Gal-Cer antibodies did not block HIV-1 infection of CD4 expressing cells [94].

More recently, new evidence is indicating the interesting possibility that some HIV-1 entry cofactors may not be proteins at all but glycolipids [83]. A neutral glycolipid, possibly with 3 sugar groups in the polar head group can serve as an alternative and/or additional cofactor in CD4-dependent HIV-1 fusion. This adds a new dimension to the ability of HIV-1 to use a variety of molecules as coreceptors.

Biological Function and Structure of the HIV-Coreceptors

The major HIV coreceptors, CCR5 and CXCR4, as well as CCR3, CCR2b, CCR1, CCR4, CX3CR1, CCR9, and CCR8 are chemokine receptors. STRL33, GPR1, GRP15, and V28 are orphan receptors but most likely are also chemokine receptors based on similarities in their primary sequences. The CMV encoded US28 is partially homologous to the chemokine receptor CCR1 and possesses multiple chemokine ligand binding ability [55] but is of unknown biological function. The major biological function of the chemokines and their receptors is to regulate trafficking of immune system cells throughout the body (reviewed in [75, 82]).

The chemokine receptors form a distinct family within the seven-transmembrane domain superfamily [75]. They interact with their ligands in a complex multistep process. The N-terminus and the three extracellular loops appear to act in concert to bind the chemokine. However, recently it has been convincingly demonstrated that binding of the CC chemokines RANTES, MIP-1α and MIP-1β to CCR5 is critically determined by a single domain — the second extracellular loop [109]. The intracellular domain of the receptor is comprised of three loops and the C-terminus, which are involved in the transduction of the chemokine-mediated signal. The most critical domains for their function are the first N-terminal 10–20 and the last C-terminal 10–15 amino acid residues. The first event in the signal transduction is the induction of G protein activation, in which an exchange occurs in the G protein α subunit from a ''GDP bound'' to a ''GTP bound state'' which results in the dissociation of the α subunit from the βγ subunits. The G protein α subunit then activates phospholipases which in turn induce the production of second messengers that mobilize intracellular calcium and activation of protein kinase C (reviewed in [82]). It is the promiscuity and redundancy of the receptor-ligand interactions (about 50 chemokines for 14 receptors) along with the cell type-specific expression of the G proteins that add to the staggering complexity of the system. This complexity, however, appears to be an absolute requirement for effective host defense against pathogens.

The major HIV-1 coreceptors, CCR5 and CXCR4, are 352 amino acid residues in length and possess a highly acidic N-termini (Fig. 1). CXCR4 contains two potential N-linked glycosylation sites (one in the N-terminus and one in the second extracellular loop), while CCR5 has only one in the third extracellular loop. The C-termini of both molecules are rich in conserved serine and threonine residues and represent potential phosphorylation sites by the family of G-protein-coupled receptor kinases following ligand binding. The highly conserved cysteine residues that are believed to form disulfide bonds between the first and second extracellular loops, and the N-terminus and third extracellular loop, respectively, may confer on them a unique barrel shape by bringing the extracellular domains into closer proximity.

The 3-dimensional (3D) structure of the HIV-1 coreceptors is presently unknown. A theoretical 3D model of the HIV-1 coreceptors CXCR4 and CCR5 was developed by S. Durell (*personal communication*) based on the physically determined structure of both bacteriorhodopsin and rhodopsin, as well as analysis of the amino acid sequences of related G-protein coupled receptors (Fig. 2). The barrel shape and close positioning of the extracellular loops can be noted in the side views of the molecules. The model highlighted differences in the electrostatic potentials of the extracellular portions of the

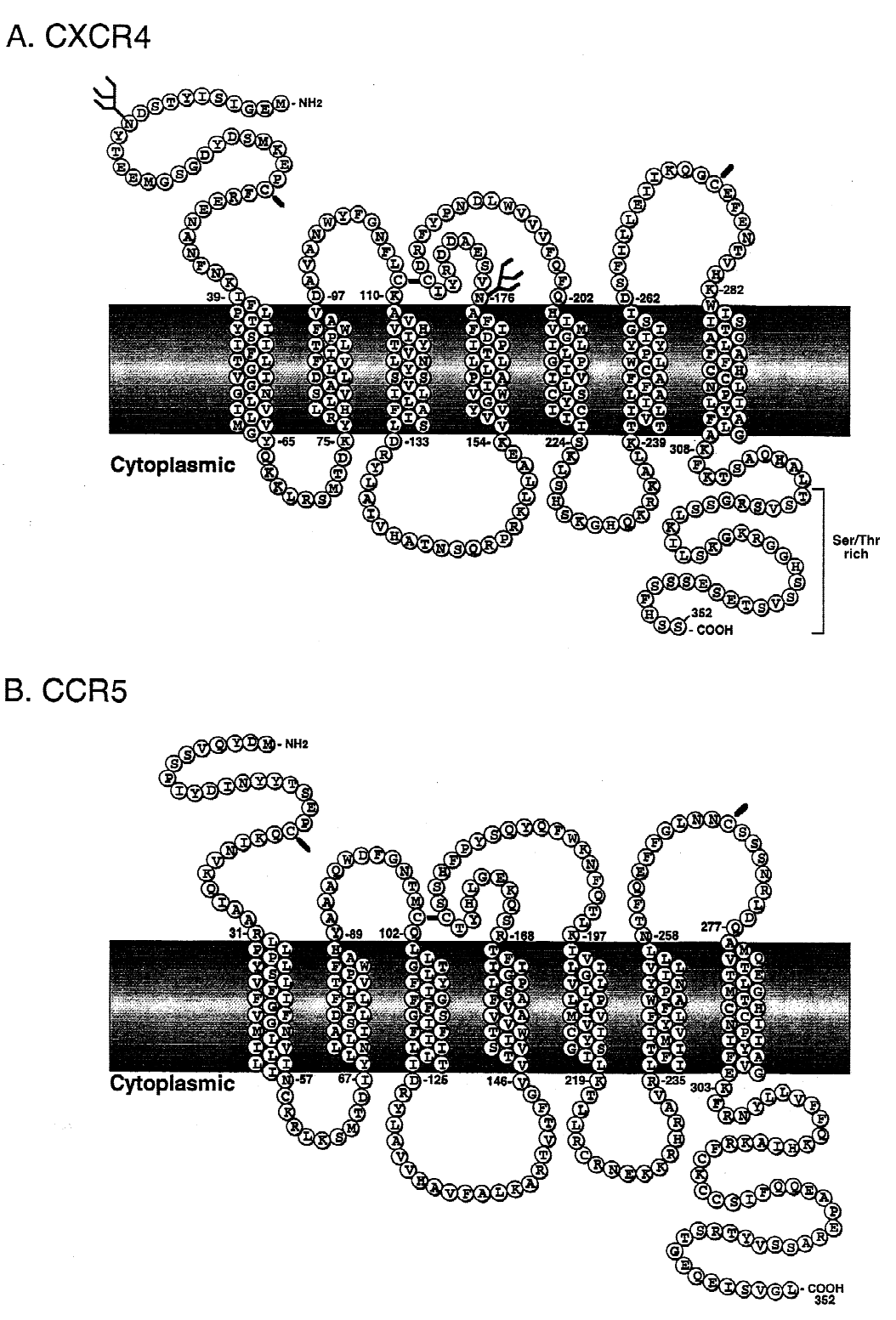

Fig. 1. Primary sequences and predicted membrane topology of the HIV-1 coreceptors CXCR4 **(A)** and CCR5 **(B).** Courtesy of R. Doms.

molecules, which may be important for virus tropism. The darker shading of the CXCR4 surface (top view) indicates a more negative charge at the extracellular surface. In contrast CCR5 is less negatively charged. The overall charge of the HIV-1 Env V3 loop, which is an important determinant of the virus tropism, is positive, with the T-tropic Env V3 loop regions being more positively charged than the respective M-tropic sequences.

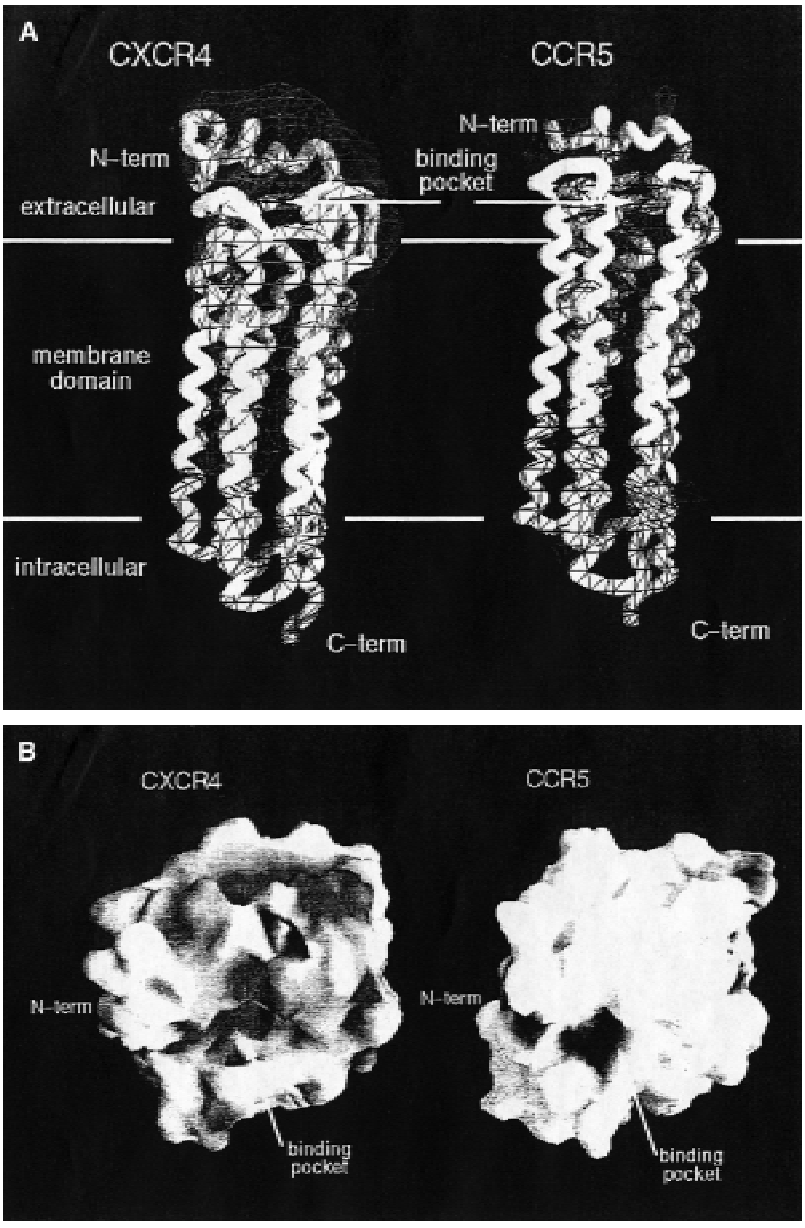

Fig. 2. Theoretical molecular models of the HIV-1 coreceptors CXCR4 and CCR5. **(A)** Side views. **(B)** Top views. The dark and lighter contour meshes indicate electrostatic potential values of −5.0 and 5.0 kT/e, respectively. Courtesy of Stewart R. Durell.

Obviously this suggests a simple explanation for the preferential interaction of T-tropic Envs with CXCR4. However, as discussed in the next section, the Env-CD4-coreceptor interactions are complex and other factors could significantly contribute to the free energy of the ligand-receptor interactions.

How do Coreceptors Help in Mediating HIV Entry?

The high-affinity interaction between gp120 and CD4 is critical for the process that ultimately results in merging of the virion and target cell membranes, thus permitting virus entry. Postbinding molecular events in Env-mediated fusion have not been fully elucidated, but are likely to be triggered in part by specific conformational changes induced by receptor and perhaps coreceptor binding. Both Env and CD4 undergo conformational changes after binding, as evidenced by increased exposure of antibody epitopes on gp120, gp41 and CD4 or the acquisition of novel combinatorial epitopes, enhanced sensitivity of gp120 to proteolytic cleavage, gp120 dissociation from gp41, and biochemical and immunological alterations in CD4 structure. These structural changes presumably lead to the exposure of the hydrophobic amino-terminal (fusion) peptide of gp41 and, ultimately, membrane fusion (reviewed in [13, 33]).

A simple model for the involvement of the corecep-

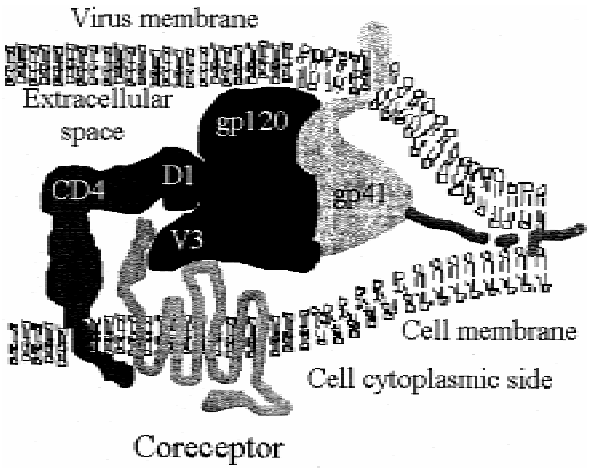

Fig. 3. A sketch of possible interactions between the CD4-gp120-gp41 complex and HIV-1 coreceptors (CXCR4, CCR5 or else) resulting in fusion. An attempt was made to represent the size and shape of the participating molecules in proportion to their real size and known 3D structure and surface topology. Although the structure of the HIV-1 coreceptors is most likely circular (*see* Fig. 2, where a 3D model is shown), here it is represented as a linear array of transmembrane domains for the purpose of illustration. The interactions involve oligomeric surface associated complexes of gp120-gp41 with CD4 molecules and coreceptors (Fig. 4). While only one subunit of this oligomeric complex is shown here, three fusion peptides are depicted on the right side of the picture, two of them belonging to other subunits of the oligomer or other structural units of the fusion complex.

tors in the viral Env-mediated membrane fusion is that they interact directly with the conformationally altered Env following its binding to the CD4 molecule. An indication for such a mechanism is that separate molecules mediate entry of T-tropic *vs.* M-tropic isolates and that the viral determinants for this selective tropism are located in the Env, with the V3 loop playing a central (although not exclusive) role (reviewed in [33]). The chemokine receptors may interact with regions of Env distinct from those involved in CD4 binding, perhaps with the V3 (and/or V2 and V1) loops or related epitopes [23, 98]. Even before the identification of the HIV coreceptors we demonstrated that mAbs against the V3 loop inhibit co-downmodulation of CD4 and fusion cofactors (later shown to be CXCR4) [51]. Another indication for a direct interaction between gp120 and coreceptors is the finding that HIV-1 gp120 can bind CXCR4 even in the absence of CD4 although about 10–100-fold weaker than in the presence of CD4 [7, 54].

We have been hypothesizing that the very first step of HIV entry involves the formation of a trimolecular complex between gp120, CD4 and coreceptor molecules [31, 51] (Fig. 3). We speculated that the coreceptor has at least two binding sites for the CD4-gp120 complex — one of them related to the N-terminus and the other one to the extracellular loops, particularly the second loop (Fig. 3) [31]. We also proposed that the evolving HIV picked up as a second receptor a molecule that was already associated with the other receptor molecule and therefore the coreceptor may associate or be at close proximity to the CD4 molecule (Fig. 3). The recent solution of the crystal structure of the entire extracellular portion of CD4 [107] has provided new opportunities to study the high affinity virus-receptor interactions in greater detail. The oligomeric HIV Env could cross-link the dimeric CD4 potentially leading to formation of large multimeric complexes (Fig. 4). Since CD4 binding induces conformational alterations in the oligomeric Env structure, perhaps the coreceptors induce additional changes that ultimately trigger fusion. Alternatively, it is possible that the coreceptors interact relatively weakly with CD4 and that the interaction is increased upon binding to gp120, leading to conformational changes required for fusion. Finally, these coreceptor mechanisms might be indirect and involve G protein signaling and the activation of downstream pathways, but studies to date suggest that the ligand-responsive cell signaling activity of CCR5 is not required for HIV-1 entry cofactor function [5, 63].

In support of the hypothesis that the initial step of HIV entry involves the formation of a trimolecular complex we demonstrated that the gp120 of the HIV-1 Env, CD4 and the HIV-1 coreceptor CXCR4 can be coimmunoprecipitated [59]. By using a displacement assay it was also shown that in presence of CD4, gp120 shares binding sites with chemokines on the other major HIV-1 coreceptor, CCR5 [98, 108]. It was also observed that CD4 colocalizes with CXCR4 in the presence of gp120 by using confocal laser scanning microscopy [100]. Although the results of these studies demonstrated the existence of a complex between gp120, CD4 and coreceptor molecules, it was unclear whether CD4 interacts directly with the coreceptors, and whether such interaction is important for the formation of the trimolecular complex and the mechanism of virus entry.

Several observations hinted that the CD4-coreceptor interactions could play a role in the formation of the trimolecular complex. By using an immunoprecipitation assay and confocal microscopy we and others found that in some cell lines CD4 could associate, although weakly, with CXCR4 even in the absence of gp120 [59, 100]. It was also noted that a soluble form of CD4, containing its first and second domain, can displace the chemokine MIP-1α, indicating possible interactions between CD4 and CCR5 even in the absence of gp120 [108]. However, the soluble form of the entire extracellular portion of CD4 was much less efficient in displacing MIP-1α raising questions whether the native membrane-associated CD4 is able to associate with CCR5 [108]. Recently, we demonstrated that membrane-associated CD4 strongly interacts with CCR5 even in the absence of

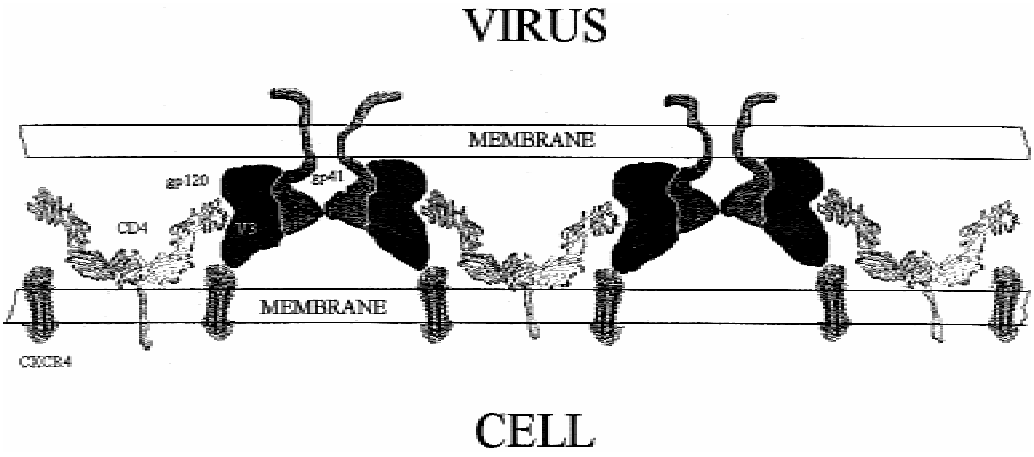

Fig. 4. A sketch of possible interactions of the oligomeric HIV-1 Env with dimeric membrane-associated CD4 and coreceptors. The oligomeric (possibly trimeric) Env could ''cross-link'' the dimeric CD4 forming large membrane-associated complexes which may be important for the subsequent stages of entry.

gp120, and that this interaction may play a critical role in the entry process (X. Xiao, L. Wu, Y. Feng, S. Ugolini, D.J. Chabot, Z. Shen, C.C. Broder, Q.J. Sattentau and D.S. Dimitrov, *submitted*) (Fig. 5). Interestingly, CXCR4 interaction with CD4 in the same cell lines (NIH3T3CD4+) was much weaker than that of CCR5, but the CXCR4-CD4 coimmunoprecipitation increases dramatically by gp120 from several different T-tropic HIV-1 strains including IIIB, MN and RF (X. Xiao, Y. Feng and D.S. Dimitrov, *unpublished observations*) (Fig. 5).

Although the affinities of membrane-associated coreceptor-CD4 interactions are presently unknown, an interesting concept is the possibility that competition for use of CD4 leads to changes in tropism. Our preliminary experiments have indicated that primary macrophages in which CXCR4 is overexpressed will allow entry of T-tropic viruses. One possible interpretation of these results is that at low CD4 concentration CCR5 out competes CXCR4 for CD4 [17] (Q. Sattentau, *personal communication*). However, recent preliminary results does not seem to support the hypothesis that CXCR4 and CCR5 share the same binding site on CD4 (X. Xiao and D.S. Dimitrov, *unpublished data*). Even if this is the case it may be that at high CD4 or CXCR4 concentrations, CXCR4 could associate with CD4 allowing entry of T-tropic viruses. One might hypothesize that virus entry efficiency may depend on the surface concentration of preformed complexes between CD4 and coreceptors. By increasing the surface concentration of either receptor or coreceptor molecules one can increase the concentration of the receptor-coreceptor complexes leading to more efficient entry until a saturation level is reached. This hypothesis seems to be supported by recent observations that the concentrations of CD4 and CCR5 required for efficient infections by macrophage tropic HIV-1 are interdependent and that the requirements for

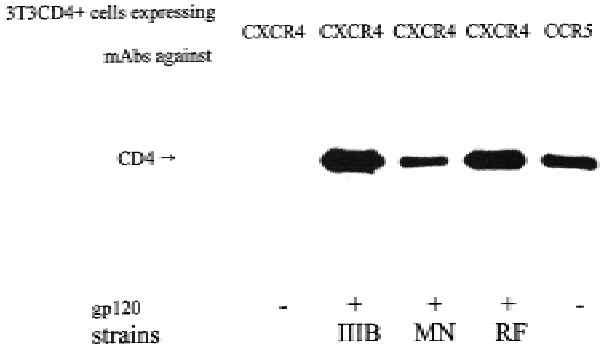

Fig. 5. Interaction of CD4 with CCR5 and CXCR4 demonstrated by their coimmunoprecipitation. CCR5 interacts much strongly with CD4 in the absence of gp120. Addition of gp120, (IIIB, MN or RF) to cells expressing CXCR4 and CD4 leads to efficient coimmunoprecipitation of CXCR4 with CD4.

each are increased when the other component is present in a limiting amount [80].

The delineation of the critical regions involved in the interactions within the HIV-1 Env-CD4-coreceptor complex are presently under intensive investigation. Several studies used chimeras between CCR5 and either human CCR2b or murine CCR5 (which do not function as virus coreceptors) to study HIV-1 entry [5, 10, 48, 89]. The exchange of different portions between CCR3 and CCR1 was also used to characterize domains responsible for the CCR3 coreceptor activity [1]. The results indicate that all or most of the extracellular regions of CCR5 and CCR3 are involved in the entry process. However, because of the similarity in the backbone structure of these molecules one might caution that various external domains are likely to interact closely, and CCR2b or murine CCR5 may not be neutral. By using a large number of mutants, chimeras and homologues of CCR5, it

has been convincingly demonstrated that the CCR5 N-terminus plays a critical role for entry of M-tropic HIV-1 and SIV [37, 41, 42, 49, 84]. The N-terminus of CCR5 is also critical for the interaction with the gp120-CD4 complex [41, 49]. The correlation between the inability of gp120 to bind N-terminus CCR5 mutants and the impairments of virus entry into cells expressing these mutants suggests that the formation of gp120-CD4-CCR5 complex is critical for virus entry but does not discriminate between inhibition of gp120-CCR5 or CD4-CCR5 interactions. Interestingly, a change in asparagine 13 of CCR5 allowed the SIV_{mac239} gp120 to bind CCR5 in a CD4-independent way [65]. This observation strongly suggests that the N-terminus interacts directly with the gp120 although the presence of CD4 could affect that interaction.

A second functional region which is important for entry includes the extracellular loops [5, 10, 63, 89]. The second extracellular loop appears to be the critical portion of the extracellular loops which is involved in the HIV entry [10, 109], interaction with the gp120-CD4 complex [109] and chemokine binding [91]. A mAb to the second extracellular loop, 2D7, was able to inhibit binding of the JR-FL gp120-CD4 complex to CCR5, efficiently block the entry of M-tropic (ADA and JR-FL) and dual-tropic (DH123) HIV-1 strains, and totally inhibit the binding of RANTES, MIP-1α and MIP-1β to CCR5 [109]. The same antibody inhibited the coimmunoprecipitation of CD4 and CCR5 (X. Xiao, L. Wu, Y. Feng, S. Ugolini, D.J. Chabot, Z. Shen, C.C. Broder, Q.J. Sattentau, D.S. Dimitrov, *submitted*), indicating that the second extracellular loop is involved in the interaction of CCR5 with CD4. Thus it remains to be elucidated whether it also interacts with gp120 in the gp120-CD4 complex. One might speculate that the second extracellular loop is involved in multiple interactions with gp120 and CD4 and this is how it plays a role in the mechanism of HIV entry.

Comparatively much less work has been performed on CXCR4 towards defining the molecule's critical coreceptor elements. These studies were similar in design to those discussed above, and primarily employed the use of small truncations in either the amino or carboxy termini and genetic chimeras. The N-terminus was the first domain proposed to play an important role from studies that showed polyclonal antibody blocking of both virus entry and Env-mediated fusion reactions [50]. A second report demonstrated that the N-terminus was indeed critical for some isolates yet not the sole element deemed important, which was not surprising in light of the information obtained from the CCR5 studies [79]. Through a more detailed analysis, employing site-directed mutagenesis, we have shown an importance of the negatively charged glutamic acid residues in the N-terminus as well as some additional charged residues in the extracellular loops (D.J. Chabot and C.C. Broder, *in preparation*). Our observations with CXCR4 correlate well with the relationship of the charge potential of extracellular domains and the Env V3 loop discussed earlier. Two additional studies independently highlighted the importance of additional extracellular domains of CXCR4, primarily the second extracellular loop, in coreceptor activity [14, 63]. However, the N-terminus appeared dispensable for at least one HIV-1 isolate (LAI) [14]. These studies also showed no apparent dependency on CXCR4 signaling for membrane fusion and virus entry, nor for any requirement of the potential glycosylation sites of the molecule. Taken together, the second extracellular loop, also the largest loop, may play a central role in defining the complex structure of CXCR4 and the other chemokine receptors. In support of this notion we have identified a homologous critical region in the second loop of both CXCR4 and CCR5. On the one hand a charge elimination of the aspartic acid at position 187 in CXCR4 by an alanine substitution reveals a cryptic function which allows CCR5-dependent M-tropic HIV-1 isolates to utilize CXCR4 as a coreceptor. Conversely, the mutation of a pair of serine residues at the corresponding position in CCR5 dramatically inhibits this molecule's ability to support M-tropic isolate fusion and virus infection (D.J. Chabot and C.C. Broder, *in preparation*).

An important difference between SIV and HIV-1 is that all SIV strains studied to date appear highly specific for CCR5 regardless of their cellular tropism (T-tropic *vs.* M-tropic) or syncytium-inducing phenotype [42]. This is consistent with earlier observations that the inability of SIVmac239 to replicate in primary macrophages was due not to a block at entry but at some downstream level, even though tropism was linked to determinants in Env (reviewed in [33]). The mechanisms that underlie Env-linked post-fusion determinants are unclear. However, cell-cell fusion experiments using cells expressing a series of CCR5/CCR2b chimeras and several T-tropic and M-tropic SIV Env proteins showed that the structural requirements for T-tropic and M-tropic SIV fusion differed [43]. In general, T-tropic SIV Envs required the second extracellular loop of CCR5 for fusion while the M-tropic SIV depended more on the amino-terminus. How this might result in differential replication capacity remains to be determined, although (as with HIV-1) chemokine receptor signaling did not appear to be required for either SIV env-mediated fusion or infection [43]. Based on data demonstrating the CD4-independent binding of SIV gp120 to CCR5 is critically dependent on the CCR5 N-terminus [65] one might speculate that in the absence of CD4 the N-terminus plays its role in the initial high affinity binding. The binding to the N-terminus could induce conformational changes in the gp120 molecule and is followed by binding to the second extracellular loop which may be re-

quired for the subsequent conformational changes leading to the exposure of the fusion peptide.

Chemokine receptors may be involved in entry by other lentiviruses as well. It has been shown that fusion between cells infected with tissue culture-adapted feline immunodeficiency virus (FIV) and human target cells could be blocked by a monoclonal antibody against human CXCR4 [105]. Although different strains of FIV exhibit distinct tropisms, this raises the possibility that related chemokine receptors may also be involved in entry of FIV in its natural target cells as well. Whether chemokine receptors are involved in entry of other lentiviruses remains to be determined.

Most of our knowledge on HIV-1 coreceptor interactions has been derived either by experiments with soluble components of the entry machinery or measurements of end results of those interactions, such as fusion, entry, or infection. By using a novel virion binding assay, Q. Sattentau and his colleagues (*personal communication*) directly measured the interactions between membrane-associated HIV-1 Env, CD4 and coreceptors. They found that T-tropic strains may require both CD4 and CXCR4 for efficient binding to cells.

Plasma membranes contain thousands of proteins, many of them being potentially capable of interacting with virus components and/or receptor molecules, especially at elevated surface concentrations. The human multidrug transporter (P-glycoprotein) is a large integral membrane protein which extrudes hydrophobic drugs and peptides from the plasma membrane. Interestingly, it also interferes with HIV-1 infection at the level of entry (M. Gottesman and C. Lee, *personal communication*). A possible explanation for its inhibitory effect is the interaction with hydrophobic portions of the HIV Env, particularly the fusion peptide. However, it was found that another large integral membrane protein (CFTR), which serves as an ionic channel, also affects fusion. Therefore another possible inhibitory mechanism may involve interactions with receptor and coreceptor molecules [32]. Although the mechanisms of the inhibitory effects of large integral membrane proteins are currently unknown, their very existence again underlines the complexity of HIV-1 entry through the plasma membrane. To add to this complexity recently, Moriuchi and colleagues found that some U937 cell lines are resistant to HIV-1 entry and that treatment with retinoic acid that induces expression of CCR5 does not remove the fusion barrier [72, 73]. Our preliminary results suggest that the block in entry could be due to a higher molecular weight (about 70 kDa) protein, possibly modified CXCR4 which cannot function as a coreceptor (X. Xiao, F. Feng, H. Moriuchi, M. Moriuchi, C.C. Broder, A. Fauci, D.S. Dimitrov, *unpublished observations*).

That the primary HIV receptor CD4 induces conformational changes in the Env-CD4 complex has been known for many years (reviewed in [33]). Another demonstration of the CD4 ability to prime the virus for the subsequent stages of entry by inducing conformational changes was the demonstration that soluble CD4 (sCD4) can promote cell fusion of CD4-negative cells (E. Berger, *personal communication*). Although it has been shown in several previous studies that sCD4 can enhance HIV-1 entry, this is the first demonstration of fusion induction in CD4-negative cells. Interestingly, a mAb, CG10, which is strictly specific for the CD4-gp120 complex, enhanced HIV-1 Env-mediated fusion and infection, possibly due to an increased exposure of intermediate structures interacting with coreceptor molecules [60]. The identification of the HIV coreceptors raised the question of whether the fusion mechanism involves further conformational changes mediated by the coreceptors. By using a novel method for detection of conformational changes in the HIV Env based on the fluorescent dye bis-ANS, which fluoresces after binding to hydrophobic regions, it was found that significant conformational changes occur only in the presence of coreceptor molecules [57]. Notably, the type of coreceptor was important for the interaction with either/or T-tropic virus: expression of CXCR4 and CD4 at the cell surface induced conformational changes in T-tropic HIV Env while CCR5 and CD4 led to conformational changes in M-tropic isolates.

With the recent achievements in elucidating the 3D structure of possible fusion intermediates, the most striking finding is the similarity in the structures involved in the conformational changes induced by receptors and coreceptors (HIV-1) [20, 103] and triggered by low pH (influenza) [18, 19]. The conformational changes leading to an increase in hydrophobicity, as detected by the fluorescent dye bis-ANS in the influenza HA after exposure to low pH, may also have some similarities with those induced in the HIV-1 Env by CD4 although the kinetics are different [57]. While coiled coils are certainly important as fusion intermediates for several viruses it appears that in some (or many ?) cases other structures could play a role. It would be interesting to find out whether coiled coils are also involved in the structure of the fusion intermediates for the TBE virus — the structure of its major glycoprotein implies close proximity to the target membrane [87] and no need for translocation of the fusion peptide. In addition, no coiled coils are predicted for some viruses, e.g., for the VSV G protein. How exactly coreceptor molecules are involved in inducing these fusion intermediates remains to be determined.

Role of Coreceptors in HIV Tropism and Pathogenesis

The capacity of viruses to infect and productively multiply in discrete tissues or populations of cells within

these tissues is referred to as tropism [99]. The receptors play major roles in determining virus tropism and, therefore, the type of cells infected by the virus and progression to disease largely depends on them. Many of the receptors for viruses, including retroviruses, appear to have a rather broad tissue distribution. Of the known retrovirus receptors, only the primary HIV receptor, CD4, is a differentiation antigen, and therefore HIV infection is more restricted [101]. With the identification of the HIV-1 coreceptors, now HIV-1 is the only virus where a molecular basis for the fine tuning of its tropism to different subsets of CD4 expressing cells has been determined (reviewed in [33]).

Pathogenesis is a process by which viruses injure discrete populations of cells in different organs to produce the signs and symptoms of disease in a particular host [99]. Production of a disease is a relatively rare outcome of viral infection possibly because it is disadvantageous from an evolutionary point of view. Virus receptors themselves may be involved in pathogenesis by at least two mechanisms: (i) cytopathic effects due directly or indirectly to the interaction of the VAP with receptor molecules, e.g., formation of syncytia, and (ii) interference with the normal function of the receptor because of the interaction with the VAP. HIV-1 isolates vary in phenotype, as defined by the cells in which they replicate in vitro. HIV-1 phenotypes also change in vivo, which has profound implications for viral transmission, pathogenesis, and disease progression. These changes are manifested in an isolate's ability to interact with the coreceptor molecules, and is a further regulatory mechanism on the types of cells which can be infected. Thus, HIV can serve as a paradigm to study the interrelations between virus entry, tropism and pathogenesis. Efforts to define the structural elements of the coreceptors that are responsible for their interactions with different HIV envelope glycoproteins have already been extensive, as detailed above. All HIV-1 strains described to date, including primary isolates, can use CXCR4, CCR5, or both. In general, it would appear that virus isolates may be better characterized by their usage of coreceptors rather than by their tropism. This is in line with an earlier (before identification of the HIV-1 coreceptors) suggestion by D. Volsky and his colleagues [25] for redefinition of the virus tropism. It appears that the classification of HIV-1 as T-, M-, and dual-tropic may be replaced by a classification based on coreceptor usage [9].

Knowledge of the HIV-1 coreceptors has shed light on some long-standing observations of virus-host interactions. Some individuals remain uninfected despite repeated exposure to HIV-1, and a mutation in the CCR5 coreceptor gene that is common in some populations has been identified as the cellular basis for resistance to HIV-1 in some of them [62, 78]. Nearly 10% of CCR5 alleles among Caucasian individuals in Europe and the US contain a 32 bp deletion that results in premature truncation of the protein [28, 62, 92]. When expressed, the mutant gene (termed CCR5Δ32) produces a protein that is not transported to the cell surface and therefore does not support HIV-1 entry [62, 85, 92]. Several large population-based studies found no infected individuals who were homozygous for CCR5Δ32, even though the homozygous genotype was seen in 1% of randomly selected HIV-negative and up to 30% of highly exposed but uninfected individuals [28, 56, 92]. In vitro, cells from CCR5Δ32 homozygotes are highly resistant to infection by M-tropic HIV-1 isolates but permissive for T-tropic or dual-tropic strains [85, 92]. These findings confirm the notion that CCR5-dependent M-tropic variants are important in person-to-person transmission. In addition, protection was seen even in individuals at risk of blood-borne transmission [28], demonstrating that a requirement for M-tropic variants and CCR5 interaction is not limited to sexual transmission of HIV-1. Not surprisingly, even though the absence of functional CCR5 confers high-level resistance, protection is not complete and rare HIV infection despite this genotype has been reported [11] [76].

Up to 16% of individuals in some populations are heterozygous for CCR5Δ32. One analysis found fewer heterozygotes among infected than uninfected groups, suggesting partial protection [92]. In contrast, others have found no evidence for protection against infection, but rather that infected individuals who were heterozygous for CCR5Δ32 had a slower progression of disease [56, 68]. Thus, polymorphism of the CCR5 allele may also be an important host genetic determinant of disease progression. Aside from the CCR5Δ32 condition, other polymorphisms of CCR5 exist but are far less common [28], and their effects on HIV coreceptor function and consequences for pathogenesis in vivo remain to be determined. These findings have also prompted a search for polymorphisms in other chemokine receptor genes that mediate HIV-1 disease progression. Most recently, a mutation (CCR2-64I) within the first transmembrane region of the CCR2 chemokine and HIV-1 receptor gene was described that occurred at an allele frequency of 10 to 15% among Caucasians and African Americans. Genetic association analysis of large cohorts has revealed that HIV-1-infected individuals carrying the CCR2-64I allele progressed to AIDS 2 to 4 years later than individuals homozygous for the common allele [96]. However, in view of the fact that only a single HIV-1 dual tropic isolate has been identified that can utilize CCR2 (CCR2b) as an entry coreceptor, the mechanism of the CCR2 polymorphism in disease progression is not clear [67].

Alternative mechanisms of protection involving the coreceptors and their ligands may also be at work. Lym-

phocytes from some exposed-uninfected individuals secrete greater amounts of the CCR5 ligands RANTES, MIP-1a and MIP-1b compared with control subjects [78], and it will be important to determine whether similar mechanisms may be involved in the long-term nonprogression seen in some HIV-infected individuals [58]. Since T-tropic isolates often appear during disease progression in association with $CD4^+$ T-lymphocyte decline, it is also possible that polymorphisms in the expression or activity of CXCR4, or overexpression of its ligand SDF-1 or related molecules, might also contribute to a favorable disease course in some patients. Interestingly, Volsky and his colleagues found recently that endogenous production of β chemokines by CD4+, but not CD8+ cells correlates with the clinical state of HIV-1-infected individuals indicating that it may constitute one mechanism of disease-free survival [90].

Additional questions regarding the possible roles of the chemokine coreceptors for HIV-1 pathogenesis have also been raised. Is there an involvement of an HIV-1 envelope-dependent chemokine receptor interaction in CD4+ cell depletion or other pathogenic manifestations? Gp120 has been observed bound to cells in vivo [97]. Perhaps shed gp120 can interact with receptors on cells mediating inappropriate signaling via these molecules which lead to cell death. The chemokines and their receptors were originally described for their abilities to mediate leukocyte migration and play a critical role in the host defense mechanism of inflammation. Interference or disruption of these pathways by HIV via its use of these receptors may also be a contributing factor in the pathogenesis of the virus. Supporting these possibilities are recent observations by Fauci and his colleagues that recombinant envelope proteins from macrophage-tropic HIV and SIV induce a signal through CCR5 on CD4+ T cells and that envelope-mediated signal transduction through CCR5 induces chemotaxis of T cells [104]. This chemotactic response may contribute to the pathogenesis of HIV in vivo by chemo-attracting activated CD4+ cells to sites of viral replication. HIV-mediated signaling through CCR5 may also enhance viral replication in vivo by increasing the activation state of target cells. Alternatively, envelope-mediated CCR5 signal transduction may influence viral-associated cytopathicity or apoptosis.

Gp120 was also shown to induce internalization of the CXCR4 receptor using a functional CXCR4-GFP fusion protein, suggesting additional possible mechanisms for chemotaxis inhibition. An interesting mechanism of the switch from M-tropic to T-tropic HIV-1 isolates during progression to AIDS is based on the observation that IL-4 downmodulates CCR5 and that IL-4 is increased in HIV-infected individuals (G. Pavlakis, *personal communication*).

It was also found that freshly isolated epidermal Langerhans cells (LC) expressed CCR5 but not CXCR4 at the plasma membrane surface; however, LC contained intracellular preformed CXCR4 that was transported to the surface during the tissue culturing [111]. Macrophages (MF) expressed high levels of both coreceptors but only CCR5 was functional in a fusion assay. These data provide several possible explanations for the selective transmission of M-tropic HIV isolates and for the resistance to infection conferred by the CCR5 deletion mutant. Bleul et al. [12] found that CXCR4 is expressed predominantly on the naive, unactivated CD26(low) CD45RA+ CD45R0-T lymphocyte subset of peripheral blood lymphocytes unlike CCR5 which is expressed on CD26(high) CD45RA(low) CD45R0+ T lymphocytes, a subset thought to represent previously activated/memory cells. CXCR4 expression was rapidly upregulated on peripheral blood mononuclear cells during phytohemagglutinin stimulation and interleukin 2 priming, and responsiveness to SDF-1 increased simultaneously. CCR5 expression, however, showed only a gradual increase over 12 days of culture with interleukin 2, while T cell activation with phytohemagglutinin was ineffective. These data suggest distinct functions for the two receptors and their ligands in the migration of lymphocyte subsets through lymphoid and nonlymphoid tissues. Furthermore, the largely reciprocal expression of CXCR4 and CCR5 among peripheral blood T cells implies distinct susceptibility of T cell subsets to viral entry by T cell line-tropic *vs.* macrophage-tropic strains during the course of HIV infection.

The discoveries of these human fusion coreceptors may also allow for a reassessment of transgenic small animal models for HIV-1 infection. Co-expression of fusion coreceptors may overcome any species restriction to virus entry. The complex role of the HIV-1 coreceptors in tropism and pathogenesis continues to be under extensive investigation.

Implications for Biomedical Research, Prevention and Treatment of Diseases

Disruption of virus-receptor interactions is an effective means for inhibition of infection and is a part of the humoral defense against viruses aimed at virus neutralization [34]. In many cases antibodies are directed specifically against epitopes in the VAPs which interact with receptor molecules. HIV-1 entry inhibitors have been recently extensively reviewed in Chapter 10 of [33] and therefore only recent developments will be discussed here.

The discovery of the HIV-1 coreceptors has stimulated new efforts for identification of entry inhibitors which prevent their interactions with the Env-CD4 complex. The identification of the fusion coreceptors provided new targets for strategies to treat HIV-1 infection

by chemokines [27], such as an N-terminally truncated form of RANTES that has anti-HIV activity in vitro [4]. A derivative of RANTES that was created by chemical modification of the amino terminus, aminooxypentane (AOP)-RANTES, did not induce chemotaxis and was a subnanomolar antagonist of CCR5 function in monocytes [95]. It potently inhibited infection of diverse cell types (including macrophages and lymphocytes) by non-syncytium-inducing, M-tropic HIV-1 strains. Thus, activation of cells by chemokines is not a prerequisite for the inhibition of viral uptake and replication. Chemokine receptor antagonists like AOP-RANTES that achieve full receptor occupancy at nanomolar concentrations are strong candidates for the therapy of HIV-1-infected individuals. Peptides from the N-terminus of CXCR4 can inhibit infection albeit at high concentrations [50]. A previously described small (9 residues) derivatized peptide (ALX) inhibits HIV-1 infection and fusion at relatively low concentrations by interfering with the use of CXCR4 but not by its downmodulation [38]. Another small molecule, AMD3100, also inhibits HIV-1 entry via CXCR4 [36]. The mechanism of action of these inhibitors has not been elucidated in detail but it appears that they bind to CXCR4. The binding could induce conformational changes which interfere with the gp120-CD4 binding to CXCR4. Although these molecules do not inhibit entry mediated by CCR5, and the virus could evolve to use other coreceptors in their presence, the development of these inhibitors is an important step as proof of the concept that coreceptors can be a target of small molecule inhibitors of HIV-1 infection. In addition, at the late stages of the HIV-1 disease, when CXCR4 usage may dominate in some patients, such inhibitors still could be useful in combination with other drugs.

A recent report of blocking the surface expression of the HIV-1 coreceptor CXCR4 by transfecting lymphocytes with an altered version of SDF-1 has opened intriguing therapeutic strategies [21]. The same approach of phenotypically knocking out the CCR5 has also been demonstrated in which a modified CC-chemokine (modified RANTES and MIP-1α) is targeted to the endoplasmic reticulum to block the surface expression of newly synthesized CCR5 [110]. These transduced lymphocytes expressing the modified chemokines (termed intrakines) were found to be viable and resistant to M-tropic HIV-1 infection. Macrophages and lymphocytes from HIV-positive patients could be genetically modified with the appropriate intrakine as a means of delaying disease progression.

A major impact of the discovery of the HIV-1 coreceptors is the demonstration that the viral tropism can be tuned by using additional molecules as coreceptors. Whether it was for the virus benefit to use coreceptors and change the tropism or the virus was forced to utilize coreceptors is unclear. However, the important point is that the HIV-1 is an example of a virus which can be targeted to relatively specific subsets of cells expressing simultaneously multiple receptor molecules. Therefore, one can imagine that viral vectors could be designed which may target very specifically a small subset of cells expressing several markers. Defective HIV-1 is an obvious candidate [77]. The development of such a new generation of viral vectors is obviously a problem whose solution requires further experimental and theoretical investigations. Only a good understanding of the interactions of the viral envelopes with their receptors and the principles of the design of the entry machinery can lead to the possibility of engineering molecules with a predetermined specificity for virus entry into cells. The recent engineering of a recombinant virus that expresses CD4 and CXCR4 and specifically targets cells expressing fusogenic HIV-1 envelope glycoprotein (Env) [45, 93] demonstrated the feasibility of such possibility. The discovery of the HIV-1 coreceptors is a milestone on the long road to achieving this goal.

Conclusions

HIVs have evolved to use several molecules as primary receptors, the most important for pathogenesis being CD4, and several coreceptors, perhaps the most important being CCR5. Interestingly, CCR5 and the other major coreceptor, CXCR4, can serve as a primary receptor for some isolates of HIV-2 and SIV. Presently, 15 molecules are known to serve as coreceptors for HIV and SIV, more than half of them being chemokine receptors and the other of unknown function. All of them contain predicted seven transmembrane domains and probably have similar design of the overall 3D structure. How exactly coreceptors help in mediating entry is presently unknown. The receptor-mediated entry of HIV into cells is a complex multifactorial process which is initiated by a relatively slow but high-affinity binding of the oligomeric virion gp120-gp41 complex to cell surface associated CD4 and coreceptors, leading to conformational changes in the multimeric association of virus and receptor molecules. The conformational changes may involve structural rearrangements in the V3 loop and in the conformationally related V1, V2 and C4 regions of gp120, as well as in the CD4 molecule possibly resulting in formation of coiled coils in gp41 which may help in the exposure of the gp41 fusion peptide. The hydrophobic fusion peptide destabilizes the cell and viral membranes resulting in the physical intermixing of their lipid bilayers and formation of a fusion pore. The fusion pore expands allowing the transfer of the nucleocapsid into the cytoplasm. Whether the coreceptors are involved in the last stages of entry, including fusion pore expansion and virus uncoating remains to be determined.

The discovery of the major HIV-1 coreceptors suggested a simple mechanism of virus tropism: T-tropic viruses require CXCR4 and M-tropic viruses use CCR5. This picture is complicated by the discovery of new coreceptors some of which can be utilized by both types of viruses. A better classification of HIV is currently suggested to be based on the type of coreceptor they use. The importance of coreceptors for pathogenesis was highlighted by the discovery that the vast majority of individuals containing a defective gene for CCR5 can not be infected by HIV-1. It appears that in addition to CCR5, CCR2b may also be involved in HIV-1 pathogenesis but this question remains controversial. Cytokines and chemokines can affect entry of HIV by altering the level of expression or inhibiting the function of the HIV coreceptors. HIV Env can also affect signal transduction and state of activation of cells expressing chemokine receptors.

While the number of molecules inhibiting HIV entry is large and growing, there are only several examples where the inhibition is very potent and highly specific. Soluble CD4 is such an example which, however, was disappointing in clinical trials although recent improvements still hold a promise. Peptide-based inhibitors and small molecules have high potential, but have not been developed to the same level of sophistication and efficacy as, e.g., the protease inhibitors. HIV-1 coreceptor molecules are currently under intensive investigation for design of potent inhibitors and possibly vaccines. Knowledge of receptors and coreceptors, and their interactions with virus envelope glycoproteins will certainly have implications also for the development of highly specific drug and gene delivery systems.

Note Added in Proof

The recent elucidation of the crystal structure of a CD4-gp120 complex (*Nature* 1998. **393**:648–659 offers new possibilities for understanding the mechanisms of HIV coreceptor interactions (*Science* 1998. **280**:1949–1953.

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