ABSTRACT

Structural and Functional Analysis of HIV-1 Coreceptors: Roles of Chrged Residues and Posttranslational Modifications on Coreceptor Actvity

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CXCR4 and CCR5 are chemokine receptors and are coreceptors for human immunodeficiency virus (HIV-1). Host cells must express CD4 and a coreceptor for optimal HIV-1 entry. The delineation of the critical regions involved in the interactions within the Env-CD4-coreceptor complex has been under intensive investigation. To define these regions we have employed an alanine-scanning mutagenesis strategy of the extracellular domains of CXCR4 coupled with a highly sensitive reporter-gene assay for HIV-1 Env-mediated membrane fusion. Using a panel of 47 different CXCR4 mutations, we have identified several charged residues that appear important for coreceptor activity for X4 Envs: mutations E15A and E32A in the N-terminus, D97A in extracellular loop (ecl)-1, and R188A in ecl-2 impaired coreceptor activity for X4 and R5X4 Envs. Mutation to alanine of one of the six tyrosines present in CXCR4, Y7, decreased coreceptor function. In addition, alanine substitution of any of the four extracellular cysteines alone resulted in conformational changes of varying degrees, while paired cysteine deletions could partially retain structure. Our data supports the notion that all four cysteines are involved in disulfide bond formation.

We have also identified substitutions which greatly enhance or convert CXCR4 s coreceptor activity to support R5 Env-mediated fusion: N11A, R30A, D187A, and D193A. Mutation of the aspartic acid at position 187 had the greatest effect on tropism.

Mutation of an analogous serine in CCR5 to aspartic acid reduced CCR5 coreceptor activity with R5X4 and some R5 isolates. Mutation of the N11, a putative glycosylation site, had the second greatest effect on CXCR4 tropism. We determined that this site has a large carbohydrate moiety that is responsible for preventing CXCR4 from supporting R5 Env-mediated membrane fusion. Our data suggests the presence of conserved extracellular elements common to both CXCR4 and CCR5 involved in their coreceptor activities. These data will help to better detail the CXCR4 structural requirements exhibited by different HIV-1 strains, and will direct further mutagenesis efforts aimed at better defining the domains in CXCR4 involved in the HIV-1 Env-mediated fusion process.

Structural and Functional Analysis of HIV-1 Coreceptors: Roles of Charged Residues and Posttranslational Modifications on Coreceptor Activity

By

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To

My Parents,

Nicki,

Jules,

and

Nicholas

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Chapter 1

INTRODUCTION

AIDS

In terms of human suffering, loss of life, and economic hardship, Acquired Immune Deficiency Syndrome (AIDS) has emerged in less than 20 years as the most devastating viral disease of modern times. While the influenza epidemic of 1918-1919 killed approximately 20 million people (103), Human Immunodeficiency Virus-1 (HIV-1), the causative agent of AIDS, has already infected about 50 million people and killed about 16.3 million, and the pandemic shows no signs of slowing. AIDS is devastating entire countries, while others countries are just beginning to be affected. While many celebrate the Centers for Disease Control finding that the U.S. death rate has fallen to just 16,000 per year from a high of about 50,000 in 1995 and 1996, the UN reports that worldwide 2.6 million died of AIDS in 1999, and 5.6 million new infections will occur in 1999, more than in any previous year. Many African countries including Zimbabwe, Swaziland, Namibia, Botswana, Uganda, Tanzania, Zambia, and Rwanda, report infection rates of over 20% of all adults. About 1500 people are infected each day in South Africa. AIDS has surpassed malaria as the leading infectious cause of death in Africa. Outbreaks are only beginning in many countries, including China, India, and the former Soviet Union. About four million are infected in India and five hundred thousand are infected in China.

While often dismissed as a disease of careless homosexuals and intravenous drug users on the fringes of society, today over 75% of transmission is through heterosexual

contact. UN statistics show that about 5% (2.6 million) of all human deaths in 1999 were due to AIDS and more than one percent of the world's adult population is infected. The protease inhibitors and nucleoside analogs that have eased fears of AIDS are losing their effectiveness, as the virus mutates (42). It is difficult to stop the spread of a virus that can remain dormant until it develops drug-resistance or until the patient becomes non-compliant to the 10 — 30 pills per day needed to keep the virus at bay. In addition these drugs are prohibitively expensive for most African countries, despite the fact that some of these countries spend about half of their health care budgets to care for AIDS patients.

HIV-1 and AIDS

HIV-1, HIV-2, and SIV are closely related lentiviruses that cause a breakdown of normal immune function. Lentiviruses are one of seven genuses of the family *Retroviridae*. Retroviruses are so named because they encode a reverse transcriptase that uses viral genomic RNA as a template for the production of DNA. Unlike the oncoretroviruses, such as HTLV-I, murine leukemia virus, and avian sarcoma viruses, which can immortalize cells *in vitro* and *in vivo*, lentiviruses are cytotoxic, slow viruses associated with immune disregulation, pneumonitis, and brain disorders. The prototypic lentivirus is visna virus, which infects sheep. Other lentiviruses are also species specific: EIAV infects horses, CAEV infects goats, BIV infects cattle, FIV infects felines, and SIV infects monkeys

The AIDS virus was first identified from a patient with lymphadenopathy in 1983 (24). AIDS begins with flu-like symptoms, associated with viremia followed by a latency phase in which the virus continues to replicate in $CD4^+$ cells though serum virus levels may be very low. There is gradual destruction of lymphoid tissue as the immune system tries to hold the virus at bay. While lymphoid tissues are the major targets of HIV, virtually all organs can become infected. Strong cellular and humoral immune responses, including neutralizing antibody, are often made against the virus, but in most cases the viral load gradually increases and the patient loses the race to replenish the cells that are either killed by the virus or by immune reactions to the virus (68, 75, 114, 137). The complexity of HIV and the devastating nature of the disease have led to a worldwide effort unprecedented in scope and resources to understand the virus and develop vaccines and treatments.

HIV-1 Structure

Like other retroviruses HIV-1 is a medium sized enveloped virus between 80 and 100 nm in diameter. Mature HIV-1 particles contain two identical copies of positive sense genomic RNA complexed to the gag nucleocapsid protein p7. The genomes of retroviruses consist of three polycistronic genes, *gag, pol*, and *env*, as well as six alternatively spliced accessory or regulatory genes. *Gag* encodes a precursor that is cleaved to form the capsid structural proteins. *Pol* encodes various enzymes required for reverse transcription, viral DNA integration into host chromosomes, and protein maturation. The major core protein, p24, surrounds the nucleoprotein complex in a cone

shape delineated by another gag protein, p6. Host tRNA (which serves as a primer for reverse transcription) and retroviral enzymes, reverse transcriptase and integrase, are also found in the core. The trimeric myristilated matrix protein, p17 (136), flanks the core and is interspersed with small gag proteins, p1 and p2, as well as HIV protease, which cleaves gag precursor into the mature gag proteins. A host-derived lipid bilayer surrounds the icosahedral capsid. At each of the 72 capsid vertices (121) is a protruding oligomeric envelope glycoprotein structure (Env) consisting of two noncovalently linked proteins, gp41, the transmembrane protein which is anchored in the lipid bilayer, and gp120 (61, 106, 176, 217, 278, 279).

CD4, the common receptor for all naturally occurring HIV-1 isolates

CD4 is present on dendritic cells, cells of the monocyte lineage, eosinophils, and helper T lymphocytes. CD4 is associated with the T cell receptor and is directly responsible for MHC class II binding (98) and antigen-induced signaling. Signaling through CD4 occurs through its C-terminus association with a src-like kinase known as $p56^{lck}$ (172). Signaling through CD4 is the first signal required for activation of helper T-cells during antigen-specific immune responses (138).

As early as 1984 it was discovered that HIV infects CD4+ T-lymphocytes and that viral gp120 interacts strongly with CD4. Coimmunoprecipitation of gp120 and CD4 was demonstrated in lysates of HIV-1 infected cells (185). Antibody to either CD4 or gp120 could block infection (78, 156, 157). CD4 is a member of the immunoglobulin superfamily; X-ray crystallography data indicate it is a 12.5 nm long rod-shaped

molecule with four extracellular Ig-like domains, D1 through D4 (161). D1, a glycosylated domain that is farthest from the membrane, binds with high affinity to gp120. CD4 can dimerize in concentrated solutions, and it may dimerize on cell surfaces in response to ligand binding (286); D4 domains of adjacent CD4 molecules bind together to form a flexible open-winged bat-shaped dimer (208, 286). The multimeric structures of CD4 and HIV-1 Env are consistent with the hypothesis that a complex set of protein-protein and protein-membrane interactions occur during membrane fusion between virus and host cell (89).

Discovery of Coreceptors

The CD4 requirement exists for all HIV-1 isolates, whether macrophage-tropic (M-tropic or nonsyncytia inducing) or T-cell line-tropic (T-tropic or syncytia inducing). The tropism of HIV-1 isolates suggested that CD4, though apparently a receptor, was not sufficient for entry, and other cofactors were needed. Alternatively, or in addition, there may be signaling requirements or negative regulators in various cells that modulate viral entry.

The first evidence for a cofactor requirement was the finding that mouse cells transfected to express CD4 remained refractory to HIV-1 infection (181). The infection block appeared to be at the level of entry, as mouse cells transfected with entire HIV-1 genomes were able to produce infectious virus (173). With some exceptions similar findings were made with other nonhuman cell lines (18). In fact some human cell lines made to express CD4 were also HIV-1 resistant (66). Again, the block appeared to be at

entry, as HIV-1 pseudotyped with Env from amphotropic murine leukemia virus was able to productively infect these cells.

To determine whether there were negative or positive cofactors, somatic cell hybrids were made between permissive human cells and nonpermissive mouse cells. In several experiments hybrid cells were permissive to HIV-1 entry or fusion with cells expressing HIV-1 Env, indicating that there was not an inhibitor of entry present in the mouse cells (47, 99, 132, 223, 277). Instead, there was a positive cofactor needed along with CD4 that was present in the human cells, and the cofactor requirement for T-tropic HIV-1 differed from that for M-tropic HIV-1. Broder et al. (45) used a novel cell-cell fusion assay to show that the cofactor requirement was determined solely by Env. Further evidence for the existence of or the requirement for cofactors or coreceptors was the determination that entry into host cells expressing tailless CD4 could be prevented by phorbol myristate acetate (PMA), a downmodulator of surface proteins (125). Unlike wild-type CD4, tailless CD4 was not normally downmodulated from the cell surface by PMA. Tailless CD4 could be downmodulated from cell surfaces by PMA if cells were preincubated with HIV-1 Env (124). This suggested PMA protection was the result of HIV-1 coreceptor downmodulation and that HIV-1 Env caused CD4 to associate with a PMA downregulatable molecule, perhaps a coreceptor.

Another clue to defining the cofactor requirement came with the identification of HIV-1 inhibitory molecules secreted by $CD8^+$ T cells. Though first described in 1989, it wasn t until 1995 that these inhibitory molecules were identified as b-chemokines. Purified MIP-1a, MIP-1b, and RANTES from cell filtrates were able to prevent infection

of human T cells by M-tropic but not T-tropic HIV-1 (70). It was fortuitous that T cells were used rather than macrophages, because, for unknown reasons, some researchers report that ß chemokines do not inhibit infection of macrophages, but may even enhance infection, depending on the timing of chemokine addition relative to HIV-1 infection (192, 246). Shortly thereafter, several groups (72, 225, 240) reported that a new 7-transmembrane protein, CCR5, functioned as a receptor for all three chemokines. This sparked a race to prove that CCR5 was the coreceptor for M-tropic HIV-1. It simply had to be shown that CD4-pos cells transfected to express CCR5 became permissive for M-tropic HIV-1 and infection could be blocked by anti-CCR5 and/or ß-chemokines.

Concurrently, Yu Feng and colleagues (117) used a HeLa cell expression library transfected into $CD4^+$ mouse 3T3 target cells to identify a coreceptor used by T-tropic HIV-1. They used a cell-cell fusion assay in which transfected target cells infected with vaccinia virus encoding T7 RNA polymerase were mixed with effector cells infected with vaccinia virus encoding T-tropic HIV-1 Env and ß-galactosidase linked to T7 promoter. ß-galactosidase activity was found in targets expressing fusin [then an orphan 7-transmembrane protein, which was renamed CXCR4 after its natural ligands, SDF-1a and SDF-1ß w ere identified (40, 200)]. They found that polyclonal antibody raised to part of the N-terminus of CXCR4 inhibited infection by X4 HIV-1 (117).

This report was followed in rapid succession by five independent reports that CCR5 functions as a cofactor for M-tropic HIV-1 (10, 67, 85, 97, 100). While MIP-1a, MIP-

1ß, and RANTES can block CCR5 coreceptor activity, CXCR4 can be blocked by SDF-1_ and SDF-1ß (40, 200).

SDF-1s appear to only bind CXCR4 and are chemoattractants for T-cells, monocytes, and $CD34^+$ hematopoietic progenitor cells (3), while chemokines that bind CCR5, MIP-1a, MIP-1b, and RANTES, can also bind to various other chemokine receptors and are chemotactic for T cells and cells of monocytic lineage. HIV-1 strains can use either CCR5 (R5 strains), CXCR4 (X4 strains), or both (R5X4 strains). CXCR4 and CCR5 only have about 30% homology including conservative substitutions (Fig.1), so, since other chemokine receptors have significantly higher homology to one or the other, it is intriguing that these two relatively dissimilar molecules are the major HIV-1 coreceptors.

Chemokines and Chemokine Receptors

Trafficking of leukocytes throughout the body, in immune surveillance, in response to inflammation, or as part of a cell maturation process, is largely dependent on a class of cytokines known as chemokines. Chemokines are small (8 — 10 KD) secreted proteins divided into two main categories, the CC (or beta) chemokines, which have consecutive cysteines at their N-termini, and the CXC (or alpha) chemokines that have a different amino acid between their N-terminal cysteines. There are two identified exceptions: lymphotactin has only one N-terminal cysteine (151), and fractalkine has three amino acids between its first two cysteines (26). The chemokine receptors are named after their ligands; CXC chemokines bind to CXC receptors and so on. While some chemokines

Figure 1. Homology between CXCR4 and CCR5. The sequence of CXCR4 is aligned above CCR5. Below CCR5 are amino acids common to both proteins. Conservative substitutions are indicated with a period. (Acidic amino acids = red; basic amino acids = blue; sulfhydryl amino acids = yellow; aromatic amino acids = brown; hydroxyl amino acids = orange; asn and gln = grey.)

Extrcellular Domain	CXCR4	CCR5
N-terminus	3.84	4.33
Ecl-1	5.95	3.43
Ecl-2	3.8	9.65
Ecl-3	4.18	4.12

Table 1. Isoelectric points of extracellular domains of CXCR4 and CCR5

can bind to more than one receptor, chemokines usually bind receptors of the same class. The only receptor known to bind chemokines of more than one class is called Duffy or DARC. Often present on the surface of erythrocytes, endothelial cells, and resting T-cells, Duffy does not associate with cell signaling molecules and, therefore, might act as a chemokine sink, deactivating excess chemokines (141). Duffy is perhaps best known as the receptor for *Plasmodium vivax,* a causative agent of malaria (130). Much as individuals who do not express Duffy are resistant to *Plasmodium vivax* infection, CCR5-negative individuals are relatively resistant to HIV-1 infection (23).

Chemokine receptors are members of a large family (of about 1000) seven transmembrane G-protein coupled receptors (GPCRs). G-proteins are comprised of [alpha]§ and [gamma] subunits and GDP (128). Ligand binding causes conformational changes in the receptor and the G-protein (257). Intracellular portions of the receptor are phosphorylated (202), and the associated G-protein releases its [alpha] subunit with its associated GDP. A GEP (GTP exchange protein) exchanges its GTP for the GDP(128). The GTP-bound [alpha] subunit as well as the ß[gamma] subunit can participate in signaling events (197). Chemokine receptors are linked to G_I proteins, whose [alpha] subunits deactivate membrane-bound adenylate cyclase. Both [alpha] and the combined § and [gamma] subunits can activate phospholipases which degrade PIP_2 to IP_3 and the second messenger diacylglycerol. IP_3, in turn, causes release of calcium, another second messenger, from endoplasmic reticulum. Second messengers activate the MAP kinase pathway and lead to rho activation. Profilin, which is released by phospholipases from the membrane, and

rho, a GTP-bound protein, cause actin polymerization, allowing movement of the cell (4). Adhesion molecules, such as integrins, are altered so that they can bind tightly to their ligands on endothelial cells, allowing for diapedesis (86).

HIV-1 Life Cycle

Following fusion of a virus with a target cell the nucleocapsid is released into the cytoplasm. Reverse transcription from genomic positive sense RNA can actually begin prior to infection, but it is completed in the cytoplasm of the new host. It is primed by a host $tRNA_{lys}$ and catalyzed by a heterodimeric protein encoded in the *pol* portion of the HIV-1 genome. As negative sense DNA is being synthesized the RNA template is degraded by the RNase H portion of the reverse transcriptase molecule, leaving just a polypurine 16-mer that is used as a primer for positive strand DNA synthesis. Reverse transcriptase, which also catalyzes synthesis of the positive DNA strand, makes approximately three errors in the production of a linear double stranded viral DNA molecule (147), allowing for a very high mutation rate.

The matrix protein, p17, and two accessory proteins, Vpr and Nef, are important for delivering viral DNA into the nucleus (259). Vpr, p17, viral DNA, and integrase, another *pol* gene product, form the preintegration complex, which is able to traverse nuclear pores (52). Integrase cuts host DNA relatively randomly and forms recessed ends on viral DNA and protruding ends on host DNA, allowing integration to occur (209). Transcription is somewhat dependent on the integration site, and it is highly regulated by host and viral proteins interacting with elements of the long terminal repeat (LTR) (31,

177). The LTR contains an NF-[kappa]B binding enhancer (152, 226), an Sp-1 binding promoter (149), and a region downstream of the transcription initiation site called TAR, the transactivating region. It is usually necessary for the HIV-1 transactivating protein (TAT) to bind the TAR element on nascent viral mRNA for stable full-length transcription to proceed (13, 150). Without a TAT-induced stem-loop structure transcription generally ends prematurely (116, 234). Another stem-loop structure forms when the Rev responsive element (RRE) of viral mRNA binds to Rev (134). Rev is required to prevent splicing of *env*-containing transcripts (62).

Aided by the myristillation of p17, large amount of Gag polyproteins are produced and make their way to the membrane (249). Another Gag protein, p7, uses its two highly conserved zinc fingers to aid in packaging of viral genomic RNA (255). The Gag proteins mature as HIV-1 protease cleaves HIV-1 polyproteins . Meanwhile, gp160 is cleaved by host proteases in the ER (102), where Env oligomers form [Earl, 1990 #3576; Dewar, 1989 #27]. These oligomers, consisting of three molecules of gp41, the transmembrane protein, and three molecules of the heavily glycosylated gp120, are transported to the cell membrane through golgi vesicles (283). Budding of maturing nucleoprotein particles occurs from Env-rich portions of the membrane. Less than 1% of these particles are infectious.

It is ironic that a virus that so thoroughly confounds the immune system uses as its receptors molecules so important for normal immune function. As part of the T cell receptor complex CD4 aids binding to MHC class II molecules and is necessary for T cell signaling (274). Chemokine receptors and their ligands are essential for the homing of

leukocytes to various organs and to sites of inflammation. They may have additional functions. For example analysis of CXCR4 knockout mice show that CXCR4, which is chemotactic for PBLs (41), is vital for B-cell differentiation (79), intestinal blood vessel formation and heart ventricle development (264, 298). SDF-1 knockout mice had similar phenotypes (195). Homozygous knockout of CXCR4 or SDF-1 results in embyonic lethality. Though CCR5 appears to be dispensable, other chemokine receptors have vital functions. CXCR5 knockout mice have B-cell homing defects (118), and CXCR2 knockout mice overproduce B-cells and neutrophils (54).

The use of particular receptors may have strategic value for HIV-1. The close association between coreceptor and CD4 that may be induced by HIV-1 Env may help the virus to exploit its host cells. In some cases binding of HIV-1 induces signaling events that may lead to cell activation, a requirement for HIV replication. HIV and SIV Env mediated signaling through chemokines receptors can result in chemotaxis and further chemokine production, perhaps leading infected cells to uninfected targets (81, 280).

Vaccines

A great deal of time and money has been put into efforts to make HIV vaccines, yet only modest achievements have been made. Perhaps the biggest obstacle is the lack of available animal models. Only very expensive and endangered primates can be infected with HIV-1, and they don t normally get sick from the infection. Unfortunately, SCID mice reconstituted with human lymphoid tissue (214) have not gained wide acceptance as

models for AIDS infections. Interestingly, transgenic mice made to express human CD4 and CCR5 are poor animal models because of an unkown post-entry replication block (51). The most commonly used animal model remains rhesus macaques infected with rapidly fatal SIVs or SHIVs (SIV with HIV Env), but the ability to extrapolate results from animals injected with laboratory SHIVS to humans naturally infected with HIV-1 remains controversial.

Many issues are unresolved. What route of infection should be used? Though subunit vaccines have not provided broad immunity, might it be useful to combine Envs from non-cross-reactive strains? As evidence shows that glycosylations may mask epitopes (229), should deglycosylated Env be used? Should oligomeric gp160 be used instead of monomeric gp120, which lacks certain epitopes and may normally function as a decoy that monopolizes the immune system? Or should fusion competent gp160 (Env that has conformational changes following interaction with CD4 and coreceptor) be used, as it appears to induce cross-clade neutralizing antibody (164).

Also unclear is how vaccines should be evaluated. Some say that prevention of disease is a good outcome of a monkey vaccine trial, but others think sterilizing immunity should be the goal. While most vaccines work by inducing strong humoral responses, a strong cellular response might be needed to kill HIV-infected cells, and while some concentrate on CTL responses, others are interested in T_H cell activity or antibody dependent cellular cytotoxicity. More recently efforts have been made to examine mucosal immunity and chemokine activity, as well as other HIV-1 suppressive factors, including a molecule recently purified from the urine of pregnant women (179).

Due to the remarkable mutation rate of HIV, attenuated vaccine strains are considered dangerous. *nef* mutations seemed particularly promising because some long-term nonprogressors are infected with HIV-1 bearing mutations in *nef* (104, 155). While a *nef* deletion mutant of SIV proved remarkably protective in adult monkeys following rectal or vaginal challenge, in some cases the vaccine strain was able to cause disease (20, 77, 148). This is remarkable, as Nef plays important roles in cell cycle regulation, infection rate, and down-modulation of surface CD4 and MHC class I molecules (215). Additional accessory gene deletions, including a virus deleted for *nef,vpr,* and *vpx,* have been constructed to further attenuate the virus, but results so far show that the more the virus is attenuated the less protective it is (148).

Another strategy has been to express HIV Env, Gag, and/or Pol proteins in other relatively nonpathogenic viruses or bacteria, including various vaccinia viruses, adenoviruses, herpes viruses, alpha viruses, *Salmonella*, or *Shigella* sp. Naked DNA vaccines in the form of plasmids and replicons are also being developed. Many trials involve a combination of strategies. For example one ongoing experiment uses an initial injection of SIV deactivated with 2,2 -dithiodipyridine, a chemical that chelates zinc from the nucleocapsid protein, followed several weeks later by injection of a plasmid encoding the entire SIV genome with deactivating nucleocapsid protein mutations (17).

Treatments

Numerous drugs, designed to alter the host response or to block various stages in virus maturation, have been tested. While drugs aimed at blocking HIV-1 protease and

poisoning reverse transcriptase activity have gained clinical acceptance, other types of drugs are in clinical trials. For example there is great hope for new drugs designed to block integrase activity, protease dimerization, Tat-TAR interaction, zinc binding to nucleocapsid protein, and RNA packaging (230). I will focus here on strategies to prevent fusion of virus or infected cells with uninfected target cells.

Recent crystal structures of portions of X4 gp41 and gp120 (162, 289) have provided clues to the nature of interactions between CD4, coreceptor, and Env that could prove useful for identification of drug targets. In particular crystal structures of gp120 revealed a conserved coreceptor-binding site in the V1/V2 structure that is important for both R5 and X4 Envs. Thus, the V1/V2 region might also be a suitable drug target. Another likely target is a knob on the D1 domain of CD4 located at Phe43 that fits into a hydrophobic cavity of gp120 (190). A drug that could fill this cavity might block viral adhesion and entry.

This is reminiscent of a hydrophobic cavity found on the human rhinovirus VP1 coat protein, which binds to ICAM-1, which, like CD4, is a member of the Ig superfamily. Soluble ICAM-1 can deactivate rhinovirus by inducing premature virion uncoating (140). Unfortunately, soluble CD4 may actually enhance HIV-1 infectivity by inducing gp120 conformational changes needed for coreceptor binding. However, rhinovirus interaction with ICAM-1 might have some use as a model for HIV-1 interactions with CD4 (88).

Gp41 is the target of T-20, a 36 amino acid peptide corresponding to an extracellular portion of gp41, which is having some success in clinical trials (153). Conformational

changes in gp120 that follow CD4 and coreceptor binding allow two alpha helices in the ectodomain of gp41 to form a coiled coil structure which springs the fusion domain of gp41 into the host cell membrane. T20 presumably binds to one of the alpha helices and, therefore, prevents formation of the coiled coil structure (35, 61, 279). Indeed, in my hands T20 is a potent inhibitor of both X4 and R5 fusion (data not shown). T20 appears to have other activities that affect its therapeutic function. It is a chemoattractant and activator of neutrophils. Interestingly, it accomplishes this by binding to the formyl peptide receptor (FPR), a seven transmembrane G-protein coupled chemotaxis receptor on neutrophils (263). FPR does not function as a coreceptor for R5, X4, or R5X4 HIV-1 (Chabot et al. unpublished observations).

As chemokines are natural inhibitors of HIV infection, there is a great deal of interest in their use in the treatment or prevention of AIDS. As CCR5 appears to be the most important coreceptor and it is apparently not essential for normal immune functions, it represents an obvious target. Amino-oxypentane (AOP) RANTES, a derivative of RANTES, a natural ligand of CCR5, shows particular promise and is in clinical trials not only for the treatment of AIDS but for other immune disorders. AOP RANTES is a particularly effective inhibitor of infections *in vitro* because it prevents recycling of endocytosed CCR5 to the cell surface.

Another possible target of future drug intervention is a region of interaction between coreceptor and CD4. CXCR4 and CD4 do not appear to be tightly associated in the absence of HIV-1 Env. CD4 and CXCR4 do not appreciably coimmunoprecipitate (90, 165), they have different endocytosis rates, and CD4 is not endocytosed in response to

the CXCR4 ligand SDF-1 (251). Unpublished data indicates that CXCR4 glycosylations may normally prevent interactions with CD4. In the presence of X4 gp120, however, there is significant immunoprecipitation of CD4 with CXCR4 (90, 165). Immunoprecipitation and immunofluorescence microscopy experiments with CCR5, on the other hand, show CCR5 is closely associated with CD4 even in the absence of HIV-1 Env (290). Antibodies that block this association can prevent HIV-1 entry (290). Fine characterization of the interactions between CD4 and coreceptors could lead to new targets for drug development.

Small molecule inhibitors of coreceptor activity

It has been suggested that positively charged residues of the V3 loop of X4 HIV-1 gp120 interact with negatively charged residues of CXCR4. This is supported by findings that the V3 loop of X4 isolates tends to be more positively charged than V3 loops of R5 isolates. Additionally, ecl-2 of CXCR4 is far more negatively charged than ecl-2 of CCR5 (table 1). This may explain why certain polyanionic compounds, such as heparin, dextran sulfate, and pentosan sulfate, can completely block attachment of X4 HIV-1 to HeLa CD4 cells by binding to Env glycoproteins (188), and why certain polycationic compounds, such as ALX40-4C, which consists of 9 D-arginines, can inhibit fusion of X4 viral and target cell membranes by binding to CXCR4 (180). In addition, binding of SDF-1 to CXCR4 has been partially attributed to a cluster of six positively charged amino acids in the b-sheet in the core of SDF-1 (83, 180). While a peptide derived from the N-terminus of SDF-1 could bind to CXCR4, its binding was enhanced

by the addition of basic amino acids. Finally, a class of small polycationic molecules called bicyclams is also able to bind to CXCR4 and block X4 infection of host cells. The ability of a particular bicyclam, AMD3100, which binds to ecl-2 and the adjacent forth transmembrane region of CXCR4 (163), to block both X4 and R5X4 HIV-1 infection of PBMCs suggests that R5X4 HIV-1 utilize CXCR4, rather than CCR5, on PBMCs (247). Thus, small molecule inhibitors of CXCR4 coreceptor function may have therapeutic value in blocking both R5X4 as well as X4 HIV-1. Derivatives of ALX40-4C, AMD3100, and SDF-1 peptides that lack signaling capabilities are among the many molecules that may prove useful in blocking infection.

Natural Resistance to HIV-1

Certain individuals remain uninfected despite hundreds of exposures to HIV-1. Others have been infected for many years without showing lymphadenopathy or other signs of progression to AIDS. While the latter may be ascribed to viral mutations, such as *nef* deletions, the former has been attributed to various degrees of genetic resistance to infection. Certain MHC markers have been associated with resistance, raising hope that a vaccine that stimulates CMI may be successful (182, 211). Other reports suggest that high levels of b chemokines are protective, perhaps by competing with HIV-1 for coreceptor binding sites or by downmodulating coreceptors from cell surfaces (210, 293). Another genetic marker associated with resistance is known as D32 CCR5, a 32 base pair deletion that results in a frame shift and premature termination of protein synthesis within the fifth transmembrane region (28, 174, 224). This mutation prevents CCR5

expression, and individuals who are homozygous for this mutation are much more resistant to HIV-1 infection than is the general population (84, 174, 241, 297). This is viewed by many as proof that CCR5 is the major coreceptor for HIV-1 *in vivo* (29). Heterozygous individuals are not resistant, but once they are infected they are more likely to become long-term nonprogressors.

Long-term nonprogression has also been associated a mutation known as 59029 G/A, located in the promoter of CCR5 (184), as well as a mutation in the 3 noncoding region of SDF-1 (285). The latter may result in high expression levels of SDF-1. This may slow down the progress of infection by competing with HIV-1 for CXCR4 binding sites or by down-modulating CXCR4 by receptor-mediated endocytosis.

The most controversial mutation associated with long-term nonprogression is the V64I mutation of CCR2b (14, 159, 193, 231, 254). One reason is that it is a conservative mutation that is located in a transmembrane region, rather than in an extracellular region. The main reason for the controversy is that CCR2b is not believed to be a major coreceptor; most HIV-1 isolates are not able to utilize CCR2b (97, 236).

While there are now several studies that failed to find an association between the CCR2b mutation and AIDS progression (187), there are reasons why such an association could exist. As stated the very nature of the mutation seems harmless. However, when comparing the first transmembrane region of CCR2b with that of CCR5 it was found that they differed only at position 64; remarkably, the V64I mutation of CCR2b made the first transmembrane region of both molecules identical. It has since been demonstrated that CCR2b and CCR5 can form heterodimers following chemokine binding.

Surprisingly, the same study showed that CCR2b could also heterodimerize with CXCR4 following chemokine binding. Intriguingly, an association has been found between the V64I CCR2b mutation and low PBMC expression of CXCR4 (168). The formation of heterodimers might prevent formation of the trimolecular complex — CD4, coreceptor, and Env — and may be one of many factors that effect cell susceptibility. In addition heterodimer formation in the endoplasmic reticulum may prevent surface expression of coreceptor.

Chapter 2

MATERIALS AND METHODS

Cells and culture conditions. Human HeLa cells and simian BSC-1 cells were obtained from the American Type Culture Collection, Rockville, Md., while the human glioblastoma cell line U373-MG and its U373-MG-CD4^{+} derivative cell line were provided by Adam P. Geballe, Fred Hutchinson Cancer Research Center, Seattle, WA (132). Cell cultures were maintained at 37˚C in a humidified 5% CO2 atmosphere. HeLa and BSC-1 cell monolayers were maintained in Dulbecco's modified Eagle's medium (Quality Biologicals, Gaithersburg, MD.) supplemented with 10% bovine calf serum (BCS), 2 mM L-glutamine, and antibiotics (DMEM-10). U373 cell monolayers were maintained in the same way except 15% BCS was used. U373-MG-CD4^{+} cell monolayers were also supplemented with 200 g of G418 (Calbiochem, La Jolla, CA)/ml. Cell lines that stably express mutant or wild-type CCR5 were prepared following DOTAP (Roche Molecular Biochemicals, Indianapolis, IN) transfection of U373-MG-CD4+ cells with pcDNA3.1 Hygro+ (Invitrogen, Carlsbad CA) containing coreceptor gene linked to T7 promoter. At 48 hours posttransfection media was replaced with DMEM-15 containing 200 mg/ml G418 and 200mg/ml hygromycin B (Life Technologies, Inc., Gaithersburg, MD). Media was changed three times per week, and surviving cells were cloned and expanded in DMEM-15 containing 200 mg/ml G418 and 100 mg/ml hygromycin B.

Plasmids and recombinant vaccinia viruses. For Env expression, we employed a battery of plasmids and recombinant vaccinia viruses encoding the Env genes from several R5, X4, and R5X4 HIV-1 isolates. The following recombinant vaccinia viruses expressing gp160 from different HIV-1 isolates (names in parentheses) were used: vCB-28 (JR-FL), vCB-32 (SF162), vCB-34 (SF2), vCB-39 (ADA), vCB-41 (LAV), vCB-43 (Ba-L), vCB-52 (CM235), vCB-53 (CM243) (45), and vDC-1 (89.6 (71) gp160 linked to a strong synthetic vaccinia virus early-late promoter (pSC59 (60)). Purified vaccinia virus stocks were used at a multiplicity of infection of 10 PFU/cell. Plasmids encoding functional gp160, from a variety of HIV-1 primary isolates, linked to the T7 promoter were obtained from the NIH AIDS Research and Reagent Program (Rockville, MD). They include 93BR019.10 (F/B), 92UG975.10 (G), 93BR029.2 (F), 92TH022.4 (E), MA301965.26 (C), 92BR025.9 (C), 91US005.11 (B), 92BR020.4 (B), 92UG037.8 (A), and 92RW020.5 (A). For CD4 expression, we used recombinant vaccinia virus vCB-3 (47). Bacteriophage T7 RNA polymerase was produced by infection with vTF1-1 (P11 natural late vaccinia virus promoter) (6). The Escherichia coli *lacZ* gene linked to the T7 promoter was introduced into cells by infection with vaccinia virus recombinant vCB21R-LacZ, which was described previously (9). For coreceptor expression, we employed recombinant vaccinia viruses or one of two alternative plasmid expression protocols. Vaccinia virus vHC-1 encoding CCR5 (also vvCCR5-1107) was described previously (290). Vaccinia viruses encoding wild type and mutants of CXCR4: vHC-3

(wild type CXCR4), vHC-5 (N176A CXCR4), vHC-6 (˜ C-terminal), vHC-7 (N11A/N176A CXCR4), and vHC-8 (N11A/D187A) were prepared by subcloning the appropriate cDNA into the *Sma*1 site of pMC1107 (55). The recombinant viruses were then obtained using standard techniques employing *Ecogpt* selection (48). For cell fusion assays, we either infected cells with the appropriate vaccinia virus encoding a chemokine receptor linked to 7.5k vaccinia virus promoter or we transfected cell monolayers with plasmids containing coreceptor genes linked to a strong synthetic vaccinia virus early-late promoter (pSC59) (60) followed by infection 2 h later with the Western Reserve (WR) wild-type strain of vaccinia virus, and transfection of monolayers was performed with DOTAP. For virus infection assays, cells were transfected with coreceptor genes linked to the cytomegalovirus (CMV) promoter in pCDNA3 (Invitrogen, Carlsbad, CA), and transfection was performed by the DEAE Dextran procedure, as described later.

Mutagenesis. CCR5 and CXCR4 mutations were made by using a QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA.) in accordance with the manufacturer's instructions. Two mutagenic polyacrylamide gel electrophoresis-purified oligonucleotides were used per mutation. The identities of all CXCR4 mutant constructs were confirmed by DNA sequencing.

Cell-cell fusion assays. Fusion between Env-expressing and receptor-expressing cells was measured by a reporter gene assay in which the cytoplasm of one cell population contained vaccinia virus-encoded T7 RNA polymerase and the cytoplasm of the other

contained the *E. coli lacZ* gene linked to the T7 promoter; ß-galactosidase (ß-Gal) is synthesized in fused cells (198). Vaccinia virus-encoded proteins were produced by incubating infected cells at 31¡C overnight (30). Cell-cell fusion reactions were conducted with the various cell mixtures in 96-well plates at 37˚C. Typically, the ratio of CD4-expressing to Env-expressing cells was 1:1 (2 X 10^5 total cells per well, 0.2-ml total volume). Cytosine arabinoside (40 g/ml) was added to the fusion reaction mixture to reduce nonspecific b-Gal production (30). For quantitative analyses, Nonidet P-40 was added (0.5% final) at 2.5 h and aliquots of the lysates were assayed for ß-Gal at ambient temperature with the substrate chlorophenol red-D-galactopyranoside (CPRG; Roche Molecular Biochemicals). Fusion results were calculated and expressed as rates of ß-Gal activity (change in optical density at 570 nm per minute X 1,000) (198).

HIV-1 infection studies. U373-MG-$CD4^+$ target cells were prepared in 48-well plates and transfected with the desired coreceptor-encoding plasmid by the DEAE dextran method. Briefly, 0.2 g DNA mixed in 110 l DMEM-2.5 with 100 M chloroquine diphosphate and 1.1 l of DEAE dextran stock (10 mg/ml) in PBS was added to each well of semiconfluent cells. After four hours media was replaced with 10% DMSO/PBS for two minutes. Monolayers were then washed with PBS and incubated overnight in DMEM-15. Viral infection assays were performed with a luciferase reporter HIV-1 Env pseudotyping system (73). Viral stocks were prepared as previously described by transfecting 293T cells with plasmids encoding the luciferase virus backbone (pNL-Luc-

ER) and Env from HIV strain JR-FL (201) or NL4-3 (LAV) (2), (221). The resulting supernatant was clarified by centrifugation for 10 min at 2,000 rpm in a Sorvall RT-7 centrifuge (RTH-750 rotor) and stored at 4¡C. Monolayers were infected with 100 l virus containing 8 g/ml DEAE dextran. After two hours 0.5 ml DMEM-15 was added to each well. Cells were lysed at 72 hours post-infection by resuspension in 105 l of cell lysis buffer (Promega, Madison, WI), and 50 l of the resulting lysate was assayed for luciferase activity, using an equal volume of luciferase substrate (Promega).

Western blot analysis. BSC-1 cell monolayers were infected overnight at an MOI of 10 with vaccinia virus encoding wild-type or mutant CXCR4. Western blot detection of CXCR4 was performed essentially as described previously (117) but with some modification. Cells were extracted with 0.5% Triton X-100 in 20 mM Tris-HCl, pH 8.0, 100 mM NaCl and the nuclei removed by centrifugation. Extracts from 5 x 10^4 cells (total) were loaded per well onto a 10% SDS-PAGE gel, samples were incubated 30 min at 37• C and not boiled, as boiling often induces aggregation of 7TM proteins. Following transfer to nitrocellulose paper, the blot was probed with 4G10, an anti-CXCR4 MAb (107). The blot was then incubated with HRP-conjugated rabbit anti-mouse IgG and developed with the Pierce SuperSignal chemiluminescence kit (Rockford, IL).

Cell surface staining. Coreceptor expression levels were determined by fluorescent antibody cell-surface staining. Appropriate cells were transfected or infected as described, and incubated overnight. Cells were then kept on ice and 10^6 cells were

washed twice with PBS, and once with PBS containing 2.5% bovine serum, and incubated in PBS with 2.5% bovine serum and 4 µg/100 l 2D7 MAb for CCR5 or 2 g/100 l 12G5 or 4G10 MAb for CXCR4, incubated one hour, washed three times with PBS, incubated in PBS with 2.5% goat serum and 10 l/100 l phycoerythrin labeled goat anti-mouse IgG for 45 minutes, and washed three times with PBS, and fixed with 2% paraformaldehyde in PBS. Fluorescence was measured with the Coulter EPIC XL flow cytometer (Miami FL).

Molecular modeling. Theoretical 3-dimensional structures of the HIV-1 coreceptors CXCR4 and CCR5 were based on the physically determined structures of both bacteriorhodopsin and rhodopsin (245, 270, 271), as well as analysis of the amino acid sequences of related G-protein coupled receptors (88, 91). A molecular modeling software package (Insight II 98.0, Molecular Simulations, Inc., San Diego, CA) was used to add a hypothetical 3 branched N-linked carbohydrate structure with a molecular weight of 6 kDa (based on the analysis of the CXCR4 non-N-linked glycosylated mutants) to the N-terminus of the CXCR4 molecule.

Chapter 3

Identification of CXCR4 Domains Important for Human Immunodeficiency Virus Type 1 X4 Isolate Envelope-Mediated Membrane Fusion and Virus Entry

RESULTS

Generation and expression of mutant CXCR4 molecules. It has been established from numerous reports that a major determinant of HIV-1 cellular tropism for infection was the envelope glycoprotein, with special emphasis on a role by the V3 loop of gp120 (reviewed in (189, 218)). More recently these earlier observations have led to the development of a model for Env-CD4-coreceptor interaction whereby the V3 loop is proposed to directly interact with CXCR4 or CCR5 (87), with the notion that the electrostatic charge of the V3 loop may be at least one important factor in this interaction (88). Because of these observations we initially focused our mutagenesis efforts on the predicted charged extracellular residues in CXCR4. We chose an alanine-scanning mutagenesis strategy, in this case charged-to alanine scanning mutagenesis (123), because it is a well accepted technique for mapping or identifying potential residues involved in particular protein-protein interactions. Shown in Fig. 2 is a representation of the CXCR4 molecule indicating the locations of the altered amino acid residues used in the present study. In addition to charged amino acid substitutions we also included a set of alanine substitutions for the four extracellular cysteine residues in both single and paired configurations, two N-terminal deletion constructs, and alanine substitutions for the two asparagine residues predicted to be sites of N-linked glycosylation. One point

mutation, F201A, was made by mistake but still included in this study.

Depending on the assay employed we expressed coreceptor genes using a vaccinia virus promoter or a cytomegalovirus (CMV) promoter based system, with a plasmid transfection protocol. With few exceptions, most notably some of the cysteine substitutions, analysis of cell surface expression by cell surface antibody staining and flow cytometry indicated that the majority of the mutant CXCR4s in our panel were expressed at levels comparable to wild-type CXCR4 (Tables 2 and 3). While elimination of amino acids 2 through 16 (N-Term Del-15) did not sharply reduce CXCR4 expression, expression was drastically reduced by elimination of amino acids 2 though 37 (N-Term Del-36) from the N-terminal domain. A double aspartic acid N-terminus substitution, E14A/E31A, also showed drastically reduced surface expression, whereas each of these mutations individually had approximately wild-type levels of cell surface expression. Several other combinations of mutations were attempted but had poor surface expression and were not included in our analysis (data not shown). However, for the purposes of quantifying coreceptor activity and comparing the mutant CXCR4s to wild-type CXCR4, all cell-cell fusion data (Fig. 3) was first corrected for variations in cell surface expression, i.e. if a particular mutation exhibited a marked reduction in coreceptor activity yet was poorly expressed as compared to wild-type CXCR4 it would potentially be misleading.

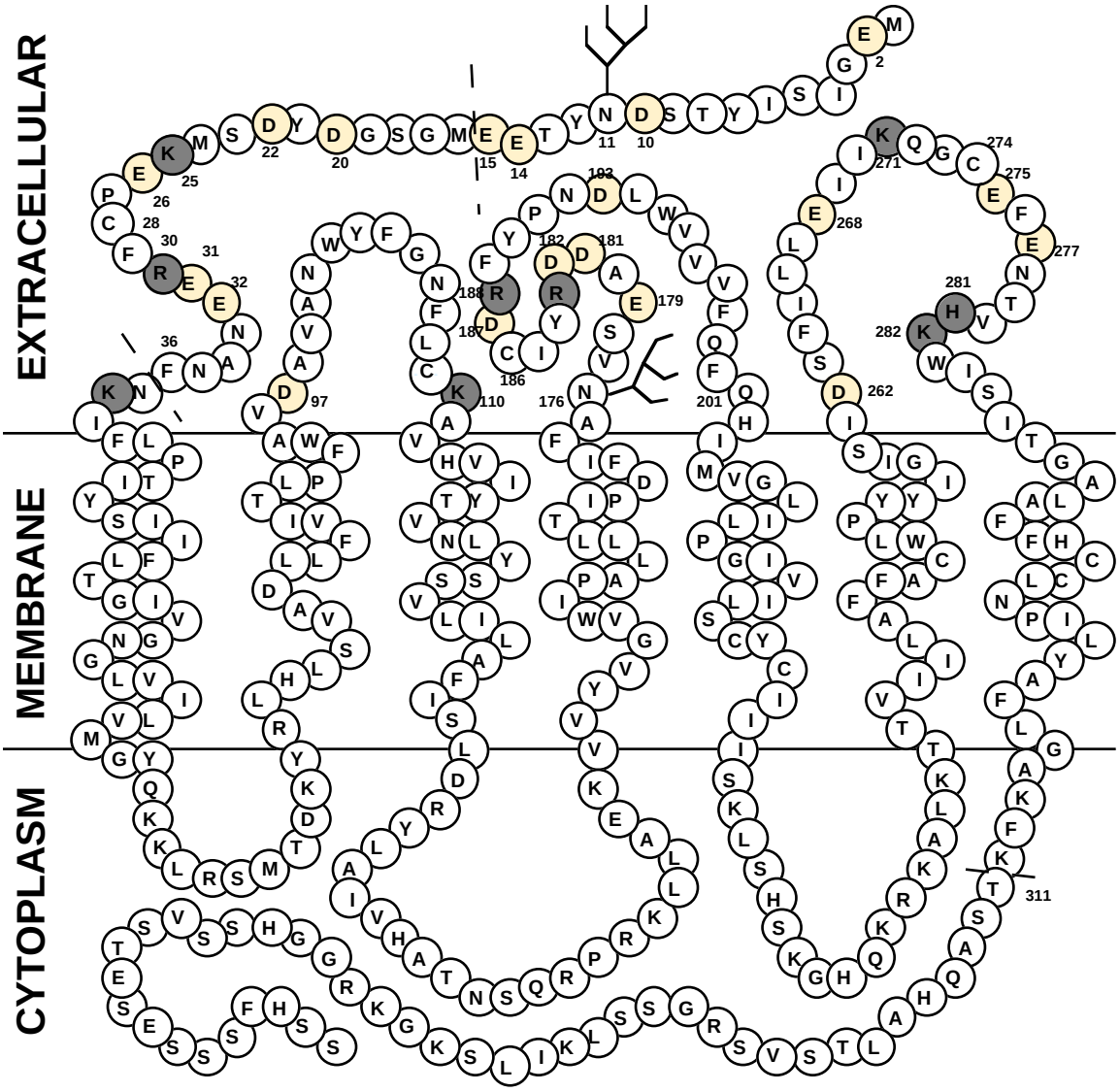


Figure 2. Bubble diagram of CXCR4. Predicted extracellular, transmembrane, and cytoplasmic regions are indicated. Residues that have been altered are numbered. Acidic residues are lightly shaded. Basic residues are darkly shaded. Extracellular cysteines are highlighted. Dashed lines indicate positions where N-terminal and C-terminal deletions were made.

Coreceptor activities of mutant CXCR4s for X4 Envs. We used a well characterized cell-cell fusion assay to determine coreceptor function for a panel of 33 CXCR4 point mutations. Vaccinia virus-encoded HIV-1 Envs were expressed in HeLa effector cells co-infected with a vaccinia virus encoding the *E. coli lacZ* gene linked to the T7 promoter (vCB21R-LacZ). U373 target cells were transfected with plasmids encoding wild-type or a mutant CXCR4 gene linked to a vaccinia virus promoter. After overnight expression, target and effector cells were mixed and fusion was allowed to proceed for 2.5 h and assessed as described in the Methods section. Results (Fig. 3) are adjusted to reflect cell surface expression levels, as determined by flow cytometry analysis with 12G5, a conformation dependent MAb to CXCR4, and the data is presented as an average of the percentage of wild-type CXCR4 coreceptor activity derived from three independent experiments performed in duplicate. The bars in the Fig. 3 data represent the range of the three calculated percentages over their average since the data is a calculation based on wild-type CXCR4 activity being arbitrarily regarded as 100%. For the X4 Envs: LAV and IIIB (Fig. 3, panels A and B respectively), and the R5X4 Envs: SF2 and 89.6 (Fig. 3, panels C and D respectively), we found a >50% reduction in coreceptor activity with three alanine substitution mutants for the negatively charged glutamic acid residues in the N-terminus (E14A, E15A and E32A). Previously, we proposed that the N-terminus of CXCR4 may play a role in the HIV-1 Env-mediated fusion event based on studies demonstrating that a rabbit polyclonal antiserum raised against a synthetic peptide corresponding to the entire predicted extracellular domain could block both CXCR4 supported cell-cell fusion and virus infection (117). The present results further support

this initial observation and suggest that several negatively charged residues may be specifically involved, perhaps by directly associating with elements in Env. It was unfortunate that further analysis of a double mutation (E14A/E31A) was not expressed at appreciable levels on the cell surface to warrant any additional supportive conclusions as to the importance of these negatively charged N-terminal residues in CXCR4 coreceptor activity. We also analyzed two N-terminal deletion CXCR4 mutation constructs, one 36 residues in length and the other 15. The 36 residue N-terminal deletion CXCR4 mutant was poorly expressed having only 19% the level of wild-type CXCR4, and we felt was unsuitable to include in our analysis. However, the 15 residue N-terminal deletion CXCR4 mutant was expressed on the cell surface at about 70% the level of wild-type CXCR4 (Table 1), but was only moderately defective in coreceptor activity with the exception of Env 89.6.

CXCR4 is capable of signal transduction after appropriate ligand stimulation. The C-terminus is rich in conserved serine and threonine residues and represent potential phosphorylation sites by the family of G protein-coupled receptor kinases, and there is a conserved DRY motif in the intracellular loop 2 which is believed to be a site of G protein interaction (reviewed in (219)). Our substitution mutant to remove the DRY sequence was not expressed on the cell surface (Table 1). However, we found no diminution in CXCR4 coreceptor activity with a 42 residue C-terminal deletion suggesting no role for this domain in Env-mediated fusion in cell lines, findings in agreement with others.

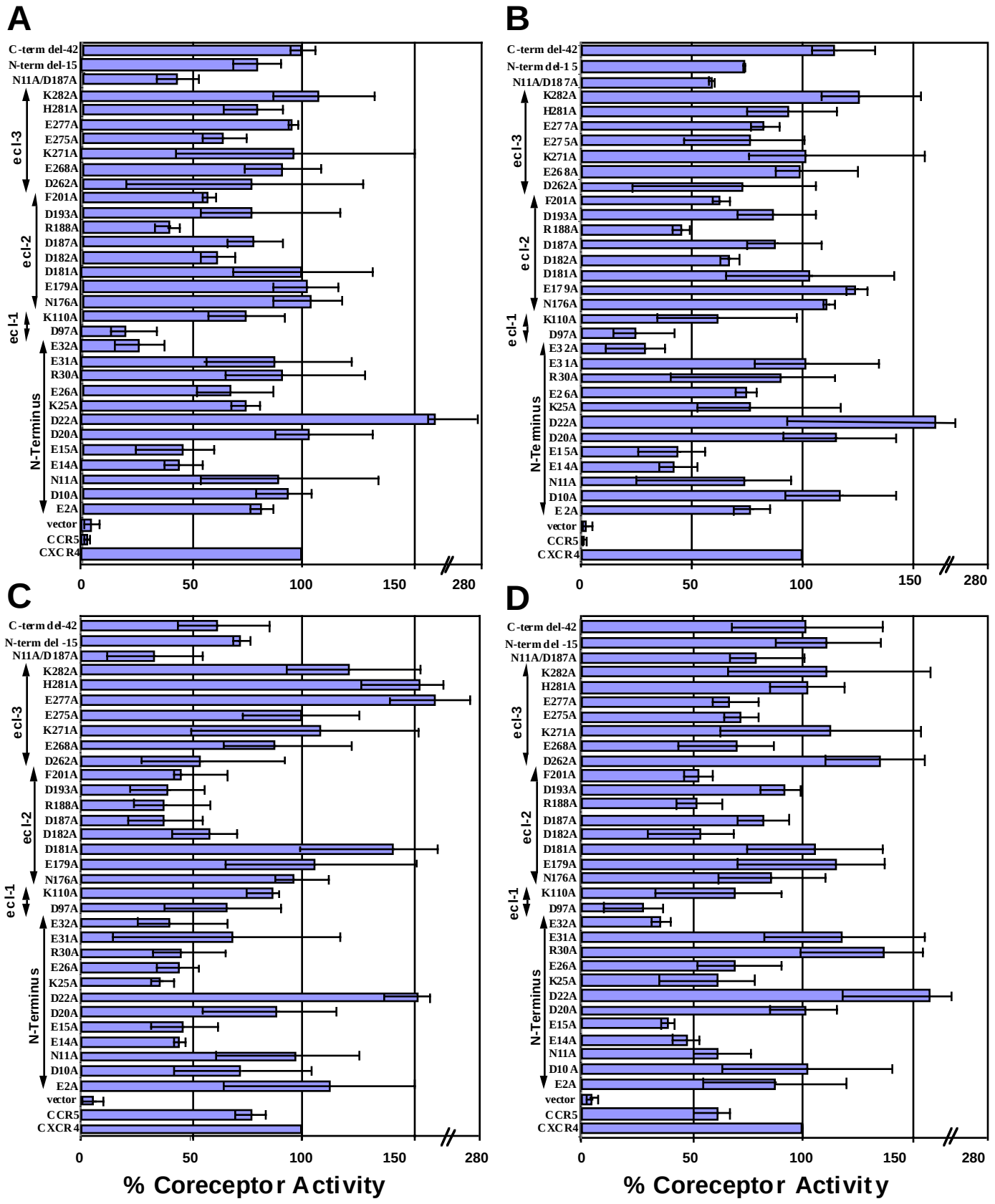

A
B
C
D
C-term del-42
N-term del-15
N11A/D187A
K282A
H281A
E277A
E275A
K271A
E268A
D262A
F201A
D193A
R188A
D187A
D182A
D181A
E179A
N176A
K110A
D97A
E32A
E31A
R30A
E26A
K25A
D22A
D20A
E15A
E14A
N11A
D10A
E2A
vector
CCR5
CXCR4
ecl-3
ecl-2
ecl-1
N-Terminus
0
50
100
150
280
% Coreceptor Activity

Figure 3. Coreceptor function of mutant CXCR4s in cell-cell fusion assays with T cell-line tropic (X4) and dual tropic (R5X4) HIV-1 Envs. U373 target cells were transfected with a plasmid encoding the wild-type or a mutant CXCR4 gene linked to a vaccinia virus promoter and infected with vCB21R-lacZ and vCB-3 (CD4). HeLa Env-expressing effector cells were infected with a vaccinia virus encoding T7 polymerase (vTF1-1) and one encoding A: LAV (vCB-41); B: IIIB/BH10 (vCB-40); C: SF2 (vCB-34); or (D) 89.6 (vDC-1). Cell mixtures (duplicates) were incubated at 37• C for 2.5 h. Fusion was assessed by measurement of b-Gal in detergent cell lysates. The activities of the mutant CXCR4s are presented as a percentage of wild-type CXCR4 activity after adjusting for the level of cell-surface expression as detailed under Methods. Each mutant CXCR4 construct was tested in duplicate at three times. The averages of these results are shown in the figure. The bars in the figure represent the range of those three calculated percentages.

CXCR4 mutation	Percentage in comparison to wild-type CXCR4 (range of three experiments)	Average percentage
E2A	78-82	80
D10A	112-115	113
N11A	66-164	115
E14A	133-173	151
E15A	134-203	160
D20A	91-94	92
D22A	62-97	78
K25A	45-117	81
E26A	91-102	96
R30A	60-118	89
E31A	75-98	86
E32A	118-136	127
D97A	144-201	172
K110A	62-82	73
N176A	72-111	91
E179A	85-112	98
D181A	67-86	76
D182A	51-89	70
D187A	108-113	110
R188A	94-106	99
D193A	87-88	87
F201A	91-146	118
D262A	65-101	83
E268A	43-103	73
K271A	70-87	78
E275A	86-141	113
E277A	58-110	84
H281A	86-140	113
K282A	92-112	102
N11A/D197A	102*	102
E14A/E31A	9.6*	9.6
N-term del-15	59-79	70
N-term del-36	17-22	19
D133K/R134S/Y135E	1.9*	1.9
C-term del-42	91*	91

*single measurement

Table 2. Cell surface expression of CXCR4 mutation constructs. U373 cells were transfected with wild-type or mutated CXCR4 in the pSC59 vaccinia vector. Cells were then infected with wild-type vaccinia virus (WR), incubated with anti-CXCR4 MAb (12G5), and stained with phycoerythrin-anti-mouse IgG. Mean fluorescent intensities were determined using flow cytometry (Coulter XL-MCL Miami, FL). Values for mutant CXCR4s are the percentage of the mean fluorescent intensity of wild-type CXCR4, which was regarded as 100%. The average percentage of three experiments and the range of those percentages are shown.

The analysis of charged residue mutations in ecl-1 of CXCR4 identified additional important residues. The ecl-1 of CXCR4 is the smallest of the three outside loops and possesses only two charged residues (Fig. 2). Mutagenesis of the negatively charged aspartic acid, D97A, potently abrogated coreceptor function for X4 Envs LAV and IIIB, as well as for the R5X4 Env 89.6. Indeed, for these three Envs the D97A mutation was the most potent single CXCR4 alteration found in this panel, being slightly better at inhibiting coreceptor activity than any of the glutamic acid residues in the molecule s N-terminus (Fig. 3, panels A, B, and D). Whereas the elimination of the single positively charged residue in ecl-1 (K110A) had little effect. The SF2 Env (Fig. 2, panel C) appeared unaffected in an ability to employ these ecl-1 mutant CXCR4s as functional coreceptors. These results support the notion of a significant role for the ecl-1 of CXCR4 for both X4 and one R5X4 Env-mediated fusion.

Mutagenesis of ecl-2 and ecl-3 of CXCR4 yielded more variable results (Fig. 3). Only substitution of the positively charged arginine residue in ecl-2 (R188A) had significantly reduced fusion activity for all four Envs examined. Although some other individual mutations of both positively and negatively charged residues in ecl-2 and ecl-3 had minor inhibitory effects on coreceptor activity for LAV, IIIB, or 89.6, we did not observe any consistent pattern and in no case was the inhibition greater than or equal to a 50% reduction. We also included a single phenylalanine point mutation in ecl-2 that was made in error and found that it was about 50% reduced in coreceptor activity for all Envs examined. In general, our results with LAV, IIIB, and 89.6 were quite similar, but as a set they differed somewhat from the results achieved with SF2, the second R5X4 Env. A

number of additional single amino acid substitutions caused reduced coreceptor activity specifically with the SF2 Env: E26A and K25A in the N-terminus and D187A and D193A in ecl-2. As a whole, the data suggests that SF2 appears more dependent on ecl-2 rather that ecl-1 in conjunction with the N-terminus. These observations indicate that an individual Env glycoprotein may exhibit specific or somewhat unique coreceptor structure dependencies.

While the HIV-1 Env-mediated cell-cell fusion assay presents a reliable model of HIV-1 Env-mediated fusion and receptor function (30), we also tested many of the CXCR4 mutations that resulted in defective coreceptor activities in HIV-1 virus infection assays as well. We transfected U373-CD4^{+} cells with plasmids encoding wild-type or mutant CXCR4s linked to a CMV promoter and, following a period of expression, infected the cells with luciferase reporter-gene HIV-1 Env pseudoviruses using the HXB2 or NL4-3 Envs. These results showed that four of the five CXCR4 mutations (E15A, E32A, D97A, and R188A) that consistently showed a significantly reduced ability to support Env-mediated fusion in the cell-cell fusion assay also had reduced activities in this virus infection assay (Fig. 4). The E14A mutation was the exception supporting infection at wild-type levels. Additionally, the lysine substitution in ecl-1 (K110A), which had 60-75% wild-type activity in the fusion assays, depending on the Env tested, was also significantly impaired in coreceptor activity for virus infection (Fig. 4). I believe that it is noteworthy that the magnitude of reduction of any one mutant CXCR4 is quite similar between these two very different assays even though the levels of

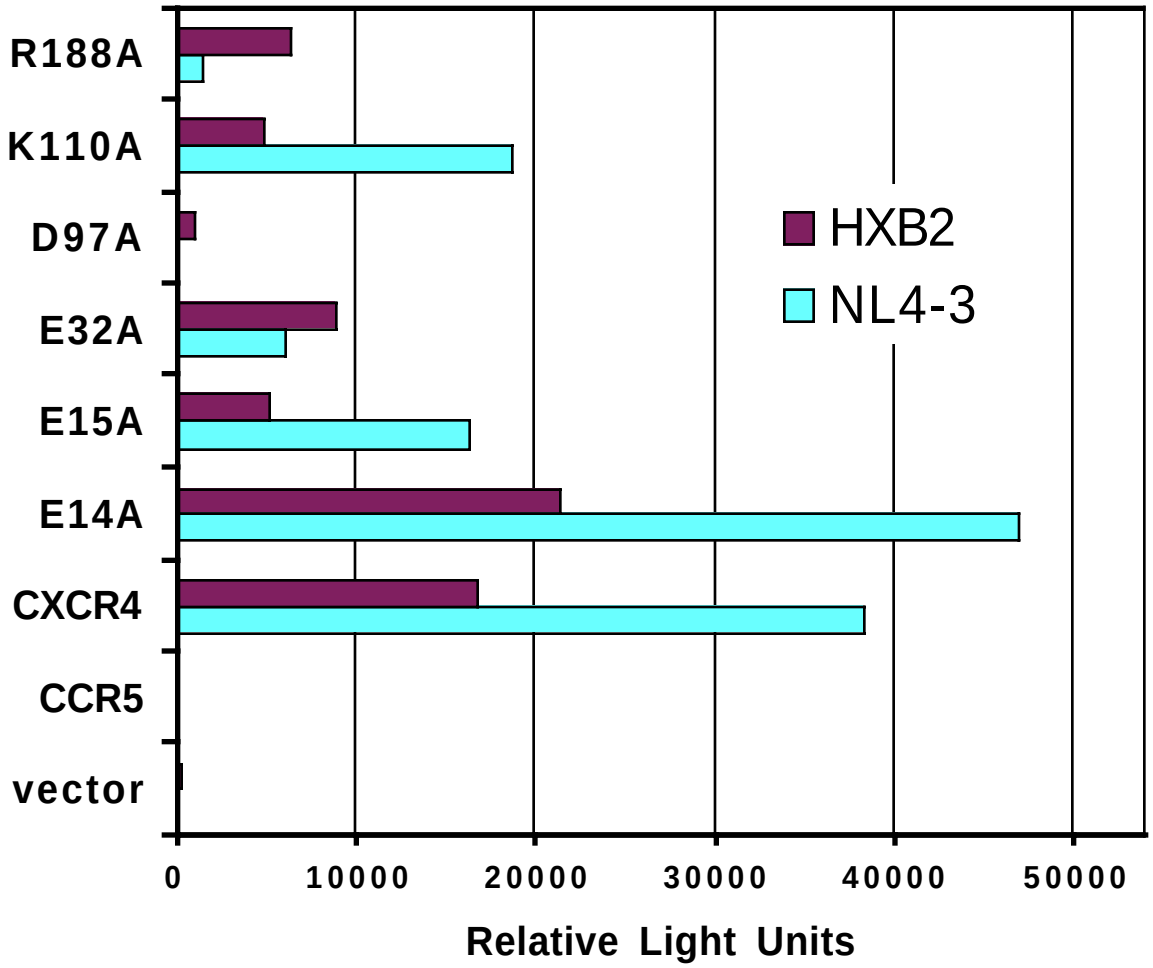


Figure 4. Coreceptor function of mutant CXCR4s in virus infection assays with X4 HIV-1 Envs. U373-CD4^{+} cells were transfected with a plasmid (pCDNA3) encoding the indicated wild-type coreceptor (CXCR4 or CCR5) or mutant CXCR4. Wells of cells (triplicate) were infected with the indicated HIV-1 Env luciferase reporter virus. Infection was assessed at day 4 by measuring the amounts of luciferase activity in cell lysates. The luciferase activity shown was obtained from separate samples in the same experiment. This experiment was repeated three times, and the data from a representative experiment is shown in the figure. All samples were tested in duplicate and the averages were plotted.

coreceptor expression is different between the vaccinia virus promoter and CMV promoter based systems. Also, it is pointed out that unlike the data in Fig. 3, and due to the assay requirements, no correction of surface expression levels is made in the data presented in Fig.4, even though some of the mutant CXCR4s (D97A and E15A) are expressed on the cell surface at levels greater than wild-type CXCR4.

Taken together these results indicate that negatively charged acidic residues in the N-terminus and ecl-1 are required for optimal coreceptor activity for several T-cell line tropic X4 and R5X4 Envs. These amino acids appeared to be the most important of the charged residues examined because their elimination resulted in a marked reduction of CXCR4 coreceptor (greater than 50%) activity for all Envs tested. Also, an additional positively charged residue in ecl-2 was important for coreceptor activity. This later observation could be interpreted as evidence that the sites of interaction between the CXCR4 coreceptor and HIV-1 Env are most likely a complex, three-dimensional array of specific contact sites dependent on both positively and negatively charged residues in the molecule s extracellular domains. In summary, the loss of activity associated with loss of acidic residues supports the hypothesis that Env tropism is determined, at least in part, by ionic interactions between the extracellular domains of CXCR4 and Env; mutation of the Env V3 loop to a more positive overall charge has been associated with a shift from R5 to X4 coreceptor usage (82, 119). Similar results were obtained with this panel of mutants when expressed in BS-C-1, 3T3, and RK-13 cells (data not shown).

Coreceptor activities of mutant CXCR4s for R5 Envs. During the course of our cell-cell fusion experiments we included a prototypic R5 HIV-1 Env glycoprotein expressing effector cell as one of our negative controls and unexpectedly we found four amino acids in CXCR4, out of the entire panel, which when substituted with alanine allowed CXCR4 to serve as a coreceptor for an R5 Env. These residues were in the N-terminus (N11A and R30A) and in ecl-2 (D187A and D193A), and they consistently supported fusion, over background, with JR-FL, as well as several other prototypic R5 Envs, SF162 and Ba-L (Fig. 4) and ADA (data not shown) (93). This phenomenon was not an artifact of the target cell line, as similar results were seen with monkey BSC-1, human U373-CD4^{+}, mouse 3T3, and rabbit RK13 cells (data not shown). This activity could be blocked by 12G5, an antibody to CXCR4 (data not shown). The two most potent single amino acid alterations were D187A and N11A with the D187A mutation being the better of the two. The importance of the D187 residue upon substitution with valine or alanine in allowing CXCR4 usage by HIV-1 R5 Envs was also recently reported by Wang et al. (276) in a mutagenesis study of the ecl-2 of CXCR4 with a similar cell-cell fusion assay employing the same HIV-1 Env-encoding recombinant vaccinia viruses. When combined, our two most potent mutants, D187A and N11A, showed a greater than additive effect in supporting R5 Env-mediated fusion. As shown in Fig. 5, the N11A/D187A mutant supports a JR-FL Env-mediated cell-cell fusion activity that exceeds the activities produced by the X4 Envs LAV and IIIB with either wild-type CXCR4 or the mutant N11A/D187A.

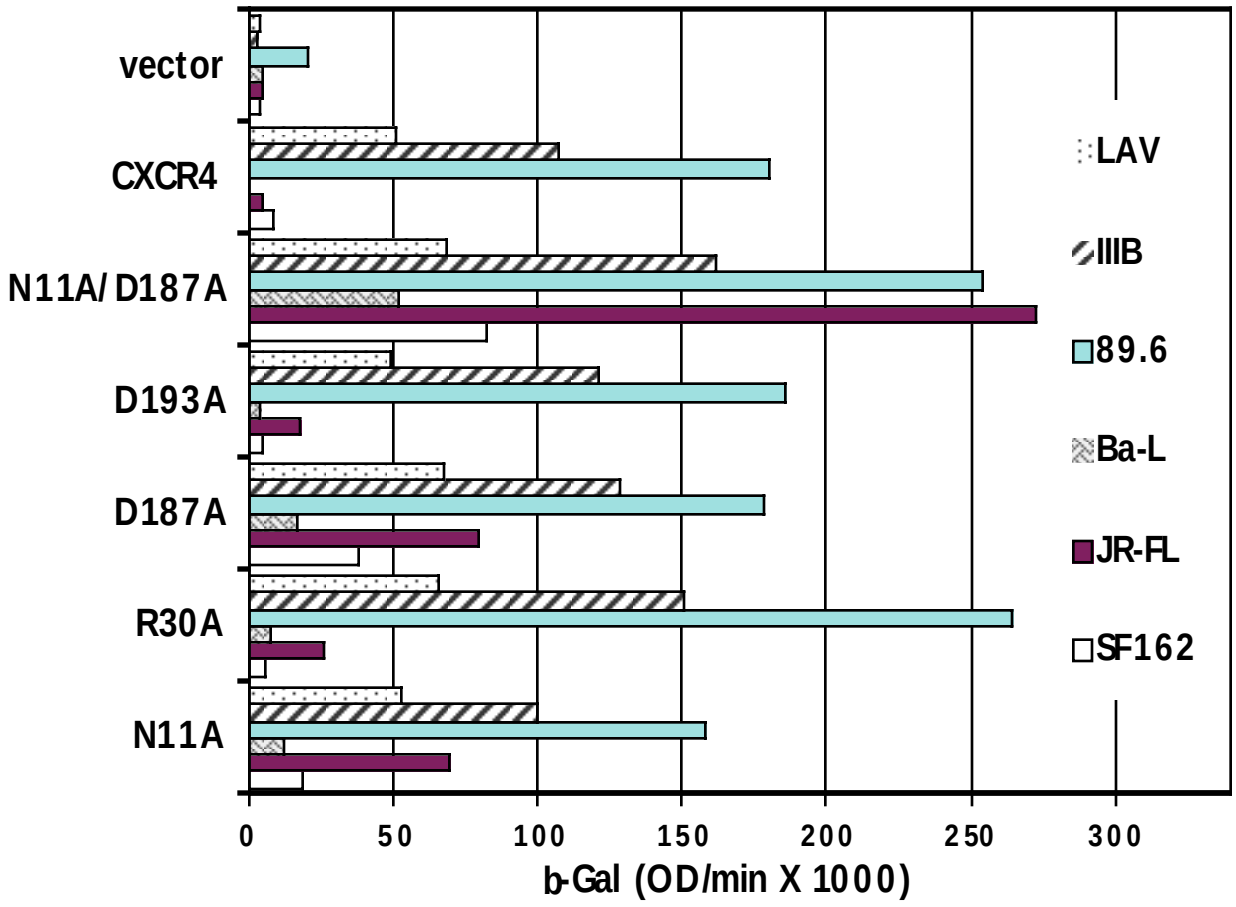


Figure 5. Coreceptor function of mutant CXCR4s that support R5 Env fusion. BS-C-1 target cells were transfected with a plasmid encoding the wild-type or the indicated mutant CXCR4 construct linked to a vaccinia virus promoter and infected with vCB21R-lacZ and vCB-3 (CD4). HeLa effector cells were infected with vTF1-1 and either LAV (vCB-41), IIIB/BH10 (vCB-40), 89.6 (vDC-1), Ba-L (vCB-43), JR-FL (vCB-28), or SF162 (vCB-32). Cell mixtures (duplicates) were incubated at 37ß C for 2.5 h. Fusion was assessed by measurement of ß-Gal in detergent cell lysates. The rates of ß-Gal activity shown were obtained from separate samples in the same experiment. This experiment was performed three times, and the data from a representative experiment is shown in the figure. All samples were tested in duplicate and the averages were plotted. OD: optical density.

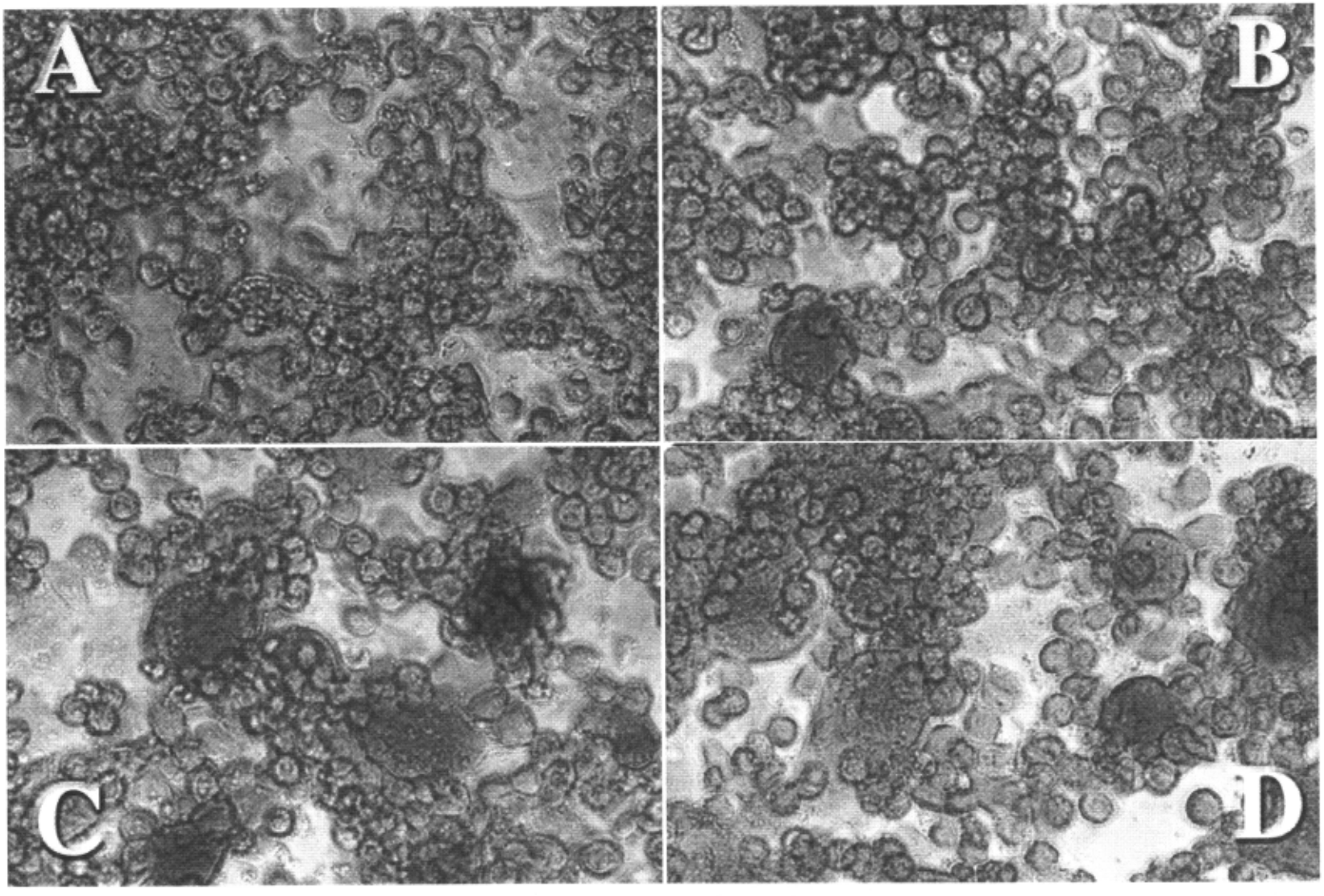

A
B
C
D

Figure 6. Coreceptor function of mutant CXCR4s that support R5 Env fusion in syncytia assay with the R5 isolate JR-FL Env. U373 target cells were transfected with a plasmid encoding the wild-type or the indicated mutant CXCR4 construct linked to a vaccinia virus promoter and infected with vCB21R-lacZ and vCB-3 (CD4). A: wild-type CXCR4; B: CXCR4-D187A; C: CXCR4-N11A/D187A; D: wild-type CCR5. HeLa effector cells were infected with vaccinia virus encoding the JR-FL Env (vCB28). Cell mixtures (duplicates) were incubated at 37• C for 6 h. Fusion was assessed by staining with crystal violet and light microscopy (200X).

To ensure that the cell-cell fusion we were observing between R5 Env-expressing cells and the mutant CXCR4 CD4+ cells was equivalent to that observed with any X4 Env-mediated fusion event, and not in someway restricted to an early fusion intermediate such as a fusion pore (194) that was simply allowing for the activation of the *lacZ* reporter system via the transfer of T7 polymerase, we also performed syncytia assays with these mutant CXCR4s. The results from these experiments paralleled the cell-cell fusion assay findings (Fig. 6). Syncytia formed when U373-CD4$^+$ cells expressing coreceptor were mixed with HeLa cells expressing the HIV-1 R5 JR-FL Env (Fig. 6, panel B), and syncytia were much larger with U373-CD4$^+$ cells expressing the N11A/ D187A mutant CXCR4 than with cells expressing the D187A mutant (Fig. 6, panel C). Indeed, syncytia with the N11A/ D187A mutant CXCR4 were essentially equivalent as compared with U373-CD4$^+$ cells expressing CCR5 (Fig. 6, panel D). Because of these surprising results we thought it important to examine some SIV Envs for ability to employ these R5 Env fusion-supporting CXCR4 mutants as functional coreceptors since almost all SIV Envs examined to date are CCR5-dependent. We found that none of the CXCR4 mutants that could function for HIV-1 R5 Envs supported fusion mediated by the SIV Envs, mac239, mac316, or mac316mut (108) (Data not shown).

To confirm these findings we also performed virus infection experiments with luciferase reporter HIV-1 R5 Env pseudoviruses with the D187A, N11A and N11A/D187A mutant CXCR4s. In general, we found similar results to that achieved with the cell-cell fusion assay, i.e. the combination mutant N11A/D187A was the best at supporting R5 Env-mediated fusion (as measured by virus entry) as compared to N11A

or D187A alone with pseudoviruses composed of JR-FL, ADA, Ba-L, or SF162 (Fig. 7). We repeated this experiment numerous times in several suitable target cell types and achieved essentially the same results, with the relative light unit values more than a log lower compared to CCR5 for the N11A/D187A mutant and approximately three logs lower for the D187A mutant. Indeed, the single N11A mutant CXCR4 which weakly supports cell-cell fusion mediated by JR-FL Env did not consistently support R5 Env pseudovirus entry. These HIV-1 pseudovirus infection results with the D187A mutant is in contrast to recent results reported by Wang et al. (276) who found very substantial virus entry luciferase signals with a D187A mutant CXCR4 expressed in U87-MG cells. It is possible that the discrepancy between the results obtained from our two types of assays could be the result of variations in the levels of CD4 and/or coreceptor expression in the two assays; vaccinia virus promoters were used to express CD4 and coreceptor in the cell-cell fusion assay, while CMV promoters were used in the virus infection assay. To address this possibility we performed the cell-cell fusion assay with CMV promoter-driven coreceptor and CD4 expression. Although the §-Gal levels were much lower, reflecting the fact that the vaccinia expression system yields much higher gene expression levels, the results again showed that the N11A/D187A CXCR4 mutant functioned nearly as well as CCR5 as a coreceptor for R5 Envs (Fig. 8). In regards to the minor discrepancy between our results, with the D187A mutant and virus infection, and that of Wang et al. (276) it may be a cell type phenomenon whereby the mutant CXCR4 is better recognized by R5 Envs in U87-MG cells. However, we think this to be unlikely since the U373-MG cells used in our assays are quite similar to the former.

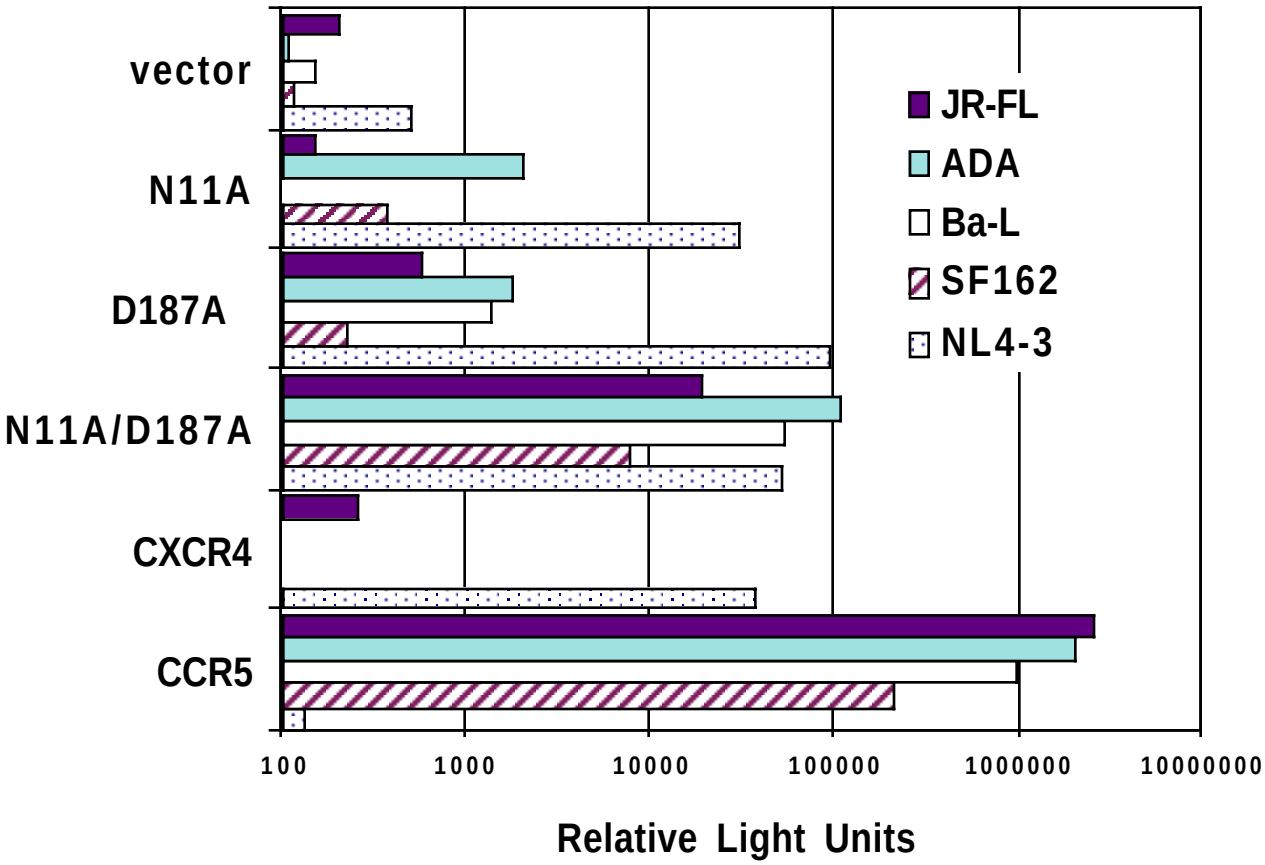


Figure 7. Coreceptor function of mutant CXCR4s in virus infection assays with R5 HIV-1 Envs. U373-CD4^{+} cells were transfected with a plasmid (pCDNA3) encoding the indicated wild-type coreceptor (CXCR4 or CCR5) or mutant CXCR4. Wells of cells (triplicate) were infected with the indicated HIV-1 Env luciferase reporter virus. Infection was assessed at day 4 by measuring the amounts of luciferase activity in cell lysates. The luciferase activity shown was obtained from separate samples in the same experiment. This experiment was performed three times, and the data from one experiment is shown in the figure. All samples were tested in duplicate and the averages were plotted.

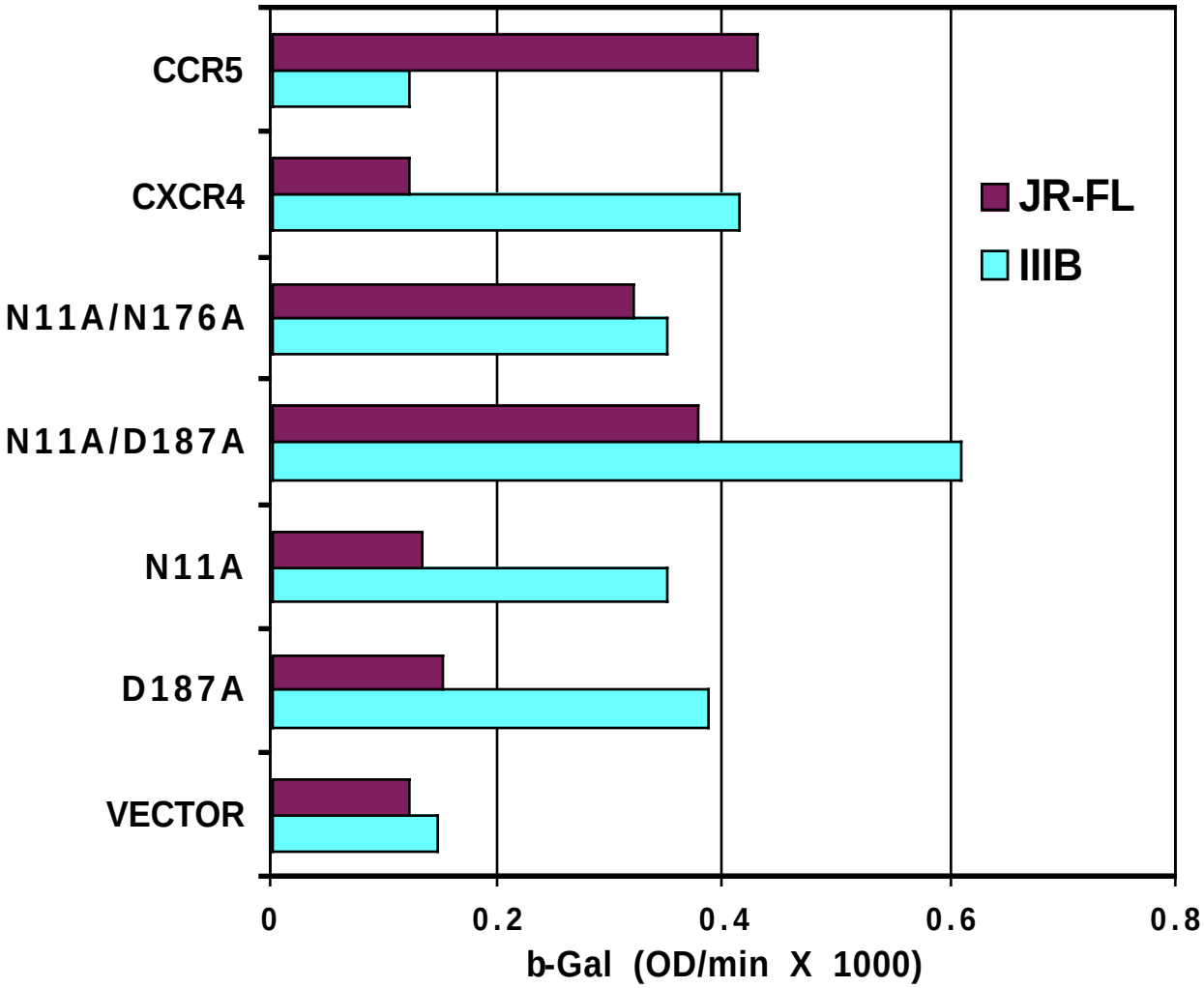


Figure 8. Coreceptor function of mutant CXCR4s in cell-cell fusion assays with low level expression. U373-CD4^{+} cells were transfected with a plasmid (pCDNA3) encoding the indicated wild-type coreceptor (CXCR4 or CCR5) or mutant CXCR4, and after overnight expression the cells were then infected with vCB21R-LacZ. HIV-1 effector cells were infected with vTF1.1 (T7 polymerase) and either vaccinia virus encoding the JR-FL Env (vCB28) or the IIIB/BH10 Env (vCB40). Fusion was assessed by measurement of §-Gal in detergent cell lysates. All samples were tested in duplicate and the averages were plotted.

Alternatively, this difference could reflect an as yet to be identified post-binding function of coreceptor during virus infection.

Roles of extracellular cysteines. The cysteine residues in ecl-1 and ecl-2 are highly conserved among the 7TMGPCRs and are believed to form a disulfide bond between each other (261). Our findings support the hypothesis that this pair of cysteines in CXCR4 forms a disulfide bond and is probably critical for proper folding and cell surface expression. Both the C109A and C186A individual CXCR4 mutations were essentially undetectable on the cell surfaces (Table 3). Substituting either of these cysteines individually with alanine abrogates cell surface expression, as determined by cell surface antibody staining with 12G5, a conformation-dependent MAb, and 4G10, a conformation-independent anti-CXCR4 MAb. Interestingly, substituting both cysteines (C109A/C186A) with alanine allowed for a small amount of surface expression (about 10% of wild-type, Table 2). This might be accounted for by the possibility that a single cysteine elimination allows for inappropriate disulfide bond formation among the remaining three extracellular cysteines. The inappropriate disulfide bonds formation may result in misfolded CXCR4 molecules that are not well-expressed on the cell surface. The examination of the second cysteine pair provided more interesting results. This second pair of extracellular cysteine residues, one in the N-terminus and another in ecl-3, is conserved among the chemokine receptors but not by other 7TMGPCR family members. Our results indicated that these cysteines may also form a disulfide bond, but that this bond is not essential for coreceptor function for HIV-1 Env-mediated membrane

fusion. Substitution of the N-terminal cysteine (C28A) alone resulted in a greater than 50% reduction in coreceptor activity with several X4 or R5X4 Envs (Fig. 8). While substitution of the ecl-3 cysteine with alanine or substitution of both the N-terminal and ecl-3 cysteines with alanine yielded a CXCR4 molecule possessing near 75% of wild-type levels of activity (Fig. 8). If there was not a bond between these two cysteines one would expect the double cysteine mutant to have less than or equal activity as compared to either of the single cysteine mutants. While the single and double cysteine mutants are expressed at wild-type levels, as determined by FACS analysis with 4G10 (a conformation-independent MAb), they all had reduced staining with 12G5 (a conformation-dependent MAb, Table 2), strongly suggesting that an alteration in conformation has occurred through elimination of this disulfide bond. As with the cysteines in ecl-1 and ecl-2, 12G5 cell-surface immunostaining indicated that substitution of both the N-terminus cysteine and the ecl-3 cysteine together resulted in some restoration of the protein s conformation, having higher surfaced-expressed levels than either cysteine mutation by itself. Because of this later observation we again speculate that mutation of a single cysteine allows its paired cysteine to form inappropriate

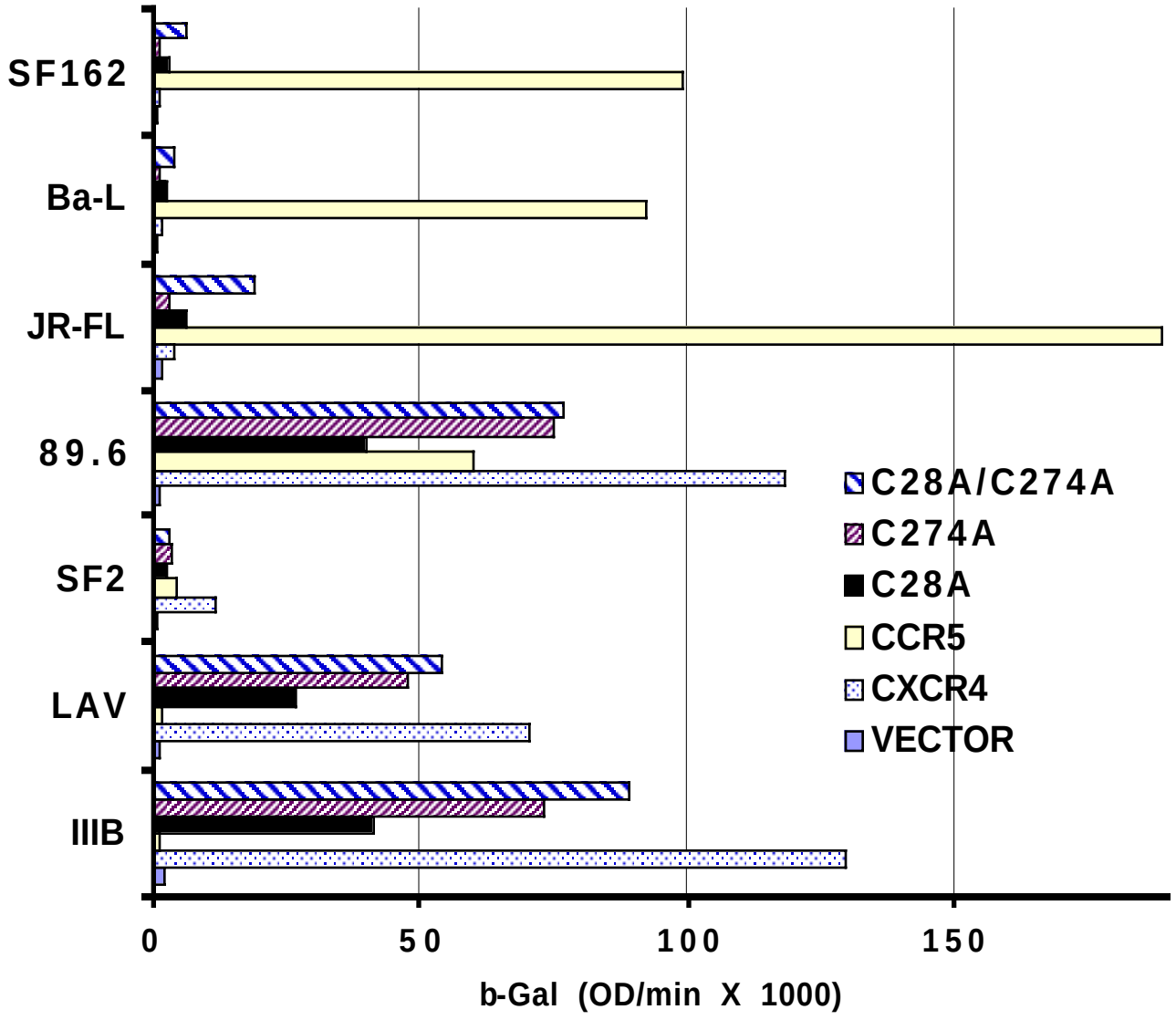

SF162
Ba-L
JR-FL
89.6
SF2
LAV
IIIB
C28A/C274A
C274A
C28A
CCR5
CXCR4
VECTOR
0
50
100
150
b-Gal (OD/min X 1000)

Figure 9. Coreceptor function of cysteine mutant CXCR4s in cell-cell fusion assays with R5, X4, and R5X4 HIV-1 Envs. U373 target cells were transfected with a plasmid encoding the wild-type coreceptor (CXCR4 or CCR5) or mutant CXCR4 construct linked to a vaccinia virus promoter and infected with vCB21R-lacZ and vCB-3 (CD4). HeLa effector cells were infected with vTF1-1 (T7 polymerase) and either vCB-32 (SF162), vCB-43 (Ba-L), vCB-28 (JR-FL), vDC-1 (89.6), vCB-34 (SF2), vCB-41 (LAV), or vCB-40 (IIIB/BH10). Fusion was assessed by measurement of ß-Gal in detergent cell lysates. The rates of ß-Gal activity shown were obtained from separate samples in the same experiment. All samples were tested in duplicate and the averages were plotted. This experiment was performed three times, and the data from a representative experiment is shown in the figure. OD: optical density.

CXCR4 Mutation(s)	% of wild-type activity with Mab 12G5	4G10
C28A	30	112
C109A	5	0
C186A	0	0
C274A	26	108
C28A C274A	53	113
C109A C186A	8	13

Table 3. Cell surface expression of CXCR4 cysteine mutation constructs. U373 cells were transfected with wild-type or mutated CXCR4 in the pSC59 vaccinia vector. Cells were then infected with wild-type vaccinia virus (WR), incubated with anti-CXCR4 MAb (4G10 or 12G5), and stained with phycoerythrin-anti-mouse IgG. Mean fluorescent intensities were determined using flow cytometry (Coulter XL-MCL Miami, FL). Values for mutant CXCR4s are the percentage of the mean fluorescent intensity of wild-type CXCR4, which is regarded as 100%.

disulfide linkages to some extent with the remaining cysteines in the molecule, resulting in protein misfolding.

Finally, in addition to reducing coreceptor activity of CXCR4 for R5X4 and X4 Envs, the N-terminal cysteine substitution (C28A) allowed CXCR4 to serve, albeit weekly, as a coreceptor for R5 isolate Envs (Fig. 8). Though the ecl-3 cysteine substitution (C274A) did not demonstrate coreceptor activity for R5 isolate Envs, combining the C28A and the C274A substitutions resulted in much greater coreceptor activity for R5 isolate Envs than the single C28A substitution. Thus, substitution of the C28/C274 cysteine pair appears to have a less drastic effect on CXCR4 conformation than substitution of just one of these two cysteines, though the C28/C274 mutant is altered enough in some fashion to allow it to function as a coreceptor for R5 Envs.

DISCUSSION

The 3-dimensional (3D) structure of the HIV-1 coreceptors is presently unknown. We have recently presented a theoretical 3D model of the HIV-1 coreceptors CXCR4 and CCR5 based on the physically determined structures of both bacteriorhodopsin and rhodopsin, as well as analysis of the amino acid sequences of related G-protein coupled receptors (88, 91). Two notable features can be derived from this model. First, the proteins are barrel shaped and there is a close positioning of the extracellular loops brought about by the two potential extracellular disulfide linkages. This would help explain the many observations from a number of groups that employed chimeric coreceptor constructs which have indicated multiple extracellular regions being involved in coreceptor function for CCR5 (19, 34, 111, 135, 178, 212, 237, 288) as well as CXCR4 (44, 178, 213).

Secondly, the models highlight the differences in the electrostatic potentials of the extracellular portions of the molecules, which may be a key element in determining usage by a particular HIV-1 isolate. Since the identification of the coreceptors for HIV-1, we and others proposed that individual HIV-1 envelope glycoproteins would have binding preferences towards a particular coreceptor mediated perhaps through interaction with the V3 loop (46, 87). The CXCR4 surface indicates a more a negative charge at the extracellular surface. In contrast CCR5 is less negatively charged. The overall charge of the V3 loop (an important determinant of cell tropism) of the HIV-1 Env is positive, where an X4 Env V3 loop region is more positively charged than an R5 Env V3 loop

sequence. This model would correlate with the coreceptor type usage depending on the type HIV-1 Env, and obviously this suggests a simple explanation for the preferential interaction of T-tropic X4 Envs with CXCR4. Indeed, recent studies have confirmed this notion by showing that specific amino acids in the V3 loop of Env can determine cellular tropism and regulate chemokine coreceptor preferences (256).

Our results with alanine substitutions of charged extracellular amino acids in CXCR4 indicate that the N-terminus and ecl-1 and ecl-2 are involved in coreceptor function for X4 and R5X4 Env-mediated fusion. Specifically, it was primarily negatively charged glutamic acid residues, E14, E15, and E32, in the N-terminus and the aspartic acid residue D97 in ecl-1, which upon removal by alanine substitution resulted in a profound impairment on the protein s coreceptor function with inhibition values greater that 50% as compared to wild-type CXCR4. Also, the glutamic acid residue mutation, E32A, in CXCR4 corresponds to the glutamic acid residue at position 18 in CCR5 that was also shown to be important for CCR5 coreceptor activity for an R5 and an R5X4 HIV-1 isolate (112). On the other hand, the elimination of the single positively charged residue in ecl-1 (K110A) was much less important for the majority of Envs examined (LAV, IIIB, 89.6) although on average there was a consistent pattern of reduced coreceptor activity of approximately 60-75% of the activity of wild-type CXCR4. The SF2 Env appeared unaffected by the ecl-1 mutations. These results suggest an important role for the N-terminus and ecl-1 of CXCR4 for both X4 and R5X4 Env-mediated fusion primarily through several negatively charged amino acid residues.

The substitution of the positively charged arginine residue in ecl-2 (R188A) was the only other charged amino acid alteration that had significant reduced coreceptor activity for all four Envs examined, and although other individual mutations in ecl-2 and ecl-3 revealed some less potent inhibitory effects on coreceptor activity for LAV, IIIB, or 89.6, no consistent pattern was evident. One particular Env, SF2, also had a reduced ability to employ CXCR4 as a coreceptor when any of four other charged residues, E26, K25, D187, or D193, were converted to alanine and this may reflect the notion that although some coreceptor residues or domains appear globally important, an individual Env glycoprotein can differentially interact with a particular coreceptor and harbor additional structural dependencies. Taken together, the SF2 Env appeared more dependent on ecl-2 rather than ecl-1, as compared to the other Envs examined. Finally, the single hydrophobic residue substitution (F201A) appeared to negatively affect all Envs tested, and this mutation was expressed at a level with near equivalence to wild-type CXCR4, as detected with the 12G5 monoclonal. In light of this observation we may pursue an extended investigation of other extracellular hydrophobic residues in our system.

In general, our data fits with other reports indicating an importance of positively charged Env residues in the V3 loop region of the previously classified syncytium-inducing X4 Envs (82, 119), and more recently work indicating that a more net negative charge in the V3 region of 4 out of 5 Envs examined correlated with an increase in CCR5 usage (256). It was unfortunate that a combination of the E14A and E31A N-terminal mutations had severely impaired cell-surface expression, and because of this result we have not yet pursued any other additive combinations of the impairing mutations we have

identified though we may do so in future experiments. Future experiments may also focus on noncharged extracellular and/or transmembrane amino acids, as several nonpolar residues have been reported to play a role in the coreceptor activity of CCR5 (112, 222), and transmembrane residues are important for ligand binding to a number of 7TMGPCRs (260).

Our results with cysteine mutations in CXCR4 provide further information on the structure of CXCR4. These coreceptor activities in Env-mediated fusion and cell surface expression data support the notion that both pairs of extracellular cysteine residues are involved in disulfide bond formations. Whereas the cysteine pair in ecl-1 and ecl-2 appear critical for proper folding and surface expression, the cysteine pair in the N-terminus and ecl-3 appeared less-so in that cell-surface expression at wild-type levels is detected with a conformation-independent anti-CXCR4 MAb. In addition, the surprising result that this cysteine pair (C28/C274), upon removal by alanine substitution, allowed for some support of R5 Env-mediated fusion suggests that the altered conformation is presenting sites of interaction on CXCR4 that are unavailable in the wild-type CXCR4 molecule.

The tropism altering substitutions in CXCR4 we found were quite surprising and has lead us to hypothesize that, although the primary sequences of the extracellular domains of CCR5 and CXCR4 are quite dissimilar, the molecules may perhaps be somewhat more similar in terms of a conformation-dependent binding site. The most potent single substitution we discovered was the elimination of the negatively charged aspartic acid residue 187 in ecl-2. We found that this single change had profound effects

in that it allowed for the CXCR4 protein to serve as a functional coreceptor for R5 HIV-1 Envs (57). We speculate that the removal of this key negatively charged residue in ecl-2 reduces the CXCR4 domain s net negative charge allowing for an appropriate region of an R5 Env, perhaps including the V3 loop, to associate with the CXCR4 extracellular surface that is otherwise repelled when the aspartic acid residue is present at position 187. Similar results were recently reported by Wang et al. (276). However, among our CXCR4 mutations we also discovered some other single amino acid mutations that had similar effects, the second most potent corresponding to the removal of the potential N-terminus glycosylation site (N11A). The combination of N11A with D187A mutations (the two most potent tropism-altering changes) had a better than additive effect, and actually supported R5 Env-mediated cell-cell fusion and syncytia at levels similar to CCR5. We hypothesize that the absence of bulky (N-linked glycosylation) or charged groups (D187) in the tropism-altering CXCR4 mutants allows for interaction with R5 Envs. This interaction could be through CXCR4 extracellular elements of an underlying or conserved coreceptor structure that is common to both CXCR4 and CCR5. We are actively engaged in experiments to directly address the influences of the potential N-linked glycosylation sites in CXCR4 on coreceptor function.

In addition, our observation that the C28A/C274A mutant also allows for some recognition and use by R5 Envs also supports this notion. The removal of this disulfide bond between the N-terminus and ecl-3 results in a conformational alteration in the molecule, as measured by the reactivity of MAb 12G5, yet the molecule is surface-expressed at levels comparable to wild-type CXCR4 and supports X4 and R5X4 Env-

mediated fusion at near wild-type CXCR4 levels. A possible explanation that would fit with our other data is that without this linkage the CXCR4 molecule is in a relaxed or more opened state and is repositioning the N-terminal glycosylation side group thus allowing access for an R5 Env. We point out that the fusion signals generated with JR-FL Env-mediated fusion is remarkably comparable between the C28A/C274A and the N11A mutants although they are biochemically very distinct.

Interestingly, the N11A/D187A mutant CXCR4 also supported HIV-1 R5 Env pseudotyped virus entry but not to the extent of CCR5 as a coreceptor. None of the other mutant CXCR4s consistently supported infection with R5 pseudotyped virus. Future experiments may address whether the discrepancy between fusion and infection data is due to post-binding effects of CCR5, which are not significant for a cell-cell fusion event but may be important for subsequent stages during viral infection. However, we point out that to date there is no evidence that the HIV-1 Env-mediated cell-cell fusion event is mechanistically different than the Env-mediated virus infection fusion event (reviewed in (88, 189)).

In summary, the data presented here adds some detail to our understanding of what are the critical extracellular domains of CXCR4 required for HIV-1 Env-mediated membrane fusion. We provide evidence that several negatively charged residues in the N-terminus and loops are important for optimal coreceptor activity and this confirms prior observations that multiple extracellular domains of CXCR4 are involved in the Env-mediated membrane fusion process. A likely explanation for the functional roles of these residues is that they are involved directly in Env binding by serving as key residues in a

3D or conformation-dependent binding region. Alternatively, some of these residues may be required for CD4 binding or preserving the native CXCR4 structure only and thus are indirectly required. However we note that all of the most impaired mutant CXCR4s are all recognized by the conformation dependent 12G5 MAb. These data provide a framework for the delineation of the Env-CD4-coreceptor contact sites and will aid future studies towards our understanding of the complex membrane fusion process mediated by HIV-1 Env and its receptors.

Chapter 4

Identification of a Homologous Region of CXCR4 and CCR5 That Determines Coreceptor Activity for R5 and X4 Human Immunodeficiency Virus type 1 Isolates

RESULTS

Expanded coreceptor activity of mutant CXCR4 molecules. We have shown that several point mutations of CXCR4; N11A, C28A, R30A, D187A, D193A, and C274A, in combination or by themselves; allow CXCR4 to function as a coreceptor for R5 HIV-1 Envs, while retaining its function with X4 and R5X4 HIV-1 Envs (58). Additional mutations, N176A and D97A (unpublished), likewise expand the coreceptor range of CXCR4. Combining some of these mutations resulted in synergistic expansion of coreceptor activity. Combining N11A with D187A, the two most potent point mutations, resulted in approximately equal activity to CCR5 in cell fusion assays with R5 Envs, but one to two logs less activity than CCR5 in infection assays with R5 Env pseudotyped viruses. We added other mutations to the N11A/D187A mutant in an effort to see if we could achieve CCR5 — levels of coreceptor activity in pseudovirus infection assays. To the pCDNA3/CXCR4 N11A/D187A vector we added the N176A and D193A mutations. In pseudovirus infection assays addition of N176A and D193A mutations did not appreciably increase CXCR4 N11A/D187A s activity in transfected U373-CD4 cells with JR-FL, an R5 virus (Fig. 10). This does not exclude the possibility that mutations C28A, R30A, D97A, and C274A might further increase CXCR4 coreceptor activity with

JR-FL, and altering some of the mutations to residues other than alanine might result in a mutant CXCR4 molecule that functions as well as CCR5 as a coreceptor for R5 Envs in infection assays, but our results suggest that small changes in CXCR4 will not be sufficient.

Expanded coreceptor range of loop 2 mutant D187A does not extend to all HIV-1 R5 Envs. The host expanding CXCR4 mutants have been tested with only four HIV-1 R5 Envs, JR-FL, ADA, Ba-L, and SF162, all of which are clade B (58, 276). We used a well-characterized cell fusion assay to compare these Envs to Envs of other clades. CCR5, CXCR4, or the most potent CXCR4 point mutant, D187A, was expressed in U373 target cells, following transfection with pSC59/coreceptor, which contains coreceptor gene linked to a vaccinia early/late promoter. These cells were then infected with vCB3, a vaccinia virus encoding CD4 and vCB21R-LacZ, a vaccinia virus encoding *E. coli* b-galactosidase linked to the T7 promoter. HIV-1Envs were expressed in HeLa effector cells coinfected with a vaccinia virus enconding HIV-1 Env and vTF1-1, encoding T7 RNA polymerase. After overnight expression, target and effector cells were mixed and fusion was allowed to proceed for 2.5 h and assessed as described in Materials and Methods. Four of four clade B R5 Envs could efficiently use the CXCR4 D187A mutant efficiently (Figs. 11).

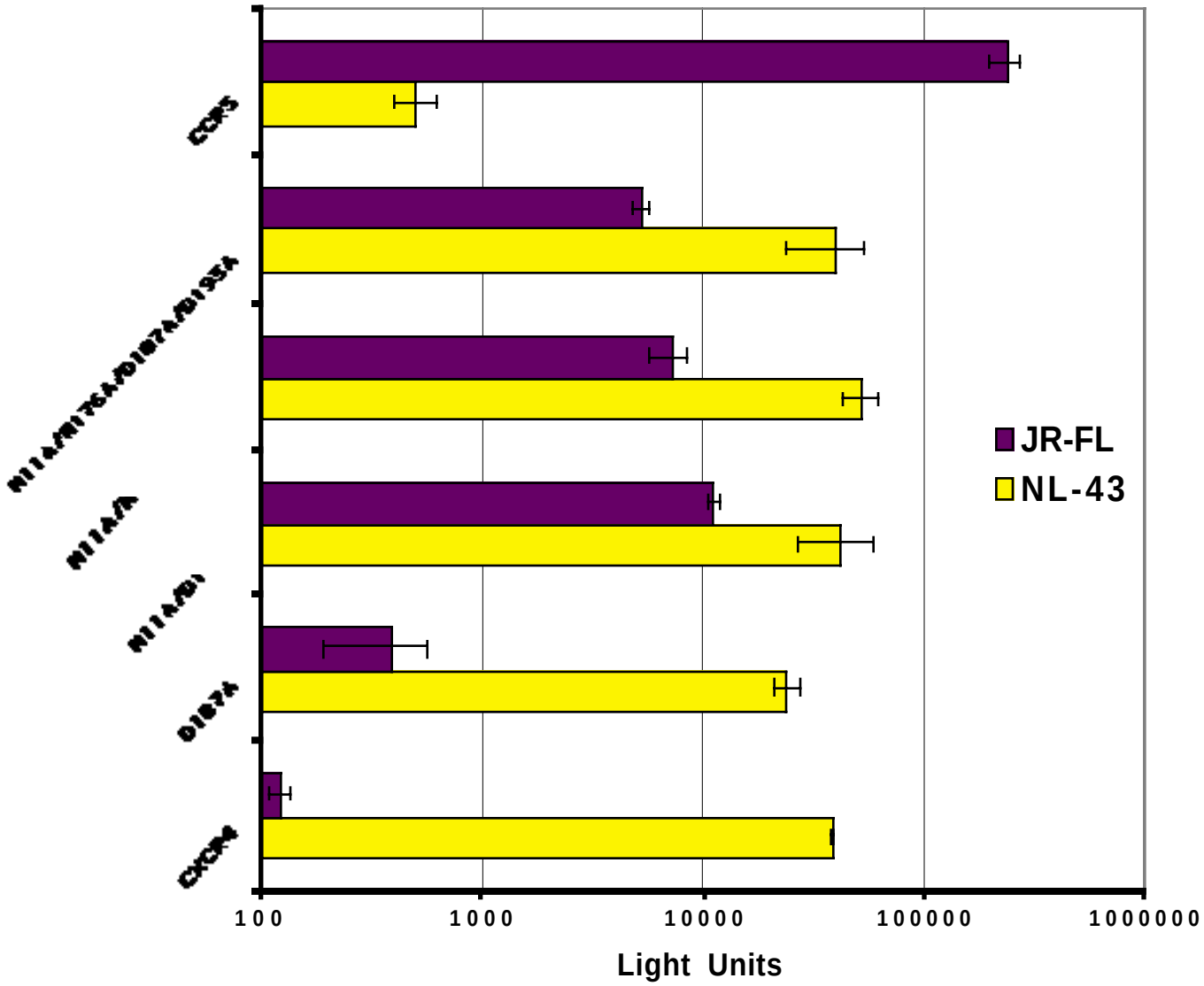

CCR5
N11A/R17GA/D187A/D193A
N11A/A
N11A/D1
D187A
CXCR4
JR-FL
NL-43
100
1000
10000
100000
1000000
Light Units

Fig. 10. Coreceptor function of mutant CXCR4 in virus infection assays with an R5 pseudotyped virus. U373-CD4 cells were transfected with a plasmid (pCDNA3) encoding wild-type or mutant coreceptor. Wells of cells in duplicate were infected with HIV-1_{JR-FL} luciferase reporter virus. Infection was assessed on day 4 by measuring the amounts of luciferase activity in cell lysates. The luciferase activities shown were obtained from separate samples in the same experiment. Error bars indicate the standard deviations of the mean values obtained for duplicate fusion assays. Data from a representative experiment are shown in the figure.

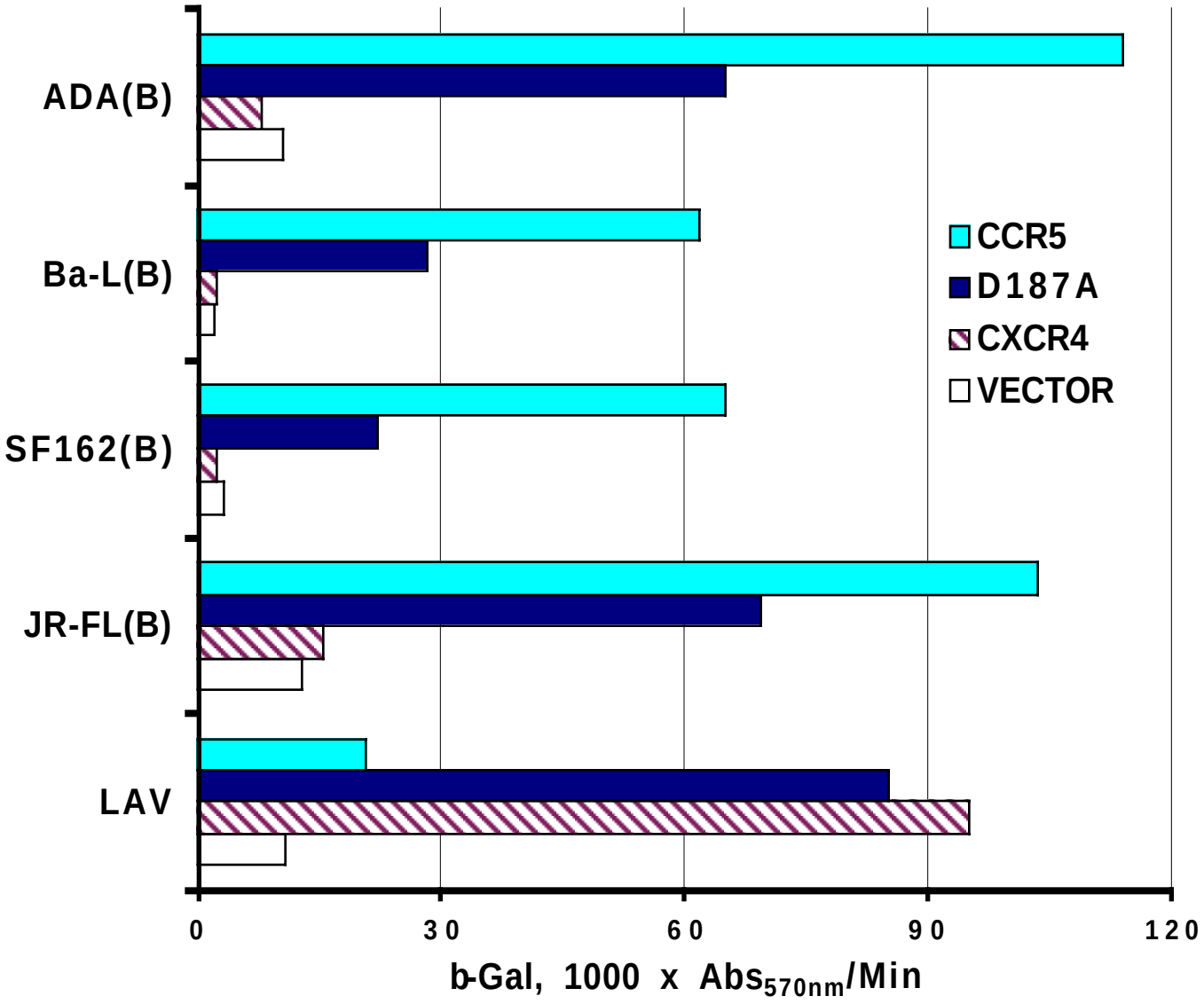

ADA(B)
Ba-L(B)
SF162(B)
JR-FL(B)
LAV
CCR5
D187A
CXCR4
VECTOR
0
30
60
90
120
b-Gal, 1000 x Abs570nm/Min

Fig. 11. Coreceptor function of CXCR4 D187A mutant in cell fusion assay with R5 HIV-1 Envs. U373 target cells were transfected with a plasmid encoding the wild-type or a mutant coreceptor linked to vaccinia virus promoter and infected with vCB21R-LacZ and vCB3 (CD4). HeLa effector cells were infected with a vaccinia virus encoding an HIV-1 Env (clade is indicated in parenthesis) and with vaccinia virus encoding T7 polymerase (vTF1-1). Duplicate cell mixtures were incubated at 37°C for 3 h. Fusion was assessed by measurement of ß-Gal in detergent lysates of cells. The rates of ß-Gal activity shown were obtained from separate samples in the same experiment. All samples were tested in duplicate and the averages were plotted. Data from a representative experiment are shown in the figure.

To determine if R5 Env utilization of CXCR4 D187A was limited to Clade B we tested a number of primary Envs in the cell fusion assay (Fig. 12). The assay was performed as above except that Envs were expressed in HeLa effector cells following transfection with pCRII/Env, which contained *Env* linked to a T7 promoter, and infection with vTF1-1. U373 target cells were transfected with pSC59/coreceptor and infected with vCB21R. Consistent with results shown in Figure 2, both clade B Envs, 92BR020.4 and 91US005.11, used D187A efficiently. A mixed clade Env (gp120 is all clade F), 93BR019.10 showed similar ability to utilize D187A. A clade A Env, 92RW020.5 used D187A as well as clade B Envs, while another clade A Env, 92UG037.8, did not use D187A. Clade C Env 92BR025.9 did not exhibit a strong ability to utilize D187A or CCR5, but it consistently used D187A more efficiently than wild-type CXCR4. Another clade C Env, MA301965.26, did not utilize D187A. The only clade F Env tested exhibited significant activity with D187A in some experiments, while the only clade G Env used was consistently negative. The X4 Env LAV was included as a control in all experiments to illustrate that CXCR4 mutants are expressed as well as wild-type (data not shown).

Expression of CCR5 mutants. Two of our findings led us to hypothesize that ecl-2 of CCR5 might play a critical role in determining coreceptor activity; first, the profound effects of the D187A mutation in allowing CCR5-dependent Envs to utilize it as a viable coreceptor and, second, 12G5, a monoclonal antibody that binds to ecl-2 of CXCR4, inhibited R5 coreceptor activity of the D187A mutant (data not shown). Similarly, 2D7,

an antibody to the ecl-2 of CCR5 inhibits CCR5 coreceptor function. In light of this we performed an alignment of the ecl-2 regions of CXCR4 and CCR5 (Fig. 13) and noted that a pair of serine residues is located in CCR5 at the position corresponding to D187 in CXCR4. Two CCR5 mutations were constructed , one was a double alanine substitution of both serine residues (S179A/S180A) and the other had a substitution of the serine residue 179 with aspartic acid (S179D). The mutations were made in the vector pSC59 and subcloned into pCDNA3.1 Hygro+, from which stable cell lines were prepared following transfection of U373-CD4 cells. Clones were screened for CCR5 expression by cell surface staining and FACS analysis. Clones with comparable expression levels were used in subsequent experiments (Fig. 14).

Coreceptor activities of CCR5 mutants. We next examined the ability of the single (S179D) and double (S179A/S180A) CCR5 mutants to support Env-mediated membrane fusion by several R5 and R5X4 HIV-1 Envs (Fig. 15). While clade B R5 Envs JR-FL and ADA were able to utilize both mutants, a clade E R5 Env was unable to use the single mutant. Two clade B R5X4 Envs, SF2 and 89.6, were also unable to use the single mutant. Similar results were achieved in pseudovirus infection experiments, where both mutants had approximately wild-type activity levels with JR-FL, while the single mutation exhibited at least a log less activity with R5X4 Envs 89.6 and SF2 (Fig.16). The single mutation in fact introduces the negatively charged aspartic acid residue, which is present in the CXCR4 protein at this location and is perhaps disrupting the molecule s

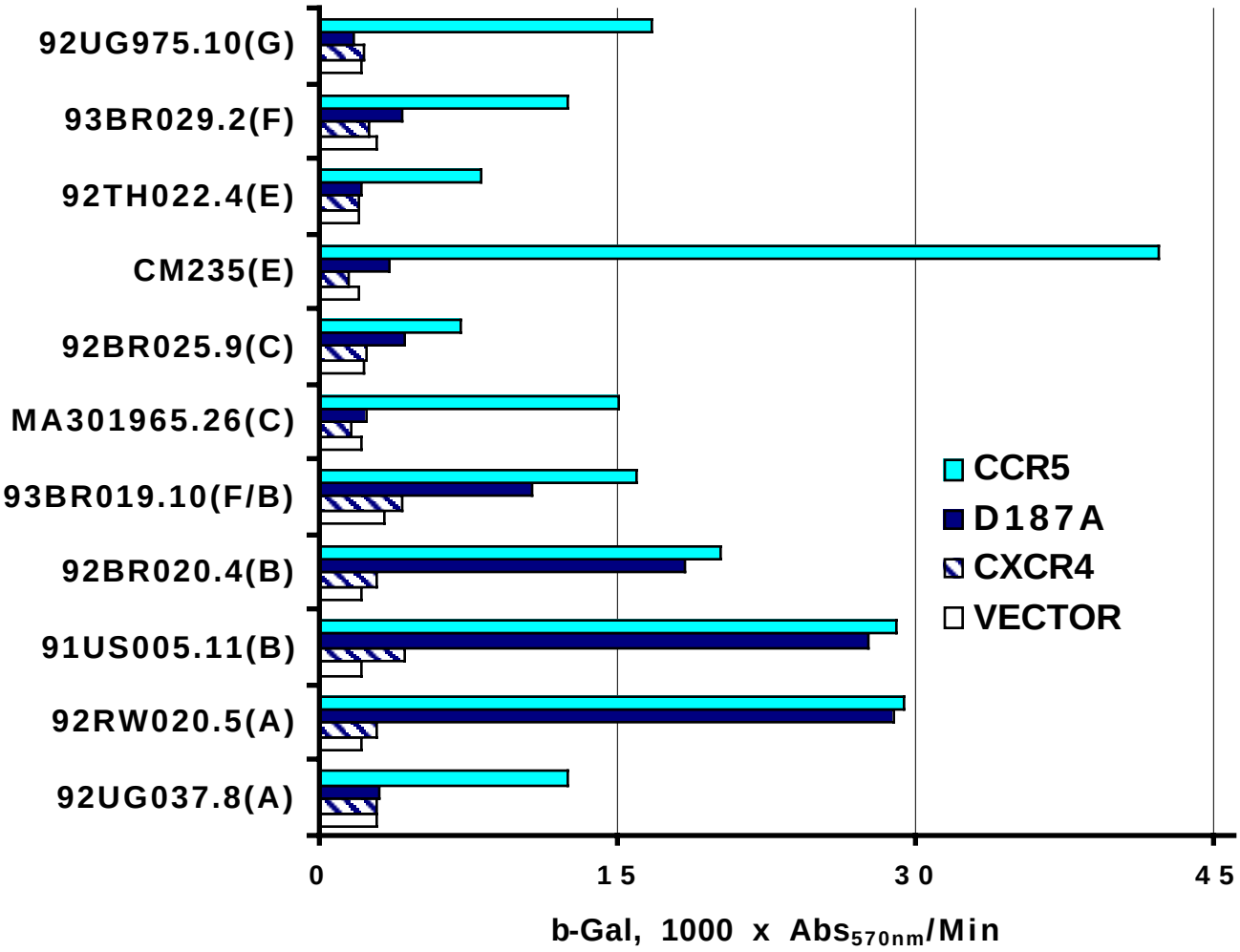


Fig. 12. Coreceptor function of mutant CXCR4s in cell fusion assays with primary R5 HIV-1 Envs. U373 target cells were transfected with a plasmid encoding the wild-type or a mutant coreceptor linked to vaccinia virus promoter and infected with vCB21R-LacZ and vCB3 (CD4). HeLa effector cells were transfected with a plasmid encoding Env (clade is indicated in parenthesis.) linked to a T7 promoter and infected with vaccinia virus encoding T7 polymerase (vTF1-1). Duplicate cell mixtures were incubated at 37°C for 3 h. Fusion was assessed by measurement of b-Gal in detergent lysates of cells. The rates of b-Gal activity shown were obtained from separate samples in the same experiment. All samples were tested in duplicate and the averages were plotted. Data from a representative experiment are shown in the figure.

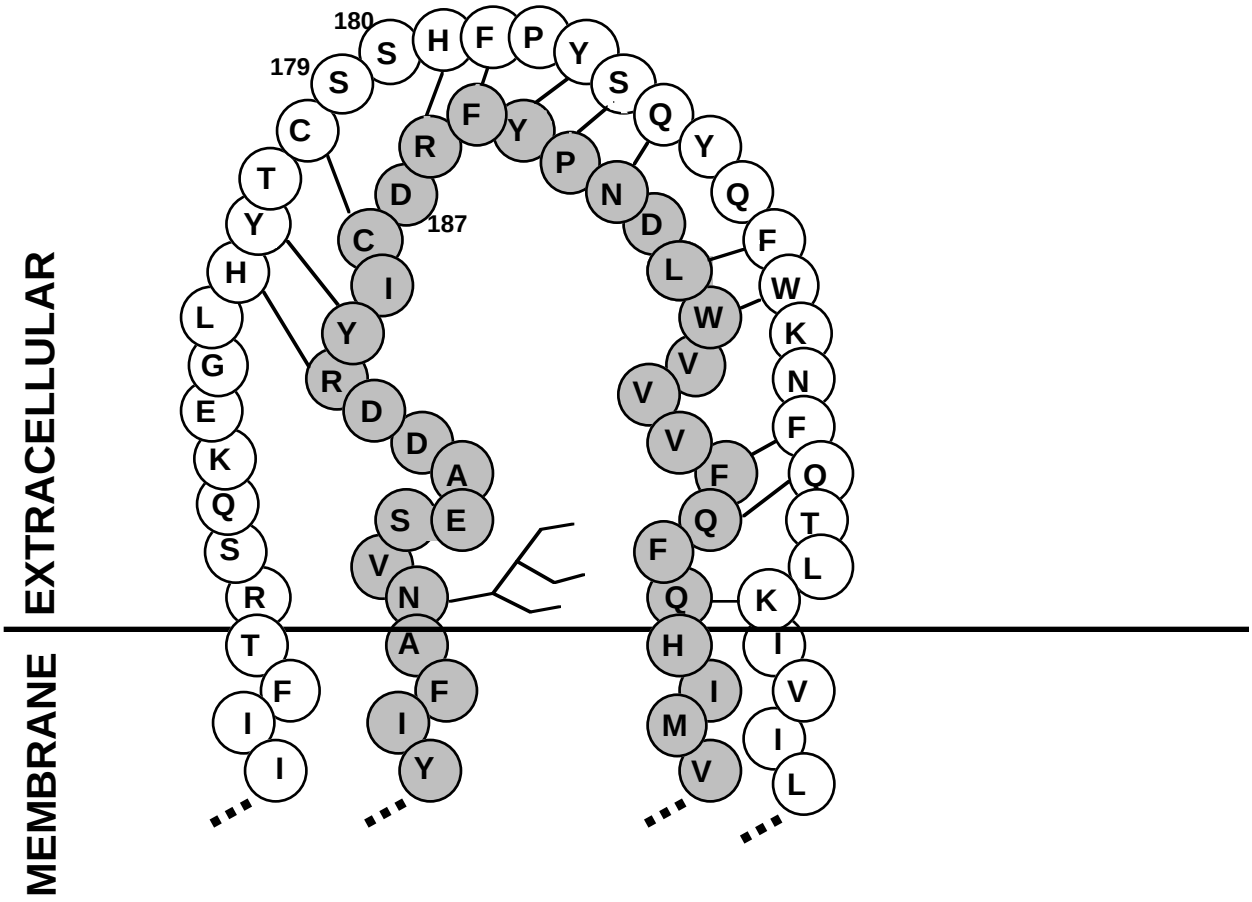


Fig. 13. Alignment of predicted second extracellular loops of CXCR4 and CCR5. Shaded circles represent the sequence of CXCR4. Open circles represent the CCR5 sequence. Flanking transmembrane sequences are shown below the horizontal line. The tropism-determining position 187 of CXCR4 appears to correspond to positions 179 and/or 180 of CCR5.

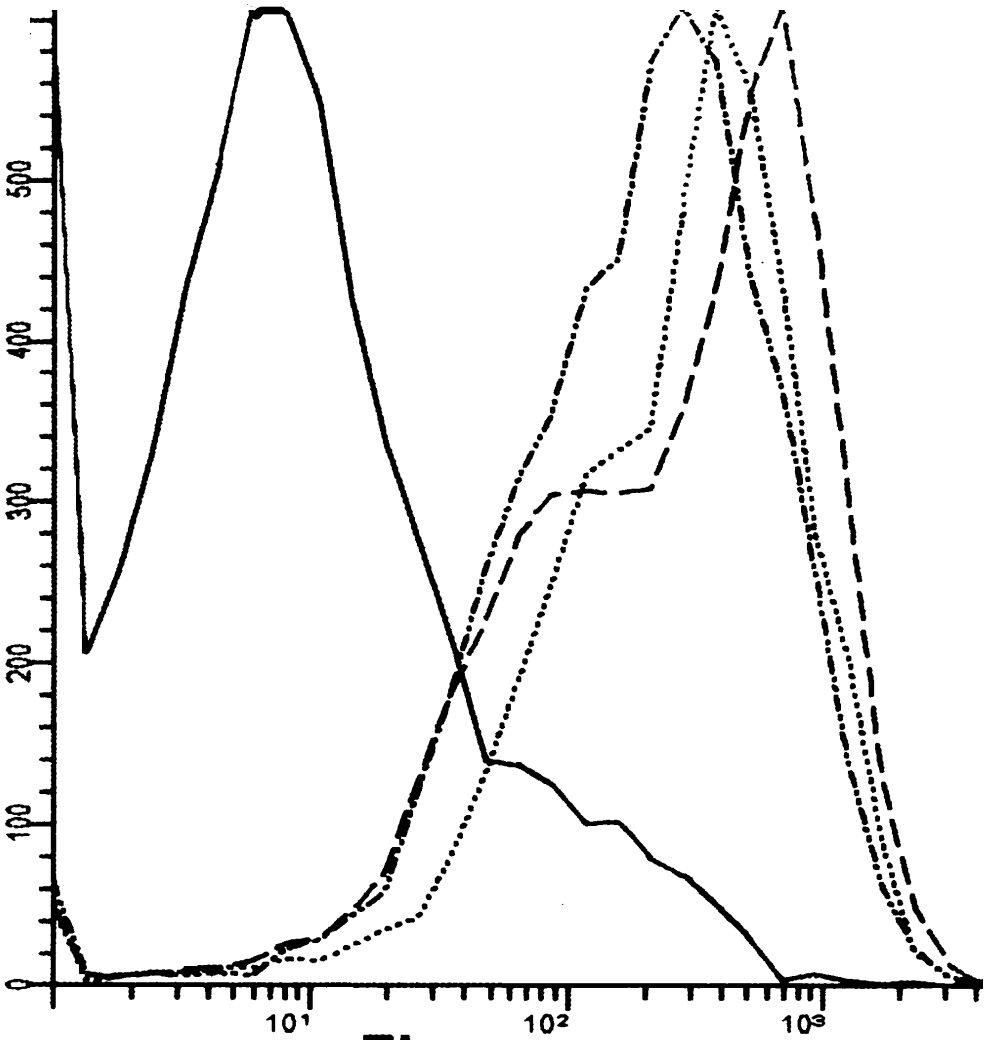


Fig. 14. Cell surface expression of CCR5 cell lines. U373-CD4 cells were stably transfected with DOTAP and plasmid (pCDNA3.1 Hygro+) encoding wild-type or mutant CCR5. Clones that survived hygromycin treatment (200 mg/ml) were incubated with anti-CCR5 MAb (2D7) and stained with phycoerythrin-anti-mouse IgG. Mean fluorescence intensities (MFI) were determined with a flow cytometer (model XL-MCL; Coulter, Miami, FL). MFI values for cell lines are compare to U373-CD4/CCR5 wild-type cells, which are regarded as 100% and U373-CD4 cells, which are regarded as 0%. The solid line represents U373-CD4 parent cells. The dashed line represents U373-CD4/CCR5 wild-type cells. The dotted line represents U373-CD4/CCR5 S179A/S180A cells (MFI = 94%). The mixed line represents U373-CD4/CCR5 S179D cells (MFI = 75%).

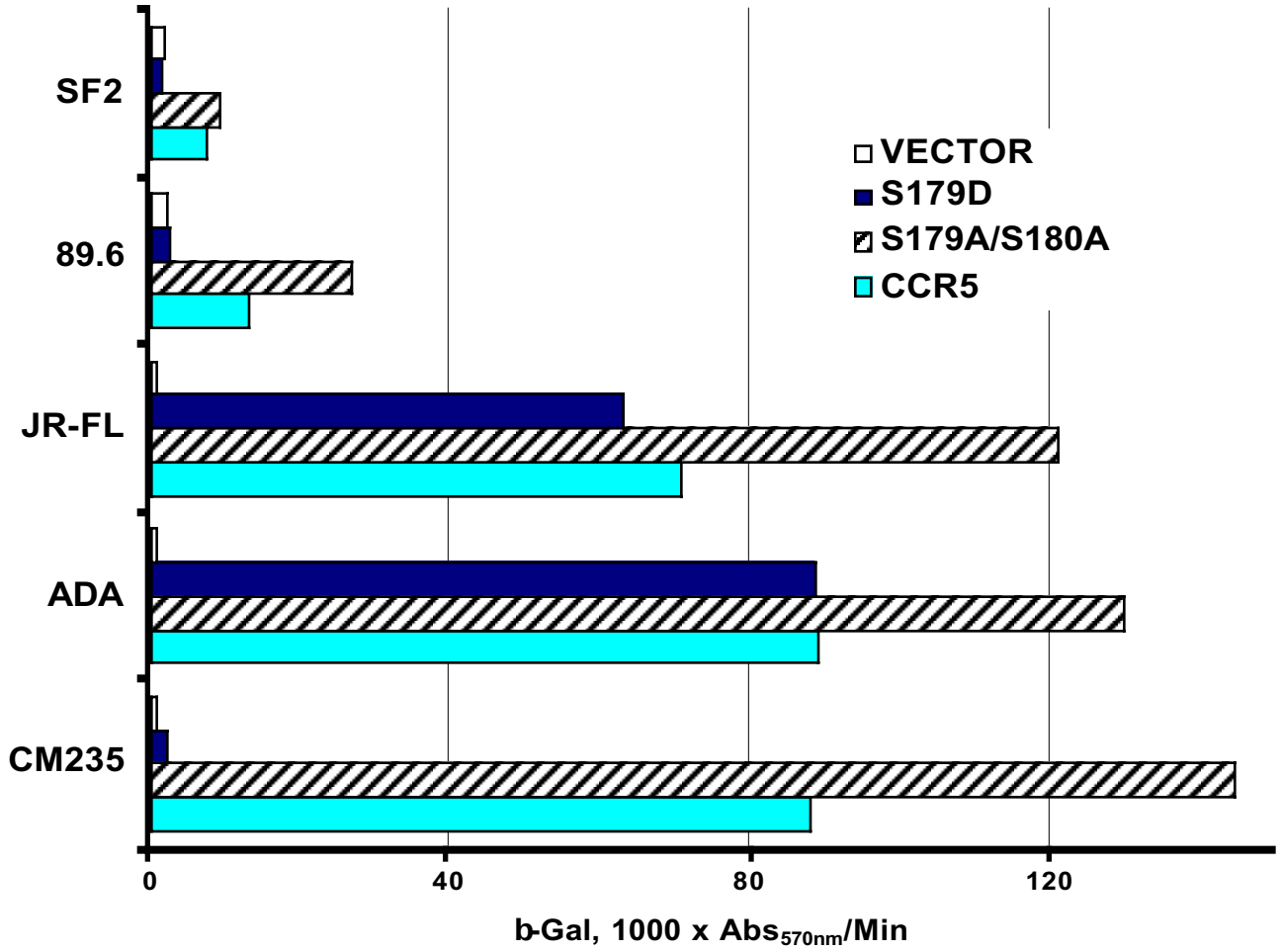


Fig. 15. Coreceptor function of mutant CCR5s in cell fusion assays with R5 and R5X4 Envs. U373-CD4 cells were stably transfected with a plasmid (pCDNA3.1 Hygro+) encoding wild-type or mutant CCR5 and infected with vCB21R-LacZ and vCB3 (CD4). HeLa effector cells were infected with a vaccinia virus encoding an HIV-1 Env and with vaccinia virus encoding T7 polymerase (vTF1-1). Duplicate cell mixtures were incubated at 37°C for 3 h. Fusion was assessed by measurement of b-Gal in detergent lysates of cells. The rates of b-Gal activity shown were obtained from separate samples in the same experiment. All samples were tested in duplicate and the averages were plotted. Data from a representative experiment are shown in the figure.

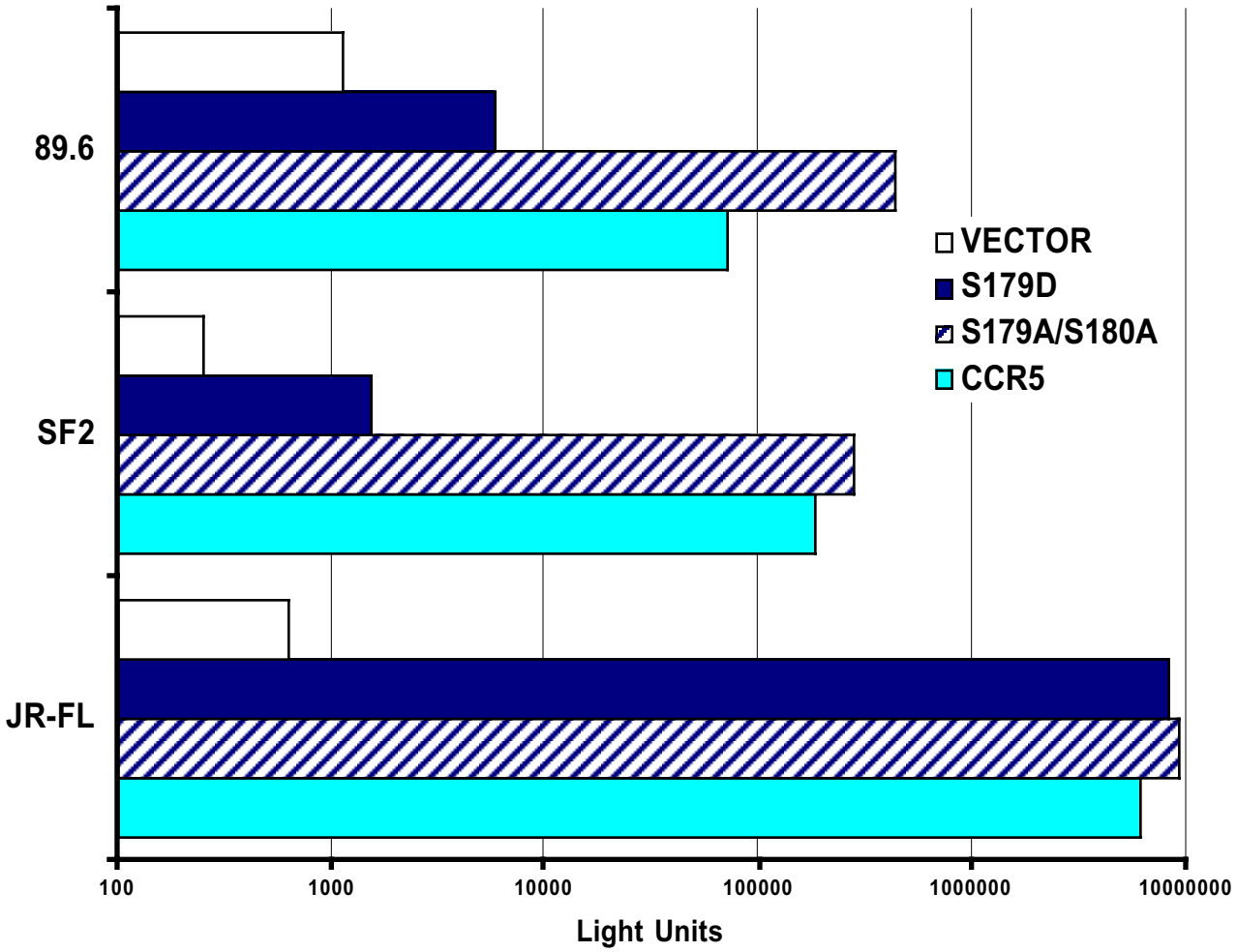


Fig. 16. Coreceptor function of mutant CCR5s in virus infection assays with R5 and R5X4 pseodotyped viruses. U373-CD4 cells were stably transfected with a plasmid (pCDNA3.1 Hygro+) encoding wild-type or mutant CCR5. Wells of cells (in triplicate) were infected with the indicated HIV-1 Env luciferase reporter virus. Infection was assessed on day 4 by measuring the amounts of luciferase activity in cell lysates. All samples were tested in duplicate and the averages were plotted. The luciferase activities shown were obtained from separate samples in the same experiment. This experiment was repeated tree times, and the data from a representative experiment are shown in the figure.

ability to correctly associate with certain Envs. We also observed that neither the single or double mutation appeared to enhance any CXCR4 dependent fusion (data not shown).

Finally we tested the CCR5 mutants for their ability to support Env-mediated fusion by a panel of primary R5 Envs and a primary R5X4 Env expressed on HeLa cells (Fig. 17). Consistent with results in figures 15 and 16, both mutants functioned well with all three tested R5 clade B Envs. Other clades showed mixed results. One of two clade A, clade C, and clade F/B R5 Envs could support fusion with the single mutant. However, in all six cases the single mutant had less than half the activity of wild-type. A clade F and a clade G R5 Env had little or no activity with the single mutant. A primary R5X4 Env, HA301593.1, was not supported by the single mutant, just as the case with the other two tested R5X4 Envs (Figs. 15 and 16).

Interestingly one clade E R5 Env, 92TH022-4, had wild-type activity with the single mutant, while other clade E R5 Envs, CM235 and CM243, had no activity with the single mutant (Figs 15, 17, and data not shown). This drastic difference between R5 Envs of the same clade may have provided an opportunity to determine Env sequences needed to use the single mutant. As shifts in coreceptor usage have been linked to small changes in the V3 region, we aligned the V3 sequences of the three clade E Envs (Fig. 18). There are no smoking guns pointed to a particular sequence present or absent only in 92TH022-4. In fact there is no residue in the V3 sequence of 92TH022-4 that is not present in either or both of the V3 sequences of CM235 and CM243. However, we speculate that presence of TÆP changes at position 10 of CM243 and position 13 of CM235 might contribute to their inability to use the single mutant. This has not been confirmed experimentally.

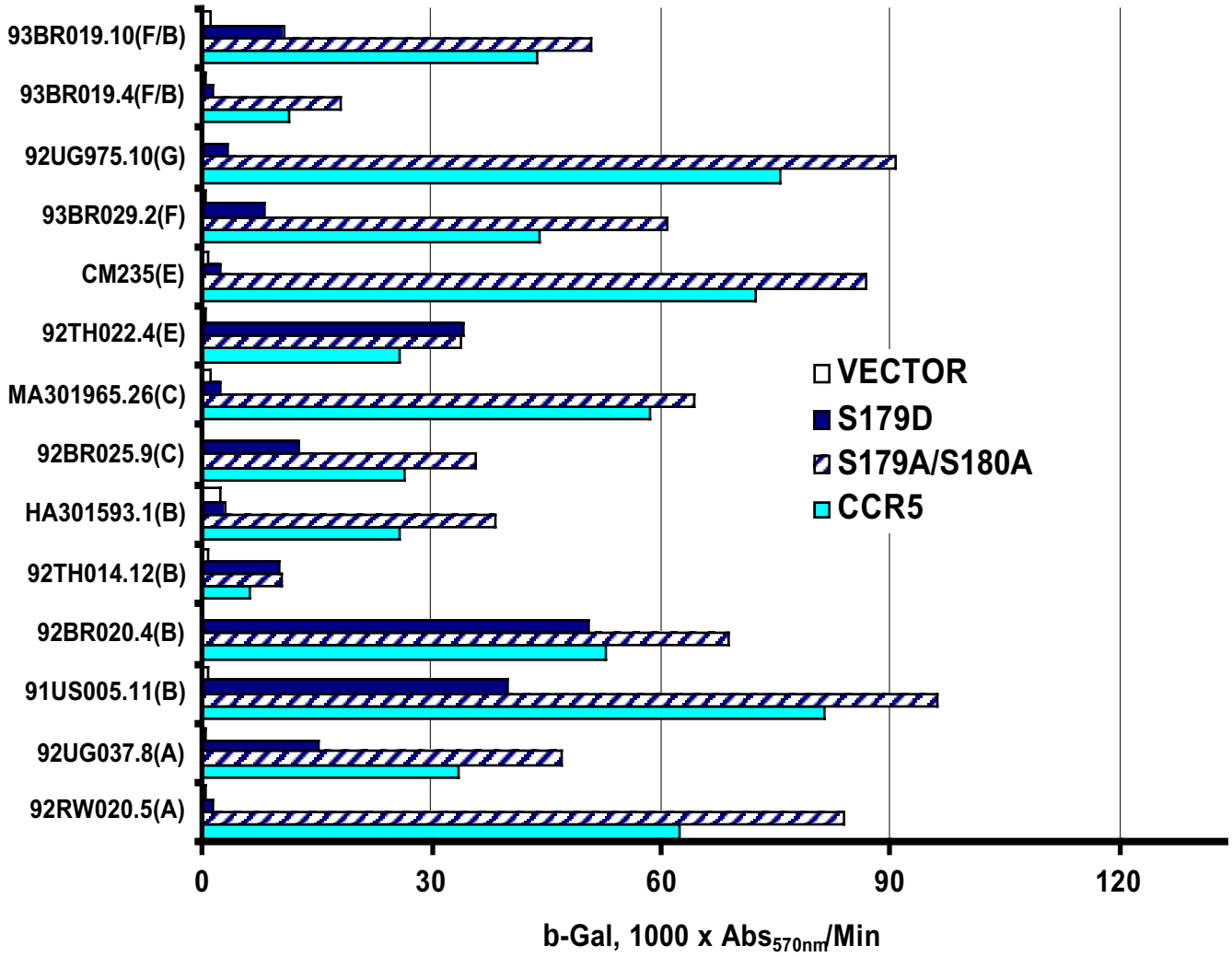


Fig. 17. Coreceptor function of mutant CCR5s in cell fusion assays with primary HIV-1 R5 Envs and a R5X4 Env (HA301593.1). U373-CD4 cells were stably transfected with a plasmid (pCDNA3.1 Hygro+) encoding wild-type or mutant CCR5 and infected with vCB21R-LacZ and vCB3 (CD4). HeLa effector cells were infected with a vaccinia virus encoding an HIV-1 Env (clade is indicated in parentheses) and with vaccinia virus encoding T7 polymerase (vTF1-1). Duplicate cell mixtures were incubated at 37°C for 3 h. Fusion was assessed by measurement of §-Gal in detergent lysates of cells. The rates of §-Gal activity shown were obtained from separate samples in the same experiment. All samples were tested in duplicate and the averages were plotted. Data from a representative experiment are shown in the figure.

```
CM235      C T R P S N N T R T S I P I G P G Q A F Y R T G D I I G D I R K A Y C
           | | | | | | | | | | | |   | | | | |   | | | | | | | | | | | |   | | |
92TH022.4  C T R P S N N T R T S I T I G P G Q V F Y R T G D I I G D I R R A Y C
           | | | | | | | | |   | | |   | | | | | | | | | | | | | | | | | | | |
CM243      C T R P S N N T R P S I T V G P G Q V F Y R T G D I I G D I R R A Y C
```

Fig. 18. Alignment of V3 loops of clade E primary isolates. Outlined letters represent residues in 92TH022.4 that are different in either CM235 or CM243.

Discussion

The structures of the major HIV-1 coreceptors, CXCR4 and CCR5, share only about 30% homology including conservative substitutions, but in the presence of CD4 they are both able to support HIV-1 Envelope-mediated membrane fusion. Macrophage-tropic (R5) Envs can utilize CCR5, while T cell line-tropic (X4) Envs can utilize CXCR4 (45). Dual-tropic (R5X4) Envs can utilize either CXCR4 or CCR5 (97). We have identified point mutations in CXCR4, N11A, C28A, R30A, D97A, N176A, D187A, and D193A, which, in the context of CD4+ target cells allow CXCR4 to support R5 HIV-1 Env mediated cell fusion. Thus, it seems likely that there is a common coreceptor structure present in CXCR4 and CCR5 that allows them to interact with both CD4 and HIV-1 Env and that certain CXCR4 amino acids block interactions with R5 HIV-1 Envs.

Combining some of these mutations had additive effects in cell-cell fusion assays and pseudovirus infection assays. Combining N11A with N176A or D187A resulted in greater activity with R5 HIV-1 Envs than any of the single mutations. The N11A/D187A mutant had approximately the same activity as CCR5 in cell-cell fusion assays with R5 Envs, but much less activity than CCR5 in pseudovirus infection assays (58). This may due to sensitivity differences between the two assays or to post-fusion functions of CCR5 that are not produced by CXCR4 mutants. Combining additional tropism converting mutations, R30A and D193A, did not increase the activity with JR-FL, an R5 HIV-1 Env, suggesting that small mutations to CXCR4 cannot allow it to acquire the full coreceptor capacity of CCR5.

The most potent of the tropism-converting mutations of CXCR4 is D187A. It functioned well in cell-cell fusion assays and pseudovirus infection assays with four different frequently used R5 HIV-1 Envs, ADA, Ba-L, SF162, and JR-FL. As with most lab strains, these were all clade B. To determine if R5 Envs of other clades can utilize the D187A CXCR4 mutant we tested it against a panel of Envs from primary isolates of different clades in our cell-cell fusion assay. As expected both primary R5 clade B Envs functioned well with the D187A CXCR4 mutant. One clade A Env, 92RW020.5 from a Rwandan patient, utilized D187A as well as the clade B Envs, while another clade A, 92UG037.8 from a Ugandan patient, was unable to use D187A.One clade C Env, 92BR025.9 consistently showed a small amount of fusion with D187A, while another clade C Env, MA301965.26, could not utilize D187A. The only clade F Env tested, 93BR029.2, exhibited significant activity in some experiments. A mixed clade Env, 93BR019.10, which was composed mostly of clade F, functioned very well with D187A. The only clade G Env tested, 92UG975.10, could not use D187A.

The location of D187 in ecl-2 of CXCR4 is intriguing because monoclonal antibodies to ecl-2 of CCR5 strongly reduce CD4 binding and coreceptor function (169, 290). We aligned ecl-2s of CXCR4 and CCR5 to determine the CCR5 amino acid corresponding to D187. Two serines of CCR5 at positions 179 and 180 aligned with D187 of CXCR4. To determine the function of these two serines in coreceptor function we converted both serines to alanine and tested this double mutant in cell-cell fusion assays and pseudovirus infection assays. Our results showed that the double CCR5 mutant did not have significantly altered function with R5, X4, or R5X4 HIV-1 Envs.

However, we had not ruled out the significance of these residues, as serine and alanine are relatively similar amino acids. A closer look at the ecl-2s of CXCR4 and CCR5 revealed major charge differences between the two molecules. The ecl-2 of CXCR4 is acidic with a pI of 3.8, while the same region of CCR5 is basic with a pI of 9.65.

Several studies have shown that the V3 loop of gp120 determines coreceptor choice (139, 256). Indeed, replacing the V3 loop of an R5 HIV-1 Env with an X4 V3 loop can convert the Env from R5 to X4. The reverse is also true; replacement of the V3 loop of an X4 Env with the V3 loop of an R5 Env will reverse the tropism of the Env. The charges of the V3 loops tend to vary with tropism; V3 loops of X4 HIV-1 tend to be more basic than the V3 loops of R5 HIV-1. Thus, a simple model is that the acidic ecl-2 of CXCR4 interacts with the more basic V3 loop of X4 Envs, while the basic ecl-2 of CCR5 interacts with the more acidic V3 loop of R5 Envs. It may als be that acidic amino acids at certain positions within ecl-2 are important.

With that in mind we converted the serine at position 179 of CCR5 to aspartic acid, the corresponding amino acid in CXCR4. Surprisingly, this mutation did not affect the ability of clade B R5 Envs Ba-L, JR-FL, and ADA to utilize CCR5, but drastically reduced the ability of R5X4 Envs SF2 and 89.6 from utilizing it. The CCR5 S179D mutant also functioned like wild-type CCR5 with primary clade B R5 Envs and had no activity with a primary clade B R5X4 Env, suggesting that the positive charge on CCR5 ecl-2 is critical for the support of clade B R5X4 Envs, but dispensable for clade B R5 Envs. Some R5 Envs of other clades were also unable to utilize the S179D mutant. One of two clade A, clade C, and mixed clade F/B (mostly F) were negative in cell-cell fusion

assays with the S179D mutant. The only clade F Env and the only clade G Env tested were both unable to utilize the S179D mutant. The S179D mutant functioned as well as wild-type CCR5 with one R5 clade E Env, 92TH022.4, but not at all with two others, CM235 and CM243.

Thus, the charge in ecl-2 of CCR5 is not only critical for R5X4 clade B Envs but also for various Envs of other clades. We have been largely unsuccessful in our efforts to identify sequences exclusive to Envs that are unable to use the S179D mutant. Though we have noted threonine to proline switches in clade E Envs unable to utilize S179D, we have yet to test their significance. It is interesting that cells expressing the D187A mutant of CXCR4 do not form syncytia when infected with CXCR4-dependent, CD4-independent FIV. Future efforts will also examine interactions of CCR5 S179D and CXCR4 D187A with CD4 and various Envs.

Chapter 5

Effects of Mutation of CXCR4 Putative Glycosylation Sites and Tyrosine Sulfation Sites on Coreceptor Function

RESULTS

R5 isolate use of altered CXCR4 molecules. Coreceptor genes were expressed using either a vaccinia promoter system or CMV promoter, with a plasmid transfection protocol depending on the particular assay employed. An alanine-scanning mutagenesis strategy (123) was performed for identifying residues involved in CXCR4 coreceptor activity (58). Shown in Fig. 19 is a representation of the extracellular domains of CXCR4 with the locations of tyrosines in gray . In CCR5 N-terminus tyrosines have been implicated in gp120 binding and HIV-1 entry. Several point mutations are highlighted: N11A, C28A, and R30A in the N-terminus; N176A, D187A and D193A in ecl-2; and C274A in ecl-3. The substitution mutation of these residues alone with alanine were noted to enhance the ability of CXCR4 to serve as a coreceptor for otherwise R5 dependent HIV-1 isolates, while retaining full function for X4 and R5X4 isolates. Only the cysteine mutations had moderately reduced X4 Env coreceptor activity (58). A combination of both the cysteine mutations (e.g. C28A/C274A) yielded a mutant CXCR4 with an enhanced coreceptor activity for R5 Envs better than either one alone. Fig. 19 also shows the locations of the two potential N-linked glycosylation sites located in the N-terminus and in ecl-2. Using 12G5 monoclonal antibody (MAb), cell surface staining, and FACS analysis, all CXCR4 mutants used in the present study had quite comparable surface-expressed levels to that of wild-type CXCR4 (85-115%) (58). Further, the non-N-linked

glycosylated CXCR4 mutants in this study reacted equally well with a panel of 6 additional conformation dependent anti-CXCR4 MAbs supplied by R&D Systems (56).

Removal of N-linked glycosylation sites expands CXCR4 coreceptor activity. The vaccinia based ß-galactosidase (ß-Gal) cell fusion assay was performed to examine the non-N-linked glycosylated CXCR4 mutants, where human U373 target cells expressing CD4 and infected with the vCB-21R-LacZ reporter virus were transfected with plasmids encoding mutant or wild-type CXCR4. Env-expressing HeLa effector cells were produced by infection with the appropriate Env-encoding vaccinia virus and a vaccinia virus encoding T7 RNA polymerase (30, 45, 198). One of the most potent, single mutations that allowed CXCR4 to serve as an R5 isolate coreceptor was N11A (Fig. 20). This alteration potentially eliminated an N-terminal N-linked glycosylation structure. Site-directed mutagenic removal of the asparagine residue of an N-linked glycosylation site motif, rather than enzymatic removal of carbohydrate, is a more definitive means for glycosylation site identification. But more importantly for the present study, it permits a functional examination of effects of glycosylation. We confirmed by mutagenesis that the N11A phenotype was likely due to the elimination of a glycosylation site by disruption of the consensus glycosylation sequence (N-X-S/T) with an alternative mutation (T13A). In Env-mediated cell fusion assays (Fig. 20) the T13A CXCR4 mutant exhibited near equivalent coreceptor activity with a panel of prototypic R5 Envs as compared to the N11A mutant. This result strongly indicates that it was the

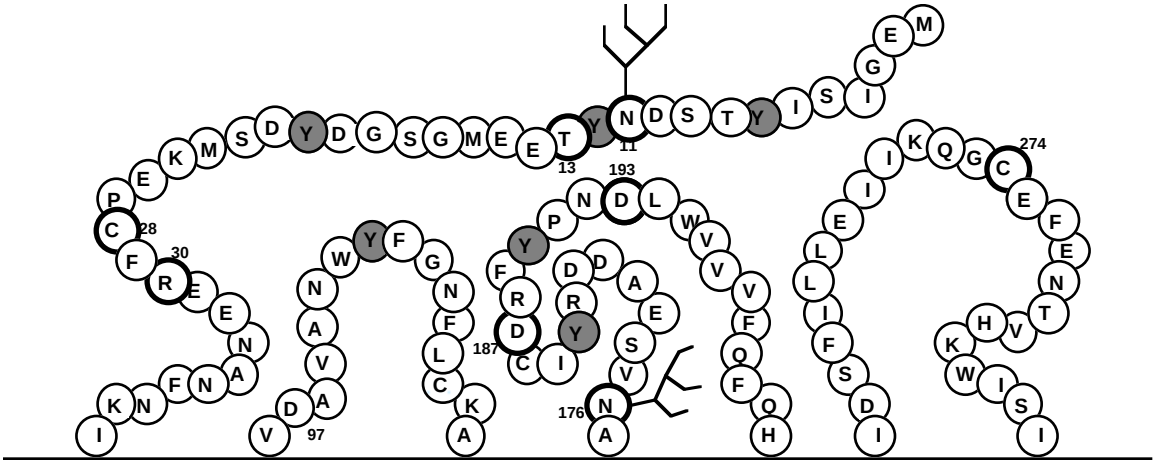


Figure 19. Diagram of CXCR4 Extracellular Domains. Glycosylation sites are indicated. Amino acids that when converted to alanine enhance coreceptor activity for R5 HIV-1 are highlighted. Tyrosines are shaded.

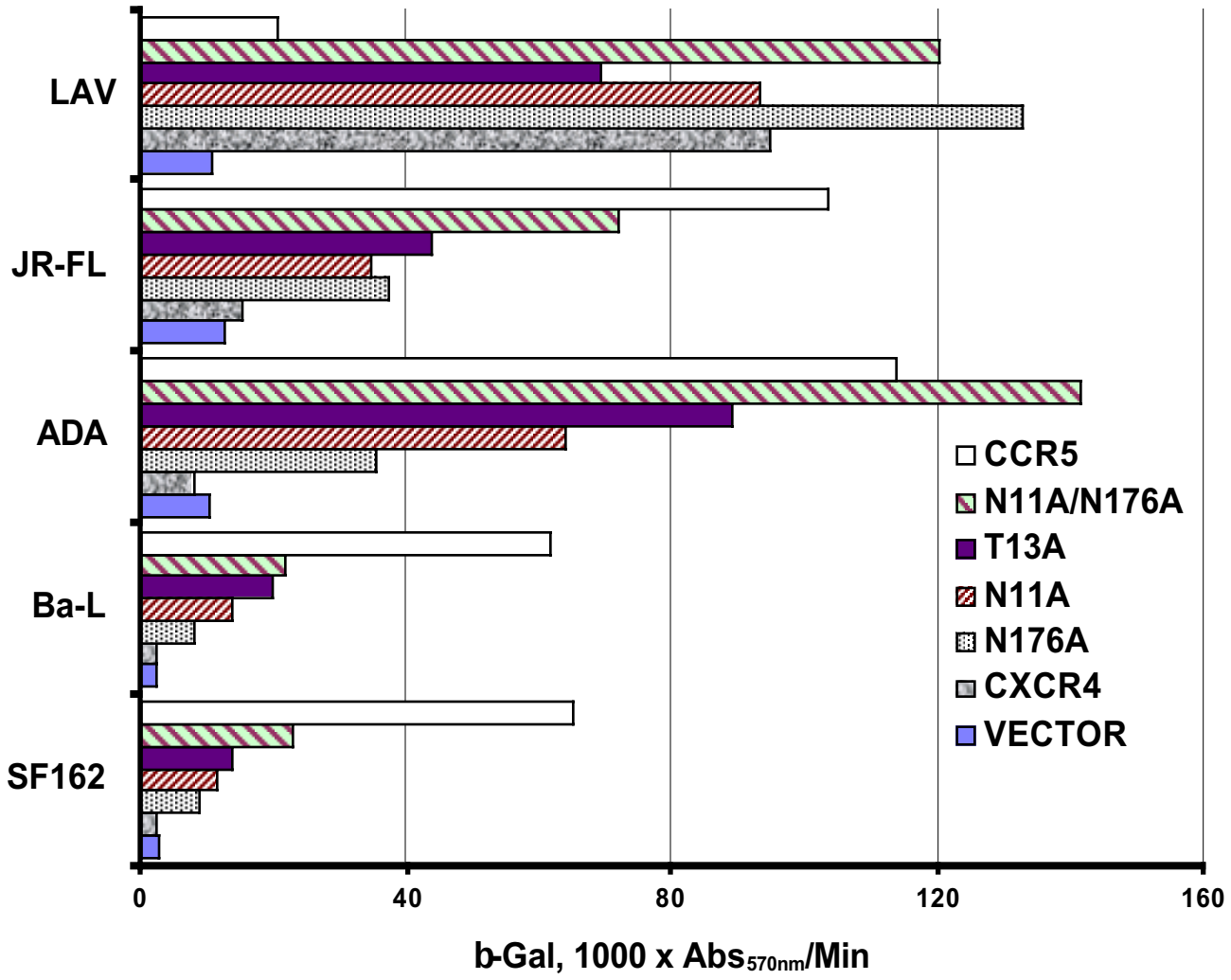


Figure 20. Coreceptor function of non-N-linked glycosylated CXCR4 mutants in cell fusion assays with R5 HIV-1 Envs. U373 target cells were transfected with a plasmid encoding the wild-type or a mutant coreceptor linked to vaccinia virus promoter and infected with vCB21R-LacZ and vCB-3 (CD4). HeLa effector cells were infected with a vaccinia virus encoding an HIV-1 Env and with vaccinia virus encoding T7 polymerase (vTF1-1). Cell mixtures (duplicates) were incubated at 37ß C for 2.5 h. Fusion was assessed by measurement of §-Gal in detergent cell lysates. The rates of §-Gal activity shown were obtained from separate samples in the same experiment. Each sample was tested in duplicate and the averages are plotted. This experiment was performed three times, and the data from a representative experiment is shown in the figure. OD: optical density.

carbohydrate modification at asparagine 11, rather than the asparagine amino acid itself, which was mediating the CXCR4 mutant phenotype of enhanced R5 coreceptor activity. We also examined the other potential N-linked glycosylation site in CXCR4, located in ecl-2, using the mutation N176A and found that it also had some enhanced coreceptor activity for R5 Env-mediated fusion but at a significantly lower level than the N-terminal site mutant. Therefore, we chose not to examine an additional S178A mutation but rather focus on the N-terminal glycosylation site and examine a double mutant both functionally and biochemically. The double non-N-linked glycosylated CXCR4 (N11A/N176A) was constructed and this non-N-linked glycosylated CXCR4 exhibited a further enhanced coreceptor activity for several R5 HIV-1 isolate Envs including JR-FL, ADA, Ba-L and SF162, over that of the single N11A construct (Fig. 20). The data shown in Fig. 20 is the actual rate of reporter activity with background levels of both vector alone, wild type CXCR4 and the CCR5 activity shown for comparison. The N11A/N176A mutant CXCR4 retained full coreceptor activity for LAV Env (Fig. 20) and with several other X4 Envs (data not shown). The expanded tropism activity of the non-N-linked glycosylated CXCR4 was quite significant, with activities ranging from 35% to 125% the level of coreceptor activity observed with CCR5 in the same experiment (Fig. 20). Similar results were achieved when these CXCR4 mutants were expressed along with CD4 in mouse 3T3 cells, rabbit RK13 cells, and monkey BS-C-1 cells (data not shown). These N-linked glycosylations could potentially block interactions between CXCR4 and certain regions within a particular Env or they might alter the conformation of CXCR4 in a way that prevents such interaction. These N-glycosylation modifications did not appear to be

required for coreceptor function with X4 or R5X4 strains, in agreement with another report (44).

In light of these surprising observations, we sought to examine the Env-mediated fusion activities of HIV-1 primary isolates and those of alternate clades. Shown in Fig. 21 are the results obtained from testing a battery of primary R5 isolate Envs with the individual non-N-linked glycosylated CXCR4 mutants in comparison to wild-type CXCR4 and CCR5 in the cell fusion assay. Two primary clade B isolates (91US005.11 and 92BR020.4), a clade F/B mosaic (93BR019.10-all gp120 F), and a clade A (92RW020.5) R5 Env could utilize the N11A CXCR4 coreceptor. The overall reporter gene signals were lower in this experiment in comparison to Fig. 2 because both the coreceptor genes and the Env genes (most driven by a T7 promoter system) were transfected as plasmids in this assay. For this reason a plasmid encoding the Ba-L Env is included for comparison. In other experiments not shown, the double non-N-linked glycosylated CXCR4 mutant yields a slightly more elevated level of coreceptor activity in comparison to the single N11A mutant for the same panel of Envs tested in Fig. 21, but the N-terminal site clearly had the greatest influence and the control mutation (T13A) also imparted this expanded coreceptor activity as well (Fig. 21). The fact that some R5 Envs from alternate clades, like the two clade C Envs examined, were not able to use the non-N-linked glycosylated CXCR4 may not be surprising because clade C X4 isolate appearance is a rarity (38, 216), and this may indicate that clade C R5 Env-coreceptor

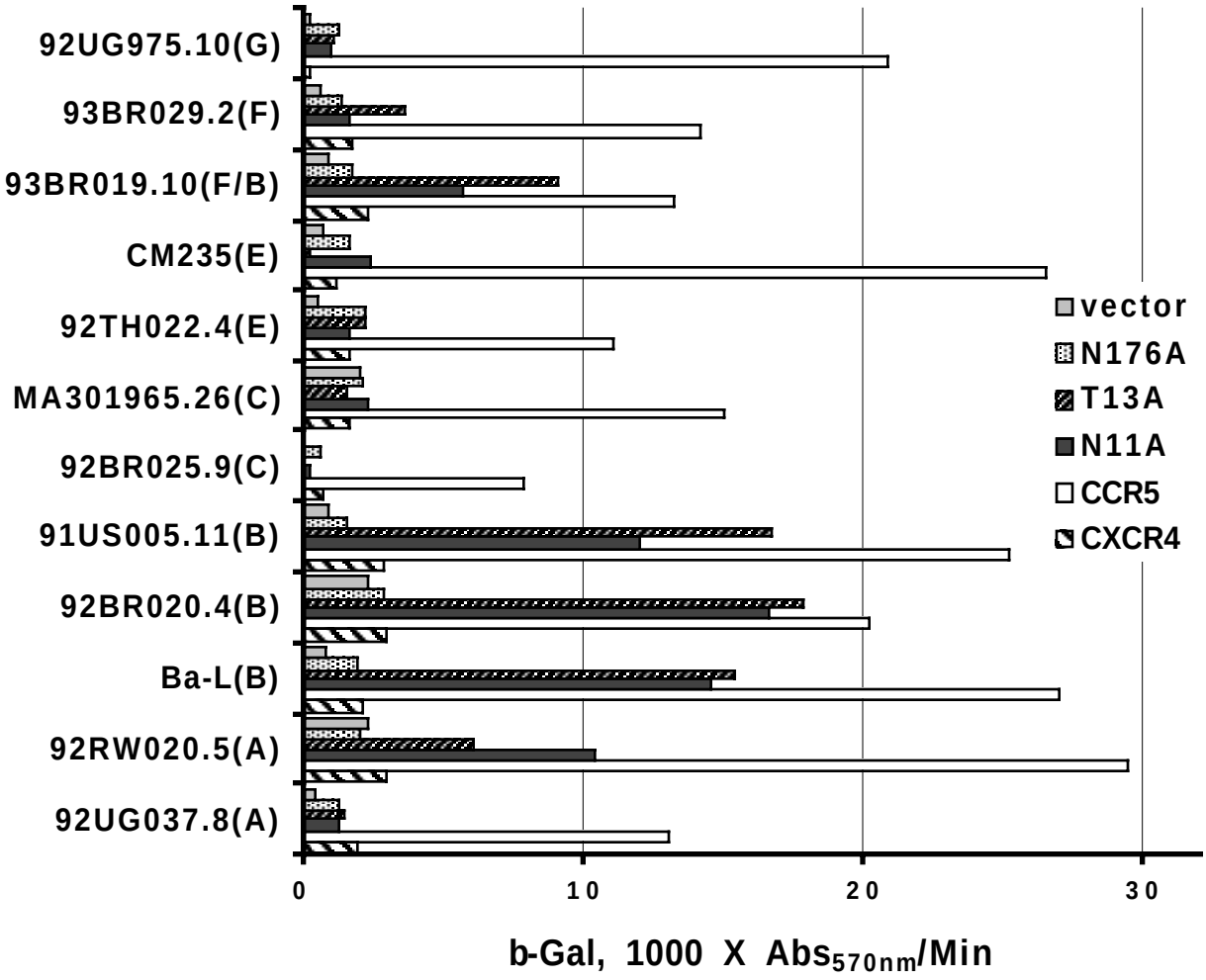


Figure 21. Coreceptor function of non-N-linked glycosylated CXCR4 mutants in cell fusion assays with primary isolate R5 HIV-1 Envs. U373 target cells were transfected with a plasmid encoding the wild-type or a mutant coreceptor linked to vaccinia virus promoter and infected with vCB21R-LacZ and vCB-3 (CD4). HeLa effector cells were transfected with a plasmid encoding Env (clade is indicated in parenthesis) linked to a T7 promoter and infected with vaccinia virus encoding T7 polymerase (vTF1-1). Duplicate cell mixtures were incubated at 37°C for 3 h. Fusion was assessed by measurement of §-Gal in detergent lysates of cells. The rates of §-Gal activity shown were obtained from separate samples in the same experiment. Error bars indicate the standard deviations of the mean values obtained for duplicate fusion assays. Data from a representative experiment are shown in the figure.

interaction is more distinct in comparison to R5 Envs from other clades like A, B, and F. The HIV-1 Env-mediated cell fusion assay presents a proven and reliable model of HIV-1 Env-mediated fusion and receptor function (10, 45, 67, 97, 100, 117, 178, 237). However, an examination of the activities of these CXCR4 mutants in an alternate assay for virus entry was also performed. The data in Fig. 22 shows that the non-N-linked glycosylated CXCR4 molecule can also support infection by a CCR5-dependent virus using an R5 JR-FL pseudotyped luciferase reporter system. Both the single (N11A) and double (N11A/N176A) non-N-linked glycosylated CXCR4 mutants could support this R5 Env-mediated virus infection, and the double mutant yielded a higher level of coreceptor activity. The background signals obtained with plasmid vector alone and with wild type CXCR4 and CCR5 are shown for comparison. In additional experiments the signals obtained with the single N176A CXCR4 mutant appeared no greater than wild type CXCR4 activity in the pseudovirus assay. In previous studies (58) we also observed differences in the relative signals obtained with an R5 Env when comparing results from the cell fusion assay versus the luciferase pseudotyped virus assay. These differences may be inherent between these two very different systems. Alternatively, the differences in the relative signals between the two assays might reflect post-binding roles of CCR5 for R5 HIV-1 isolates and be an area worthy of further investigation. Nevertheless, these two assay systems both support the conclusion that non-N-linked glycosylated CXCR4 can serve as a coreceptor for R5 Envs.

Identification of CXCR4 N-linked glycosylations. Previously we examined

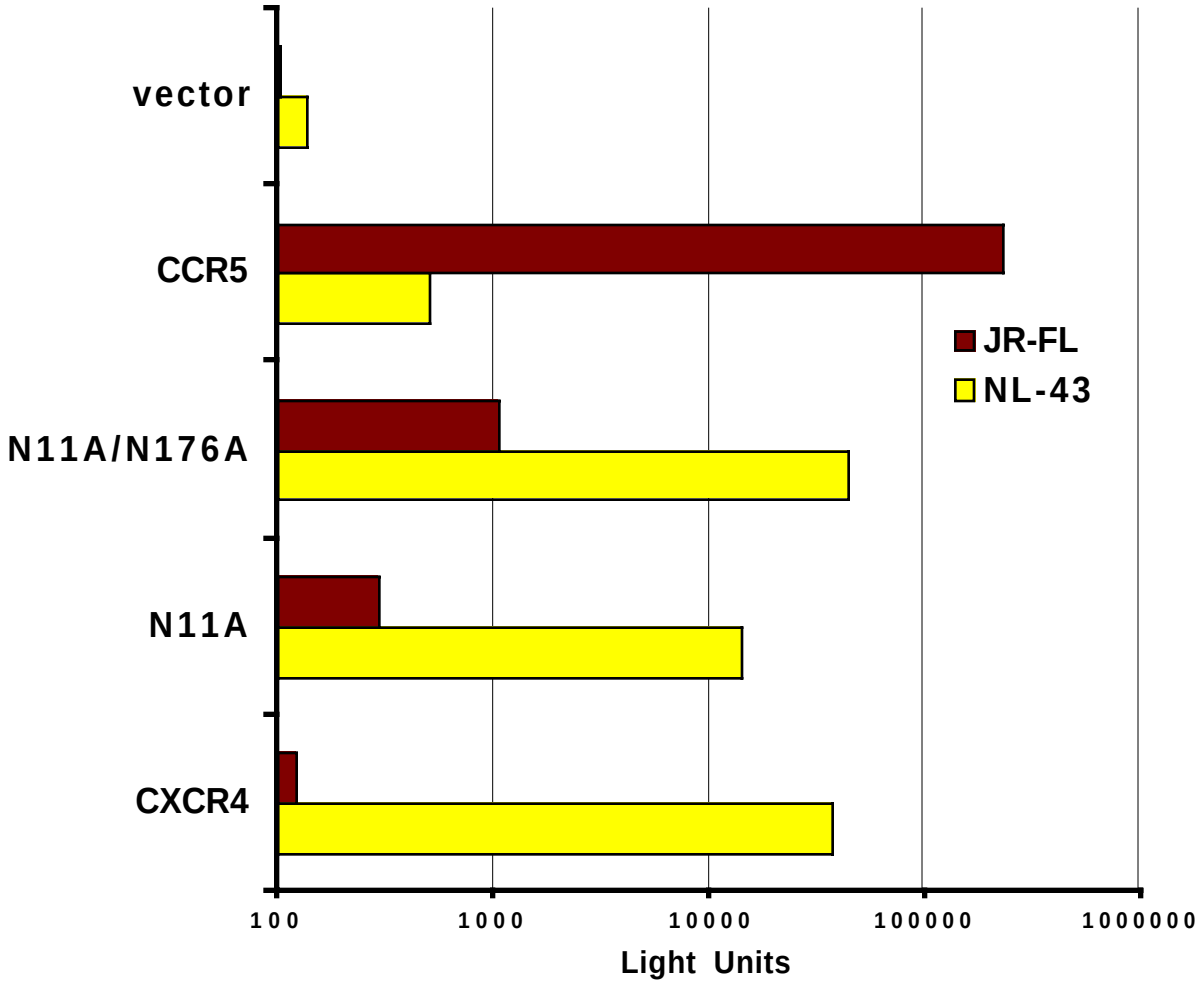


Figure 22. Coreceptor function of non-N-linked glycosylated CXCR4 mutants in a virus infection assays with an R5 HIV-1 Env. U373-CD4^{+} cells were transfected with a plasmid (pCDNA3) encoding wild-type or mutant coreceptor. Wells of cells (triplicate) were infected with the indicated HIV-1 Env luciferase reporter virus. Infection was assessed at day 4 by measuring the amounts of luciferase activity in cell lysates. Samples were tested in duplicate and averages were plotted. This experiment was performed three times, and the data from a representative experiment is shown in the figure.

recombinant vaccinia virus expressed CXCR4 by Western blot analysis using a polyclonal rabbit antiserum raised to the N-terminus of the molecule (117). In those studies, the predominant molecular species of CXCR4 was approximately 47-48 kDa. Also apparent was a second less-intense band with an apparent molecular weight of ~94-97 kDa, or double that of the predominant species. We have consistently observed this monomer/dimer pattern of CXCR4 as well as CCR5 (data not shown), and the ratios of the two bands can vary depending on the SDS-PAGE conditions. Virtually identical results were obtained by Doms and colleagues with an HA epitope tagged CXCR4 vaccinia construct (33). The predicted molecular weight of CXCR4 is 39.7 kDa, which had indicated a likely post-translational N-linked glycosylation event on the protein. The first clear evidence that CXCR4 was indeed N-glycosylated on at least one of these sites was provided from experiments using endoglycosidase F treatment of recombinant vaccinia expressed CXCR4 followed by SDS-PAGE and Western blotting (33). We wished to expand on these observations and precisely identify the sites of N-linked glycosylation and, more importantly, correlate these findings to functional activity. Therefore, we performed a biochemical analysis by SDS-PAGE and Western blot of the non-N-linked glycosylated CXCR4 mutants.

Western blot detection of CXCR4 following SDS-PAGE separation has been notoriously difficult. We and others have found analysis by SDS-PAGE and Western blotting of wild type CXCR4 or mutants that are expressed transiently using plasmid-transfected cells unsatisfactory. This is likely due to a combination of low expression and low affinity antibody, and in order to obtain unambiguous results we constructed

several new recombinant vaccinia viruses encoding wild-type and several mutant CXCR4 constructs. We analyzed lysates of cells infected with these vaccinia viruses to biochemically characterize the CXCR4 N-glycosylations by Western blot with the MAb 4G10 raised against the CXCR4 N-terminus. Binding of 4G10 to CXCR4 also appears unaffected by the N-terminal glycosylation (58) (data not shown) (Fig. 5). Using this approach, we determined that the N11A mutant had a significantly lower apparent molecular weight (monomer ~41-42 kDa) in comparison to wild type CXCR4, and was very close to the predicted size of unmodified CXCR4 (~40 kDa). There was no detectable size difference between the single N176A mutant and wild-type CXCR4, nor between the double N11A/N176A mutant and the single N11A CXCR4 mutant. The blot is purposefully over-exposed to show the two band pattern in the N11A and N11A/N176A lanes. Lower autoradiographic exposure reveals the same broad band at both positions in the other samples (data not shown), as was noted in prior work with wild type and epitope tagged CXCR4 (33, 117). Thus, if there is any N-linked glycosylation present at N176A it is too small a modification to be measurable in this assay. The non-N-linked glycosylated CXCR4 monomer was even smaller in comparison to an additional CXCR4 mutant, that was used as a relative molecular weight marker in this experiment (a C-terminal 42 amino acid (5 kDa) deletion construct with a molecular weight of ~43-44 kDa). Also, the apparent dimer bands were equally shifted lower in all cases (Fig.23). Taken together our data indicate that the principle site of N-linked glycosylation of CXCR4 is in the N-terminus and consists of an approximated 5-7 kDa carbohydrate moiety.

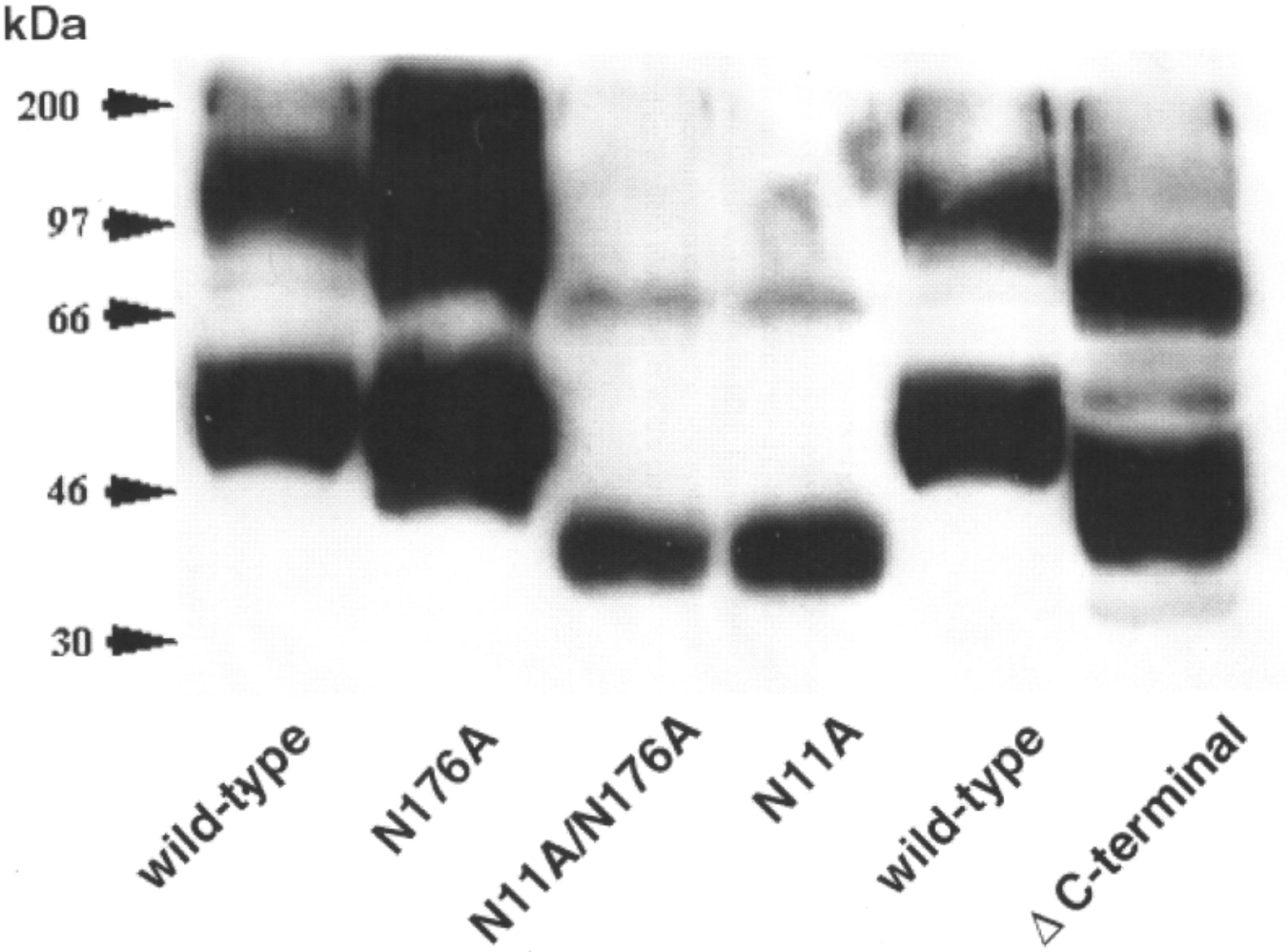

kDa
200
97
66
46
30
wild-type
N176A
N11A/N176A
N11A
wild-type
Δ C-terminal

Figure 23. Biochemical analysis of non-N-linked glycosylated mutant CXCR4s. Western blot of wild type CXCR4 and non-N-linked glycosylated CXCR4 mutants expressed by recombinant vaccinia viruses. ˘ C-terminal is a C-terminal 42 amino acid deletion construct with a molecular weight of ~46 kDa. Lysates were prepared from BSC-1 cells infected with a vaccinia virus encoding the indicated CXCR4 gene and analyzed by SDS-PAGE followed by Western blot analysis with a mouse monoclonal antibody to CXCR4 (4G10).

Using these CXCR4-encoding recombinant vaccinia viruses to express CXCR4, we observe essentially identical molecular weight patterns of monomer and dimer CXCR4 in a variety of cells including primary human macrophages, mouse 3T3, and human HeLa, U373, and HOS cell lines (data not shown). The double non-N-linked glycosylated CXCR4 mutant encoding vaccinia virus was also examined functionally (Fig. 24) and, although there may be a slight decrease in relative expression efficiencies in whole cell lysates (Fig. 23), this mutant is clearly quite efficient in supporting Env-mediated fusion by several prototypic R5 isolates in comparison to vaccinia expressed CCR5. The non-N-linked glycosylated mutants were also fully functional for a prototypic X4 (LAV) and R5X4 (89.6) Envs as well (Fig. 24).

N-terminus tyrosines have been identified as essential residues for gp120 binding HIV-1 entry (112, 113, 222). Indeed, sulfation of at least two of these tyrosines needs to be sulfated for optimal gp120 binding and chemokine binding (113). We constructed tyr to ala mutants of all six CXCR4 tyrosines so that we could test their importance in cell-cell fusion assays and in pseudovirus infection assays. Tyrosines and tyrosine sulfations do not appear to be as important for CXCR4 coreceptor function as they are for CCR5. Of the CXCR4 mutants only Y7A consistently had about a 50% reduction in activity as compared to wild-type CXCR4 with multiple X4 and R5X4 Envs. Y12A had less than half of wild-type coreceptor activity with SF2, an R5X4 Env, but only had small reductions in activity with other HIV-1 Envs.

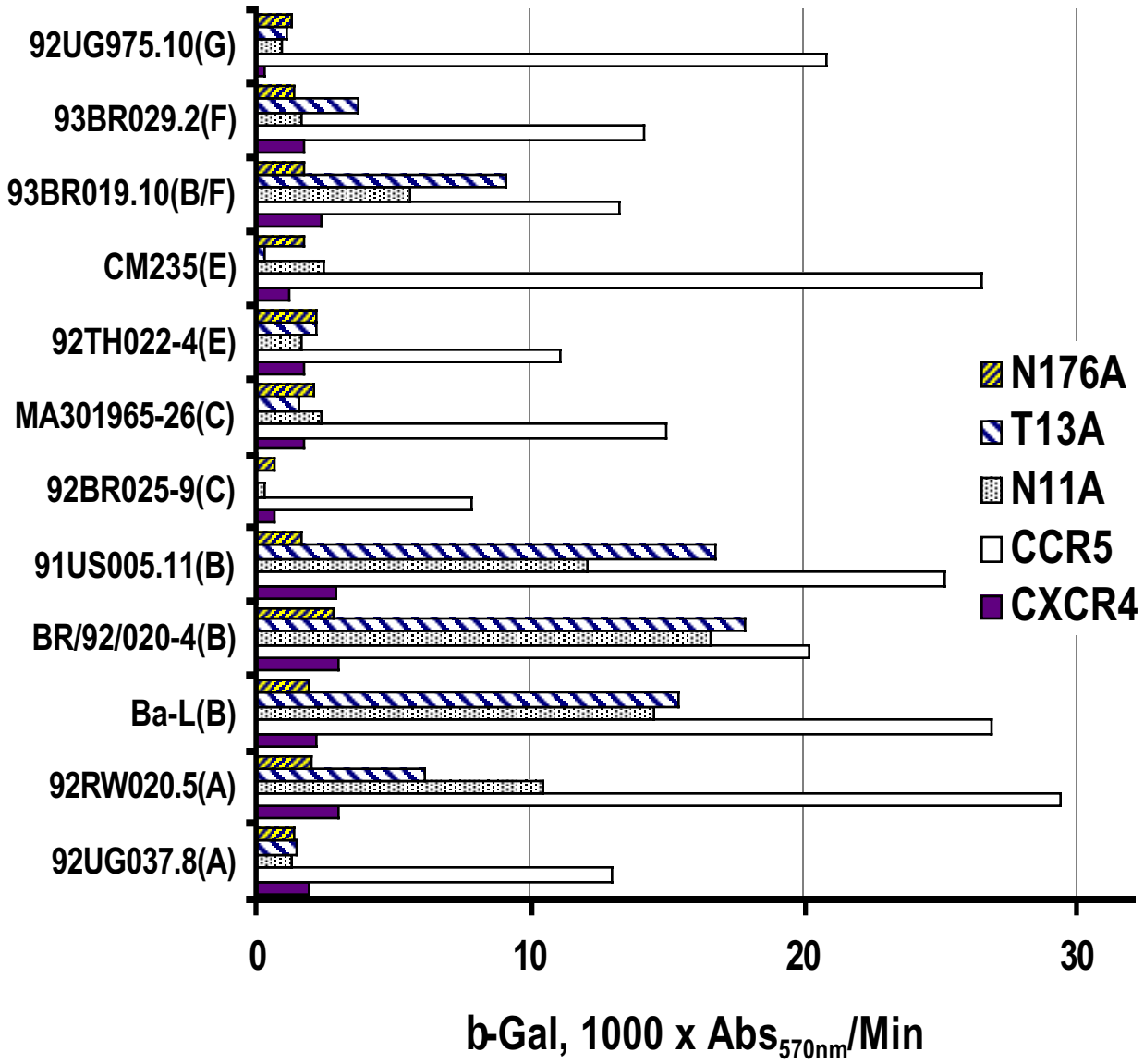
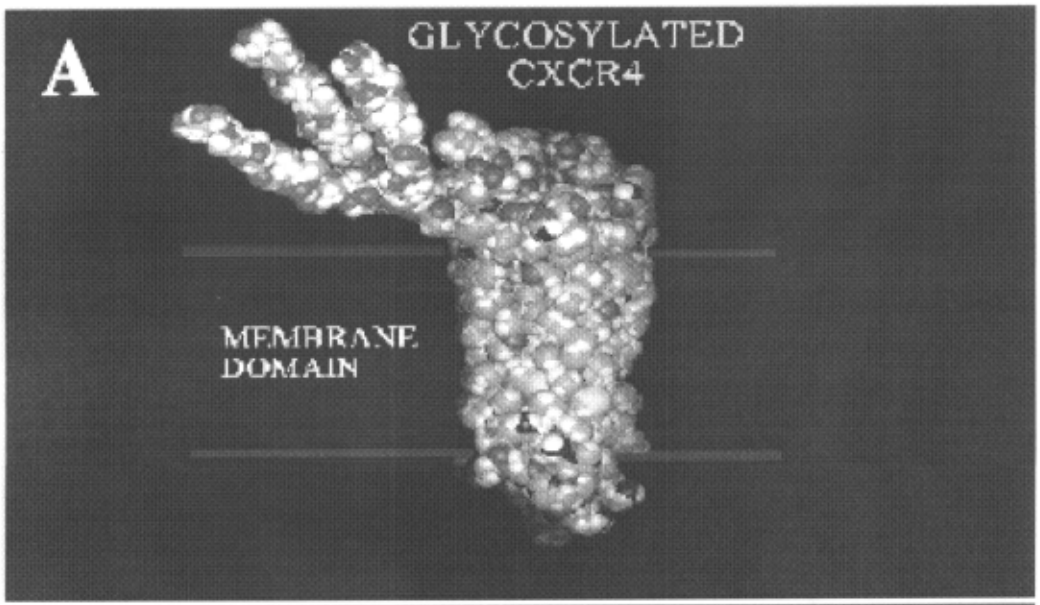


Figure 24. Functional analysis of vaccinia virus expressed double non-N-linked glycosylated CXCR4. Coreceptor function of recombinant vaccinia virus encoded non-N-linked glycosylated mutant CXCR4 in cell fusion assays with clade B Envs. Target cells were infected with vCB-21R-LacZ, vCB-3 (CD4), and either WR, vHC-7 (N11A/N176A), vHC-1 (CCR5), or vHC-3 (CXCR4). HeLa HIV-1 Env-expressing effector cells were infected with a vaccinia virus encoding T7 polymerase (vTF1-1) and the indicated Env. Cell mixtures (duplicates) were incubated at 37ß C for 2.5 h. Fusion was assessed by measurement of §-Gal in detergent cell lysates. The rates of §-Gal activity shown were obtained from separate samples in the same experiment. Samples were tested in duplicate, and averages are plotted. This experiment has been performed multiple times, and the data from a representative experiment is shown in the figure. OD: optical density.


A
GLYCOSYLATED
CXCR4
MEMBRANE
DOMAIN

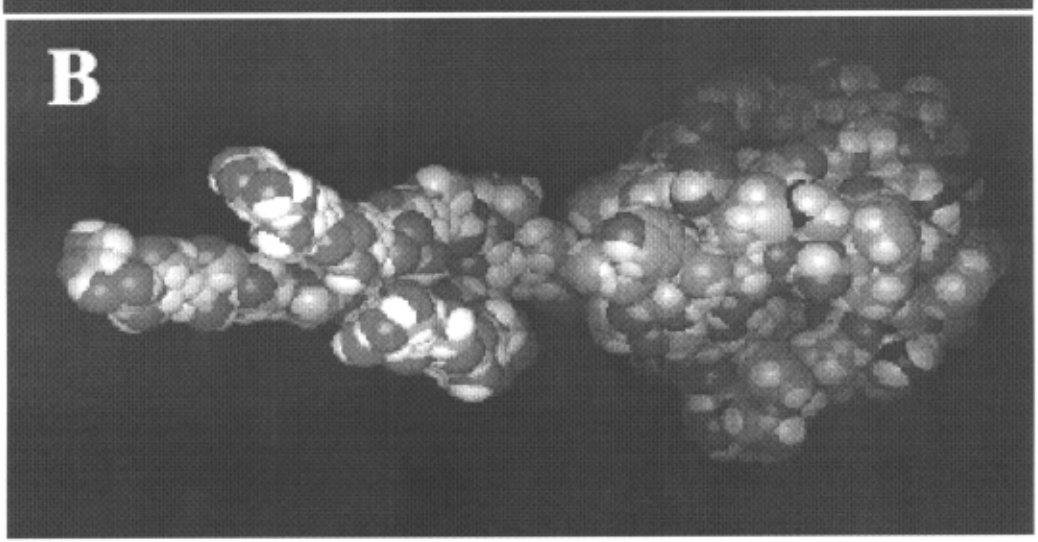

B

Figure 25. Schematic model of the HIV-1 coreceptor CXCR4 with a representation of the amino-terminal N-linked glycosylation moiety. The carbohydrate is drawn as a simple three branch structure with a molecular mass of approximately 6 kDa based on the measured molecular weight shift observed under reduced SDS-PAGE analysis. Green represents carbon. Red represents oxygen. Yellow represents nitrogen. White represents hydrogen. (A) Side view; (B) Top view.

	N-term.	ecl-1	ecl-2	ecl-3
CCR2B	14	X	X	X
CCR3	X	X	X	X
CCR5	X	X	X	268
CCR8	X	X	X	X
CCR9	20	X	X	X
CXCR4	11	X	176	X
CX3CR1	X	X	X	X
APJ	15	X	X	X
BOB	X	X	X	X
BONZO	16	X	X	X
US28	30	X	X	X
ChemR23	7	X	191	X
BLTR	2	X	165	X

Figure 26. Putative glycosylation sites of HIV-1 Coreceptors. Positions of predicted glycosylation sites are reported by domain. **x** = no glycosylation.

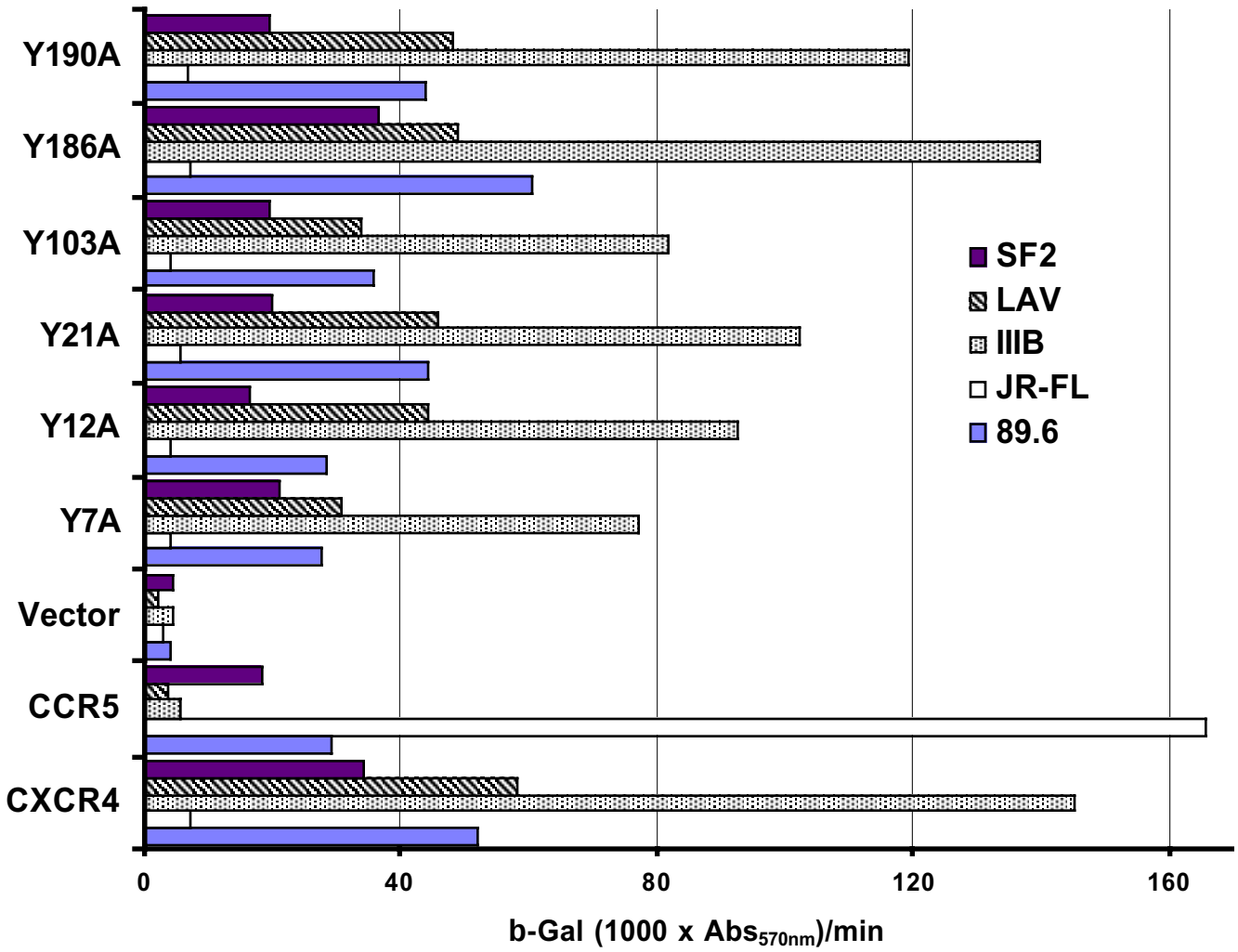


Figure 27. Coreceptor Function of Tyrosine Mutants of CXCR4 with X4 and R5X4 Envs in Cell-Cell Fusion Assay. U373 target cells were transfected with a plasmid encoding the wild-type or a mutant coreceptor linked to vaccinia virus promoter and infected with vCB21R-LacZ and vCB-3 (CD4). HeLa effector cells were infected with a vaccinia virus encoding an HIV-1 Env and with vaccinia virus encoding T7 polymerase (vTF1-1). Cell mixtures (duplicates) were incubated at 37ß C for 2.5 h. Fusion was assessed by measurement of §-Gal in detergent cell lysates. The rates of §-Gal activity shown were obtained from separate samples in the same experiment. Each sample was tested in duplicate and the averages are plotted. Results are corrected for CXCR4 surface expression following FACS analysis with the MAb 12G5. This experiment was performed three times, and the data from a representative experiment is shown in the figure. OD: optical density.

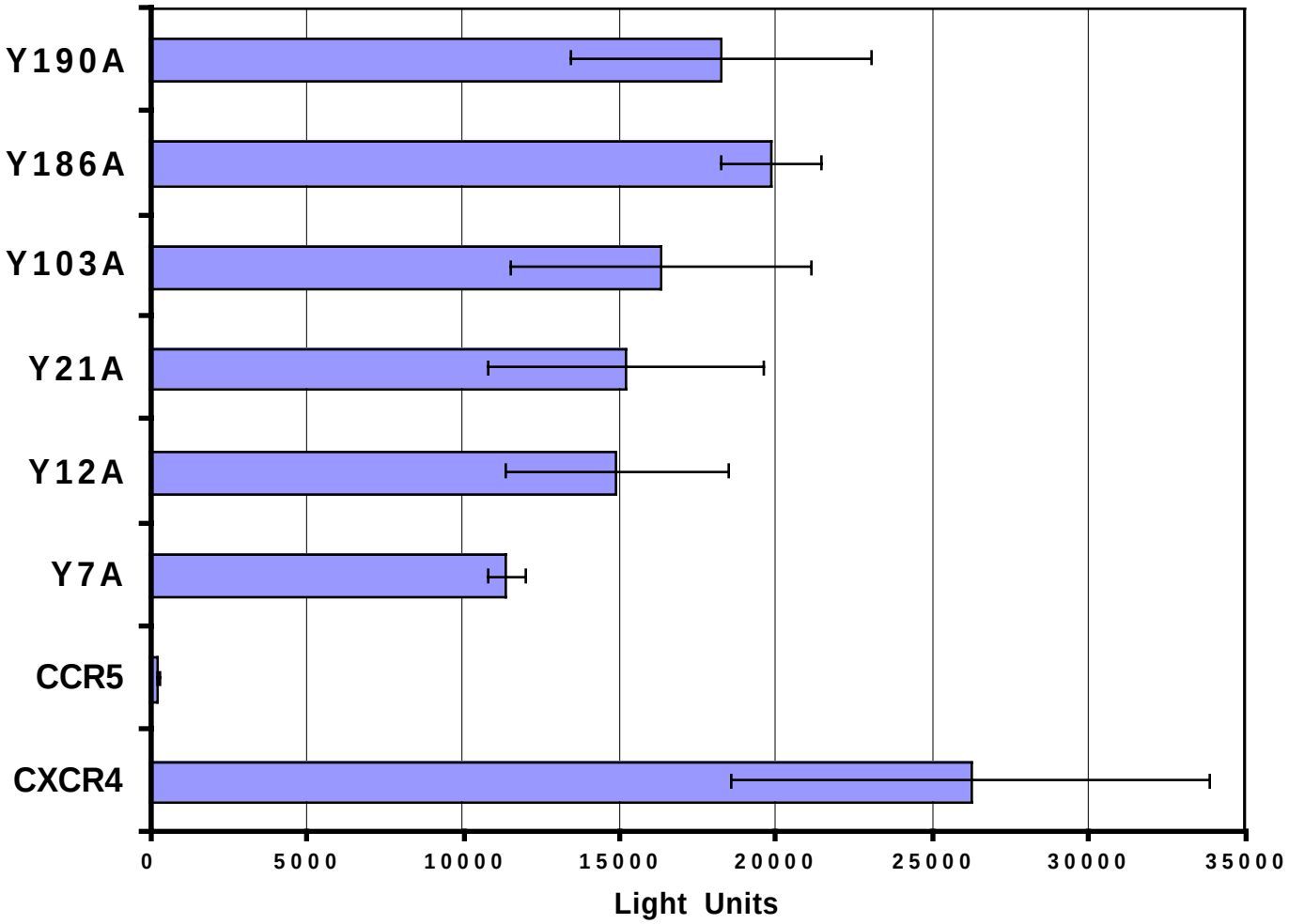


Figure 28. Coreceptor function of CXCR4 tyrosine mutants in a virus infection assays with an X4 HIV-1 Env. U373-CD4^{+} cells were transfected with a plasmid (pCDNA3) encoding wild-type or mutant coreceptor. Wells of cells (triplicate) were infected with the NL-43 HIV-1 Env luciferase reporter virus. Infection was assessed at day 4 by measuring the amounts of luciferase activity in cell lysates. Error bars indicate the standard deviations of the mean values obtained from triplicate wells.

DISCUSSION

Following the identification of the HIV coreceptors, the first proposed model to address their role in virus entry suggested that an individual Env would have binding preferences towards a particular coreceptor, mediated through interaction with the V3 loop of Env (an important determinant cell tropism) (46, 87). The CXCR4 amino acid sequence indicates a more a negative charge at the extracellular surface, while CCR5 is less negatively charged. The overall charge of the V3 loop is positive, with X4 V3 loops being more positively charged than an R5 V3 loop. Thus, the model would correlate with coreceptor type usage depending on the type Env, and obviously this suggests a simplified explanation for the preferential interaction of an X4 Env with CXCR4 and an R5 Env with CCR5.

However, our present data suggests that such an explanation may be an over-simplification. The removal of a carbohydrate moiety might affect exposure of charged portions of CXCR4, although at this time we cannot exclude the possibility that the N-linked carbohydrate structure is further modified in a way that would affect the overall charge, e.g. sialic acid addition. Along this line, it has been reported that sulfation of the N-terminus of the CCR5 coreceptor is important for function, where sulfated tyrosines contributed to the binding of CCR5 natural ligands as well as gp120-CD4 complexes (113). It was also concluded that only O-linked and not N-linked glycosylation modifications to CCR5 were evident but this was not confirmed by site-directed mutagenesis. An assay for assessing SDS-PAGE gel-shift of an exogenously enzymatically treated CCR5 or CXCR4 may not detect a modification of low mass. Our

results speak to this notion because we find that mutaion of the N-terminal glycosylation site but not the putative ecl-2 glycosylation site imparts a modification large enough to be measurable by SDS-PAGE. We cannot exclude the possibility that the ecl-2 site has a small glycosylation or one that does not bind well to SDS. Our attempts to measure the biological effects of N-linked glycosylation site removal by other means, including tunicamycin treatment of cells, was unsuccessful due apparently to cellular toxicity (data not shown). Indeed, the finding that prevention of N-linked glycosylation would allow CXCR4 to function as a coreceptor for R5 Envs in addition to retaining full function for X4 Envs was quite unexpected.

We hypothesize that removal of the carbohydrate moieties in CXCR4 is responsible for its enhanced coreceptor activity with CCR5-dependent R5 Envs by unmasking existing structures capable of interacting with these Envs. It may be that CCR5-restricted Envs are adapted to recognizing a coreceptor with a non-N-linked glycosylated N-terminus, whereas CXCR4-restricted Envs can accommodate such a configuration. Taken together, our data indicate that despite differences in primary sequence in their extracellular regions, there is perhaps an underlying conserved 3-dimensional structural similarity between CXCR4 and CCR5 and that subtle alterations in the CXCR4 (i.e. removal of carbohydrate), or mutation in Env to accommodate CXCR4 N-glycosylation, can allow R5 Envs to utilize it as a coreceptor.

We also show that glycosylation at the N-terminus is the major site of carbohydrate addition by mass to CXCR4. Removal of the N-linked glycosylation site in ecl-2 did not result in an apparent molecular weight shift. However, it was clear that the double non-

N-linked glycosylation mutant (N11A/N176A) had greater R5 Env coreceptor activity than either single mutation; the N11A/N176A CXCR4 molecule had equivalent coreceptor activity in comparison to wild-type CCR5 for some R5 Envs, so perhaps a quite small N-linked glycosylation modification is present at the ecl-2. We also note that among the set of CXCR4 mutations that exhibit enhanced coreceptor activity for R5 Envs is the disruption of the likely disulfide bond between the N-terminus and ecl-3 (58). A possible explanation of the underlying mechanism for this prior observation, in light of the present data, is that this alteration relaxes the barrel shape of CXCR4 and thereby re-positions the existing N-terminal glycosylation moiety, allowing better exposure of contact sites for R5 Env interaction.

We also point out that we have consistently observed that R5 virus entry signals with cells expressing non-N-linked glycosylated CXCR4 in the luciferase pseudovirus assay, are often lower as compared to the cell fusion assay results, and this was also observed with the charged-to alanine (D187A) CXCR4 mutant, another R5-enhancing alteration also found by another group (58, 276). On the one hand, the pseudovirus assay is dependent on Env-mediated fusion as well as reverse transcription, pre-integration complex formation and nuclear translocation, while our recombinant Env cell-cell fusion system was devised to eliminate the dependency on these post-entry events in measuring Env fusion activity. Nevertheless, we feel that these two very different assays, with wholly distinct readouts and different receptor and Env expression levels, are consistent in the data generated. It remains to be proven if there are indeed other mechanisms at work in the entry process of HIV that can account for the efficiency differences described

here, such as an event during the post-entry phase of virus replication as demonstrated with SIV (59). It should also be emphasized that our present results have a quite different significance than those of the charge mutations that broadened CXCR4 coreceptor activity reported earlier (58, 276), because here there are no amino acid alterations to the CXCR4 backbone other than those which eliminate N-linked glycosylation.

We have also consistently observed the apparent monomer/dimer pattern of CXCR4 and CCR5 (Fig. 23 and data not shown). The first suggestion of an oligomeric feature of an HIV coreceptor was shown using an immunoprecipitation assay with metabolically labeled CCR5, and a monomer/dimer pattern was reported in a low SDS environment (28). More recently, a similar SDS-PAGE pattern of CXCR4 and CCR5 has been reported (232) where the chemokine ligands were shown to induce a monomer to dimer transition. Further studies need to be performed to determine the nature and significance of these observations, and our data is derived from SDS-PAGE under reducing conditions, which suggest that strong hydrophobic interactions are involved. We feel that the high molecular weight species we observe is most likely a dimer and not an alternative or more heavily glycosylated CXCR4 molecule since we still observe a dimer in the double non-N-linked glycosylated mutant although one might speculate that the N-terminal carbohydrate moiety may play a role in stabilizing the dimer, based on the data in Fig 23.

The fact that the anti-CXCR4 MAb, 12G5, had been demonstrated to differentially inhibit HIV-1 infection in both a cell type and virus strain dependent manner (186) had earlier prompted the suggestion that the CXCR4 molecule itself is differentially processed, as in macrophages, resulting in it being utilized differently by various isolates (92). It has

been recently suggested that a high molecular weight species of CXCR4 that is defective in coreceptor activity is present in human macrophages (166) and that post-translational glycosylation could account for this observation. However, we have been unable to detect endogenous CXCR4 in macrophages and we note that recombinant vaccinia virus-expressed wild-type and non-N-linked glycosylated CXCR4 yield the identical molecular weight patterns in human macrophages as those shown in Fig 23. We are actively engaged in studies aimed at addressing the nature and biological relevance of the monomer/dimer forms of the coreceptors. Indeed, if multimeric complex formation between the oligomeric HIV-1 Env with its cellular receptors is required for fusion pore formation (194) and subsequent virus entry, then the very existence of CD4 independent strains of HIV-1, HIV-2 (142, 158, 227) and SIV (109) support the notion of oligomeric coreceptors.

A revised schematic model of CXCR4, in which we incorporate a hypothetical 3-branched 6 kDa carbohydrate structure on the molecule s N-terminal domain based on our estimated molecular weight differences, is shown in Fig. 25. In viewing this model, it becomes readily apparent how such a structure could potentially mask elements of the coreceptor s extracellular domains. Although theoretical, we feel it is quite relevant to present it in the context of this report because it dramatically shows how readily such a structure could cover underlying elements of not only the coreceptor s N-terminus but the extracellular loops as well, a feature rarely appreciated in stick-figure diagrams. As more monoclonal antibody reagents to the CXCR4 coreceptor become available to complement the growing numbers of available mutant coreceptor molecules, further detailed mapping and modeling constraints will be possible to help refine a theoretical 3-dimensional

model of the coreceptors. Our results concerning the CXCR4 N-terminal glycosylation suggest that it is the simple post-translational modification of one of the principle coreceptors (CXCR4) that is preventing many, or perhaps most, isolates from exhibiting a dual tropic phenotype (depending on the clade).

A number of other HIV-1 coreceptors also appear to be glycosylated (Fig. 26). The CCR5 coreceptor does not have an N-terminal N-linked glycosylation site, and interestingly the rarely employed CCR2 coreceptor, which is most closely homologous to CCR5, contains an N-terminal N-linked glycosylation site. However, removal of this site did not expand CCR2 coreceptor activity; only 4 fold increase to 10% the activity of CCR5 with a single R5 Env, ADA (data not shown). This is unlike several isolates which yield 35% to 125% the level of CCR5 coreceptor activity with the non-N-linked glycosylated CXCR4 (Fig. 2). Taken together, our data suggests a greater homology on a 3-dimensional level of Env interaction sites between the CXCR4 and CCR5 coreceptors, than previously thought or expected, even more so than that between CCR5 and CCR2. On the 3-dimensional level, this homology could account for (or comprise) the important contact sites for their interaction with Envs.

It is R5 HIV-1 strains that are largely responsible for virus transmission, and individuals who lack CCR5 due to a natural mutation in the gene (ccr5 ˘32 allele) are resistant to HIV-1 infection (84, 143, 241). HIV-1 X4 isolates tend to emerge much later in infection, and the tropism switch from R5 to X4 viruses correlates with progression of the infection to symptomatic AIDS (74, 248, 252). This tropism switch is in part related to genetic changes in Env sequence, but not all infected individuals who progress to AIDS

develop X4 isolates, so the reasons for the *in vivo* evolution of HIV are not completely understood. Our findings now add further complexity to this picture because they demonstrate the possibility that early stage primary R5 isolates could infect target cells via CD4-CXCR4 receptors under circumstances of differential glycosylation of CXCR4 without an accompanying genetic change in Env. The existence of non-N-linked glycosylated CXCR4 or CXCR4 glycoforms in an individual, as result of genetic or environmental influences or even from the infection process itself, that allow for R5 Env recognition could have broad reaching implications in the HIV infection and pathogenic process, and outcomes in the host. Indeed, glycoforms of a protein may be cell type or even cell-cycle dependent (238). Whether there are alternate glycoforms of CXCR4 *in vivo* remains to be determined; experiments that would certainly be highly challenging considering the difficulty in detecting endogenous CXCR4 at the present time by blotting techniques.

In our study we have shown that not only were all prototypic clade B R5 Envs examined able to utilize the non-N-linked glycosylated CXCR4 as a coreceptor but that several R5 primary isolate clade B Envs could use this modified coreceptor as well. Somewhat more variable recognition was seen across clades. We observed that in addition to the clade B isolates, a clade A and F isolate and a mosaic F/B isolate (all clade F gp120) were capable of using the non-N-linked glycosylated CXCR4 coreceptor. In retrospect, it is note-worthy that the clade C R5 Envs where not capable of using the non-N-linked glycosylated CXCR4 and indeed clade C X4 isolate appearance is a rarity (38, 50, 216), while on the other hand we would predict that a clade D R5 isolate would have been able to

utilize it had there been a cloned gp160 available for testing. Thus, some Env genetic subtypes might indeed be more distinct in their interaction with coreceptor. A larger study with a greater number of R5 isolates appears warranted in order to fully assess and validate the breath of these present findings. We are establishing a transformed $CD4^+$ cell line with the double non-N-linked glycosylated CXCR4 mutant to further examine its utility as a target for HIV-1 infection studies and this will also allow us to more extensively examine cross-clade R5 isolate use of this altered CXCR4 molecule.

In addition to N-linked glycosylations, other modifications may be important for CXCR4 coreceptor function. GPCRs have been found to be differentially spliced or posttranslational modified (N and O—linked glycosylated, palmitoylated, phosphorylated, sulfated, and disulfide bonded) (261). Protein tyrosine sulfation have been found in all animal species exained to date (144, 196). Tyrosine sulfation occurs in the lumen of the trans-golgi network, where tyrosylprotein sulfotransferase transfers a sulfate group from 3 -phosphoadenosine 5 -phosphosulfate to tyrosines (171). Tyrosine sulfations sometimes aid in protein-protein interactions (27, 196). Iin particular, tyrosine sulfations have been implicated as vital for CCR5 coreceptor function (113). We mutated all six CXCR4 tyrosines so that we could test their importance in cell-cell fusion assays and in pseudovirus infection assays. Tyrosines and tyrosine sulfations do not appear to be as important for CXCR4 coreceptor function as they are for CCR5. Of the CXCR4 mutants only Y7A consistently had about a 50% reduction in activity as compared to wild-type CXCR4 with multiple X4 and R5X4 Envs. Y12A had less than half of wild-type coreceptor activity with SF2, an R5X4 Env, but only had small reductions in activity with

other HIV-1 Envs. Future experiments should focus on the effect of CXCR4 tyrosines on coreceptor function with primary isolates of different clades, as well as whether tyrosines of CXCR4 are sulfated.

Chapter 6

Discussion

Adherence and Fusion of Virus with Host Membranes:

HIV-1 Envelope Glycoproteins

The location of gp120 protruding 9 to 10 nm from the viral envelope, besides forming an obvious target for the immune system, allows for interaction with host cells. gp120 forms a trimolecular complex with gp41, CD4, and coreceptor (269). The nature of this complex is a subject of great interest, as it may be the key to development of treatment and vaccines. The gp120 subunit of Env is an extremely complex protein. It has 23 putative N-glycosylation sites and nine disulfide bonds which divide the molecule into five variable and five constant regions (88). The extreme heterogeneity between gp120 sequences of different isolates, particularly in the variable regions, is largely responsible for the difficulty in developing broadly neutralizing antisera to HIV-1 (63, 175). Gp41, the transmembrane protein that noncovalently associates with gp120, containing four putative N-glycosylation sites, two cysteine palmitoylations, and a disulfide bond (291). gp160, the precursor of gp120 and gp41, oligomerizes, probably into a trimer, in the endoplasmic reticulum (49, 106, 206) and is cleaved by furin or other cellular proteases in the golgi (12, 105, 273, 282).

Retroviral surface glycoproteins are reminiscent of the well-characterized glycoproteins on the surface of influenza virus. Influenza virus HA-1 binds to host receptors and is analogous to gp120, and HA-2 is a transmembrane protein analogous to

gp41. Like gp120 and gp41, HA-1 and HA-2 are products of a common precursor, they remain linked following cleavage, and they form a trimeric structure (281, 284).

Adherence of HIV-1 to Host Cells

Initial adherence between HIV-1 and host is mediated by viral surface gp120 and perhaps cellular proteins obtained upon budding (205). These cellular proteins may include integrins and MHC molecules (183). They may bind to protein, such as CD4, and/or glycosaminoglycans on host cells. The process of membrane fusion begins with structural changes in gp120 triggered by high affinity interaction with CD4. These changes may occur with cell-associated CD4 or, less efficiently, with soluble CD4. The association of the N-terminus of CD4 with discontinuous regions of the core (constant regions) of gp120, particularly along the peptide backbone of gp120, rather than with amino acid side chains, results in exposure of the coreceptor binding site, which is composed largely of the V3 loop as well as V1 and V2 regions (16, 115, 167, 289). Mutagenesis studies indicate that D368 and E370 of C3 and W427 and D457 of C4 are important for CD4 binding (69, 160, 167, 203)). Physical evidence for the model comes from experiments showing coimmunoprecipitation (185, 267, 287, 290) and membrane colocalization of CD4, coreceptor, and HIV-1 Env (145, 269, 290). X-ray crystallography results showed a 9 nm^2 ridge-like structure on CD4 with a hydrophobic amino acid at its tip (F43) (239), which appears to fit into a grove on gp120 (190). More evidence for the model is illustrated by the finding that radiolabeled MIP-1[alpha] or MIP- § could be blocked from binding cell-associated CCR5 by R5 gp120 but only

following incubation of the cells with CD4. Additionally, a monoclonal antibody named 17b, which is believed to bind to the coreceptor binding site, bound with higher affinity to gp120 following interation of the gp120 with CD4. Therefore, it is not surprising that lab strains that are CD4 independent bind antibody directed to this normally concealed coreceptor-binding site on the V3 loop. The V3 loop was implicated in coreceptor binding by findings that V3 loop deletions or substitutions prevented interference by gp120 with MIP-1ß (267, 275, 287). Additionally, small changes in the V3 loop can change HIV-1 tropism from R5 to X4 or visa versa (67, 250).

Membrane fusion

CD4 and coreceptor binding results in dramatic gp41 conformational changes that bring the virus and host cell membranes together (35, 61, 279). The conformational changes in gp41 have been likened to the springing of a mouse trap; its two alpha helices, which are separated by a disulfide bond (the hinge of the trap), are suddenly free to interact, probably forming an antiparallel coiled coil (53). This forces the hydrophobic fusion peptide (the needle of the trap) to swing out into the host membrane, much as occurs with HA2, the transmembrane protein of influenza virus. While this energetically favorable reaction is triggered by an endosomal pH change with influenza virus, in HIV it is presumably triggered by gp120 conformational changes brought on by interaction with CD4 and coreceptor. With both viruses the fusion mechanism must not be triggered prematurely or the fusion peptide will likely fold in a way that will not be able to drive fusion between host and viral membranes.

Evolution of Coreceptor Use

With few exceptions transmission of HIV-1 is by R5 strains, rather than either X4 or R5X4 strains (76, 272). It has been suggested that this may be because cells initially infected, perhaps langerhans cells in mucosal epithelium or macrophages in the submucossa, do not express functional CXCR4. Indeed, a report shows that freshly isolated langerhans cells do not express CXCR4 on their surface, though they do have cytoplasmic CXCR4 (294). As discussed below it has been reported that most X4 HIV-1 strains cannot infect macrophages despite the presence of CXCR4 on their surfaces (45). However, this does not explain why R5X4 strains appear to be less transmissible *in vivo* than R5 strains. There is no evidence to suggest that the ability to use CXCR4 may be disadvantageous in early stages of infection. The explanation may be that X4 strains are more easily neutralized by serum and innate immune factors. For whatever reason, it is clear that in most cases patients are initially infected with an R5 strain and the virus replicates and mutates rapidly so that there can be evolution within a patient from R5 into R5X4 strains and then into X4 strains. It has been estimated that in about 40 — 50% of AIDS patients syncytia-inducing X4 isolates replace nonsyncytia-inducing R5 isolates as the major viral population (65, 244, 248, 265). X4 strains generally grow faster and are more cytopathic than R5 strains. The identification of X4 strains in a patient often coincides with a precipitous drop in CD4-pos T-cells and conversion to full-blown AIDS (74).

A possible driving force for this evolutionary switch is suggested by the finding that CC chemokine production is upregulated as AIDS develops in HIV-infected patients (8, 204, 243). Down-modulation of CCR5 and competition for CCR5 binding sites could result. Additionally, Kinter et al. (154) found that G-protein signaling induced by CC chemokines in T-cells resulted in a closer association between CD4 and CXCR4 on host cell surfaces as well as increased rates of X4 HIV-1 replication when low infection titers were used. Another possible advantage of CCR5 initially in infection is that signaling through CCR5 can result in T-cell activation (21), which is needed for HIV-1 replication. Interestingly, in SCID-hu Thy/Liv mice an R5 strain infected stromal cells, including macrophages, in the thymic medulla, while an X4 strain replicated much more rapidly and depleted cells in both the thymic cortex an medulla (32).

It should be noted that reports describing coreceptor evolution have generally not considered clade or geographic origin of isolates. Reports that coreceptor usage does not appear to be strongly linked to an isolate's clade (25, 37, 268, 295), have led some to conclude that coreceptor evolution is probably not be clade-specific. However, recent reports show that a disproportionate number of clade D isolates are X4 (50), while most clade C isolates are R5 (38, Broder, 1999 #3536). It might be informative to track coreceptor usage during the course of infection of individuals infected with clades C and D.

Other HIV-1 coreceptors

On occasion other molecules may be used as coreceptors. Indeed 13 seven-transmembrane proteins have been identified through *in vitro* assays to have coreceptor activity. There are many seven-transmembrane proteins on each human cell. In fact it is estimated that there are over 1000 different human seven-transmembrane proteins. They generally act as receptors and are coupled to G-proteins through which signaling events are channeled. Thus, this class of protein is often referred to as G-protein coupled receptors (GPCRs). GPCRs are categorized by their ligands: amines, amino acids and their derivatives, proteins, nucleosides, lipids, phospholipids, multistructural odorants, or retinol. All of the discovered HIV-1 coreceptors except ChemR23 (an orphan receptor that resembles formyl-methionyl peptide receptors) share greatest homology with the chemokine receptors, a class of GPCR protein receptors.

Indeed, ligands for eight of them, CCR2b, CCR3, CCR5, CCR8, CCR9, CXCR4, CX3CR1, APJ, and US28 are known to be chemokines, and other chemokine receptors, such as CCR1 and CXCR1, can function as coreceptors for various HIV-2 and SIV isolates. The ligands of HIV-1 coreceptors BOB and Bonzo are unknown. There are two major classes of chemokines. Chemokines with two pairs of consecutive cysteines in their N-terminus are known as CC chemokines, while CXC chemokines have one amino acid between both cysteine pairs. Additionally, there are two other very small classes of chemokines: C chemokines have just one cysteine at their N-terminus, and the only known CX3C chemokine has three amino acids between each pair of cysteines.

Structural comparisons of coreceptors

Even though there is a great deal of primary sequence heterogeneity among the chemokine receptors, it appears that there is some common structural feature that allows them to function as lentivirus coreceptors. This is particularly surprising given that there are four diverse classes of chemokine receptors (and chemokines). Despite the fact that CCR5 and CXCR4 have only about 30% homology with conservative substitutions (Fig.1), our work demonstrates that single amino acid mutations in CXCR4 allow it to function as coreceptor for R5 HIV-1. Thus, there appears to be a conserved R5 coreceptor framework common to CCR5 and CXCR4. In wild-type CXCR4 it is somehow prevented from functioning with R5 Env. This is likely due to a combination of features as combining these tropism-altering mutations has additive effects.

The most potent of these mutations is D187A. Conversion of the aspartic acid to an alanine allows CXCR4 to function at levels up to 95% of CCR5 as a coreceptor for R5 HIV-1 in cell fusion assays and up to 5% of CCR5 in infection pseudotyped virus infection assays. The level of activity of the mutant is cell and Env-dependent. While it functioned at levels comparable to CCR5 in cell fusion assays with multiple lab and primary isolate clade B R5 HIV-1 Envs, it did not function as well with some R5 Envs of other clades. As the V3 loop of HIV-1 gp120 can determine coreceptor usage, we looked for amino acid sequence characteristics common to R5 gp120s that were able to utilize the CXCR4 mutant versus those that were not. We were unable to find any patterns, including specific sequences or net charges responsible for the phenomenon.

However, the loss of the negative charge at position 187 of CXCR4 may be responsible for the increased host range of mutant CXCR4. A number of reports show that the evolution of R5 to X4 HIV-1 within a patient is generally coincident with increased alkalinity of the V3 loop (82, 119, 146). It was suggested that this charge difference is likely responsible for coreceptor usage, as CXCR4 is more acidic than CCR5. The most dramatic charge differences between CXCR4 and CCR5 lies in the domain of D187, ecl-2, where the pI of CXCR4 is 3.8, while the pI of CCR5 is 9.65. The mutation modestly increases the pI of CXCR4 ecl-2 to 4.0. Mutation to alanine of another acidic amino acid within ecl-2, D193, also resulted in some coreceptor activity with R5 HIV-1 Envs in cell fusion assays, though not in infection assays, and mutation of other acidic residues within ecl-2; E179A, D181A, and D182A, did not affect CXCR4 s coreceptor function. In fact mutation of a basic residue, R188A, reduced CXCR4 function with X4 HIV-1 Envs. To test whether the net charge of ecl-2 is responsible for the lack of function of CXCR4 with R5 HIV-1 Envs Z.Wang et al. mutated multiple acidic residues of ecl-2 (276). Combining D187A with D193A did not have significantly higher activity with R5 Envs than D187A by itself, and combining D187A with various other mutation combinations, E179Q/D181A/D182A, E179Q/D181A/D182A/R188A, and E179/D181A/D182A/R188A/D193A diminished rather than enhanced activity of the D187A CXCR4 mutant with R5 Envs. Thus, the overall acidity of ecl-2 is probably not sufficient to explain why wild-type CXCR4 is unable to act as a coreceptor for R5 HIV-1.

Perhaps the best test to determine if the increased activity of the D187A mutant is due to the loss of a negative charge, rather than the loss of other attributes of aspartic

acid, would be to test a D187N mutant. The experiment was carried out, and while the D187N mutant was nonfunctional or barely functional in R5 HIV-1 pseudotyped virus infection assays, it was about 50% as potent as D187A in cell fusion assays (276). Thus, it is likely that the charge as well as other attributes of aspartic acid block wild-type CXCR4 from functioning with R5 HIV-1 Envs (276). The differences between the infection data and the cell fusion data may reflect sensitivity differences between the two assays or they may indicate that the negative charge of D187 has a greater effect on postbinding events involving CXCR4 than on cell fusion.

Certain cysteine mutations also allow CXCR4 to function as a coreceptor for R5 HIV-1 Envs. Conversion to alanine of the N-terminal cysteine, C28A, especially when in combination with alanine substitution of the ecl-3 cysteine, C274A, expands CXCR4 coreceptor range. As these two cysteines probably form a disulfide bond (see below), we believe that removal of the bond opens up the barrel structure of the extracellular elements of CXCR4 allowing it to accommodate R5 HIV-1 Envs. Perhaps mutation of C28 frees up the N-terminus for R5 HIV-1 binding, much as the N-terminus of CCR5 appears to be important for initial steps in gp120 binding. C274A may enhance the activity of the C28A mutant by preventing inappropriate disulfide bond formation between C274 and other cysteines.

Other HIV-1 cofactors

There are many factors that affect fusion between virus and host membranes. Many of these factors are not HIV-1-specific. They include charged molecules, such as

glycosaminoglycans, and adhesion molecules, and they depend on the cell type that virus budded from as well as the new target cell type. Often these molecules play a more significant role in adherence prior to membrane fusion, rather than the fusion event itself. A very surprising observation was that expression of CD4 does not aid adherence of X4 virus to HeLa cells, which naturally express CXCR4 (188). And antibody to CD4 not only did not reduce X4 HIV-1 adherence to CD4-pos HeLa cells, but in some cases it increased adherence. In contrast, antibodies to various polyanions, such as heparin, dextran sulfate, and pentosan sulfate, completely blocked attachment. Heparin was also implicated in attachment by findings that heparinase also completely blocked attachment.

It should be noted that there are alternative, less efficient mechanisms of HIV-1 entry. Certain cell types, including neural and intestinal cells that do not express CD4 can be infected. For example CD8-pos T-cells can be heavily infected in late-stage AIDS. CD4-neg enterocytes are also infected in some AIDS patients. It has been suggested that this may be due to evolution of CD4-independent virus (275), and this idea is not so far-fetched considering CD4-independent strains have been developed in the lab by exposing CD4-neg cells to very high concentrations of HIV-1 (142). Another possible explanation for the infection of CD4-neg cells is that they might have a membrane glycosphingolipid, such as galactosyl ceramide (Gal-Cer) (131) or globotriaosylceramide (GB3), which can be used in place of CD4 (220). Gal-Cer apparently can replace CD4 and coreceptor as a receptor for HIV-1, albeit with much lower virus entry. Antibody directed to the galactose portion of Gal-Cer prevents HIV-1 entry and gp120 binding to CCR5-neg CXCR4-neg CD4-neg HT29 colonic epithelial cells. The role of Env was

confirmed by entry inhibition by gp41-based peptides. Incorporation of sulfatide, a naturally occurring sulfated derivative of Gal-Cer, into membranes also supports attachment of HIV-1 through interaction with Env, but it does not support virus entry, as determined by luciferace pseudotyped virus infection assays (110). In fact incorporation of sulfatide into neural and intestinal CD4-neg cell lines that can be infected with HIV-1 resulted in a dose-dependent inhibition of infection. Thus, sulfations present in sulfatide would prevent Gal-Cer from supporting fusion but not from supporting adherence.

GB3, but not Gal-Cer, incorporation into HeLa CD4 cells enhanced fusion with cells expressing X4 Env. However, GB3 did not support infection of HeLa cells that do not express CD4, so GB3 appears to function — as a coreceptor - in a CD4-dependent fashion. It has been suggested that CD4 should be considered a secondary receptor because it is likely that the requirement for CD4 evolved much later among retroviruses than the requirement for chemokine receptors. Among older viruses, such as HIV-2 and SIV there are many primary isolates that are CD4-independent, though they are better able to enter cells that do express CD4. An older virus, FIV utilizes CXCR4 and is not aided by the presence of CD4 on host cells.

Another possible method of entry would be by receptor mediated endocytosis through antibody or complement receptors (228). The high antibody titer to gp120 in AIDS patients probably results in the coating of some viral particles with antibody and complement. Cells with Fc or C receptors may endocytose there viruses. Similarly, macrophages and other cells with mannose and other sugar receptors may recognize the heavily glycosylated gp120 molecule (242).

***In Vivo* evidence of Coreceptor Use**

The biological significance of CCR5 and CXCR4 in AIDS pathogenesis is well established. As mentioned all HIV-1 strains appear able to utilize one or both. Additionally, individuals with mutations in CCR5 that prevent its expression have a high degree of resistance to HIV-1 infection (174). PBMCs from these individuals can be infected with X4 HIV-1 but not with R5 HIV-1 (210). A mutation in the 3 untranslated region of SDF-1 is associated with a longer disease latency period in infected individuals. This is presumably the result of overexpressed SDF-1 down-modulating CXCR4 or competing for CXCR4 binding sites with X4 HIV-1. It is not known if other coreceptors play significant roles in AIDS. Due to high levels of CCR8 in thymus cells it has been proposed that CCR8 may play a role in infection of this key organ of the immune system. It was recently found that APJ was widely used by primary isolates from blood and lung, but the same report found that, while APJ and other secondary coreceptors functioned with many Envs in cell-cell fusion assays, they generally did not function in infection assays (253). This discrepancy may be due to the relative sensitivities of the assays or to postbinding effects of coreceptors that are essential for infection but not for membrane fusion. Evidence that CCR3 may be an important coreceptor in the brain and other organs is provided by reports that anti-CXCR3 and eotaxin, a ligand of CXCR3, block infection of microgial cells and monocyte-derived dendritic cells (133, 235). However, a more recent report finds that 7B11, an antibody to CCR3 and 12G5 an antibody to CXCR4, do not appreciably inhibit infection of microglial cells by three primary brain

isolates from dementia patients, while antibody to CCR5, 2D7, dramatically inhibited infection (5).

CXCR4 in Macrophages

As mentioned the suggested presence of nonfunctional isoforms of CXCR4, either due to posttranslational modifications or splice variations, could explain why X4 HIV-1 often fail to fuse/infect macrophages despite the presence of cell surface CD4 and CXCR4. Another possibility is that the tight association of limited amounts of CD4 with CCR5 (90) limits the availability of CD4 for interaction with CXCR4. However, the situation may be more complicated. Recent evidence shows that while cell culture adapted X4 HIV-1 cannot infect macrophages, a number of primary X4 HIV-1 isolates can (292). It has been suggested that this may be due to Env-specific signaling events, perhaps through CD4, that allows CD4 to be freed from CCR5 so that CXCR4 can participate in a fusion complex with CD4 and Env (Tzanko Stanchev— personal communication). This argument is largely refuted by work with CCR5-neg macrophages; macrophages from ˜32/˜32 CCR5 individuals could not be infected with cell culture adapted X4 HIV_{NL43} despite the absence of CD4-CCR5 complexes. However, other Env-mediated signaling events may also help to explain why SIV_{mac239} cannot infect macaque macrophages, yet they can infect cell lines transfected to express CD4 and CCR5 (191). And Env-mediated signaling may be responsible for the finding that HIV-1 luciferace virus pseudotyped with Dbl Env could not infect macrophages

despite being able to infect two cell lines expressing CD4 and either CCR5 or CXCR4 (64).

There are many other possible explanations for the reduced coreceptor function of CXCR4 in macrophages. One possibility is that there is a molecule other than CCR5, such as the closely related CCR2b, that blocks CXCR4 interaction with CD4. Another possibility is that CXCR4 is expressed differently in macrophages than in other cells. As discussed below it may be differentially spliced or posttranslationally modified. It has been reported that macrophage CXCR4 appears to be 90 kD by western analysis, about the size of dimeric CXCR4 seen in other cell types, while monocyte CXCR4 appears somewhat smaller (166). The authors present CD4 coimmunoprecipitation data suggesting that macrophage CXCR4 does not bind CD4 as strongly as monocyte CXCR4. It is unfortunate that the authors did not use several CXCR4 monoclonal antibodies or N-terminal protein sequencing to eliminate the possibility that they were looking at cross-reactive proteins, rather than CXCR4 isoforms.

Postbinding Functions of Coreceptors

Inhibition of HIV-1 fusion or entry by coreceptor-specific ligands has been a valuable tool in identification of coreceptors. However, a pitfall of this strategy could be that signaling events resulting from these ligands might affect host cell permissiveness for fusion/infection. Ligand binding to coreceptor can induce calcium mobilization and tyrosine phosphorylations that are required for chemotaxis (81, 120, 280), and these signaling events might bias experiments. RANTES is able to activate T-cells (21),

affecting chemokine release and chemokine receptor expression, as well as HIV-1 replication. However, this seemed to be an unwarranted criticism as initial studies appeared to indicate that signaling through chemokine receptors did not affect fusion or infection. Coreceptor mutations which prevent signaling, such as C-terminal deletions and DRY sequence mutations had no affect on fusion/infection, nor did pertusis toxin, which ADP-ribosylates the a subunit of G_I-proteins, thus inhibiting signal transduction (11, 15, 95, 111, 126, 178). More recent evidence, however indicates that signaling caused by chemokines can affect fusion/infection by other ways than competition with HIV-1 for coreceptor binding sites or by coreceptor down-modulation. An example is the controversial finding that MDC, a ligand of CCR4 (which is not a coreceptor), can prevent HIV-1 replication in PBMCs. This inhibition may have resulted from signaling events. Alternatively it could have resulted from receptor mediated endocytosis of CD4 or CCR5; it s conceivable that CCR4 might closely associate with CD4 or heterodimerize with CCR5 following MDC binding.

Another indication that coreceptors have postbinding effects comes from data presented here and elsewhere that indicates that some CXCR4 mutants and some minor coreceptors function well in cell-cell fusion assays but poorly in HIV-1 and SIV infection assays (58, 253). So, while these coreceptors support membrane fusion they might not support subsequent CXCR4 or CCR5 activities. This very point may explain findings by Chackerian et al. (59). They found that macaque sMAGI cells expressing human CD4 and simian coreceptor (CCR5 or CXCR4) could be infected by SIV isolates, but these cells were blocked for HIV-1 replication at either pre- or post-reverse transcriptase steps,

depending on the HIV-1 isolate. The block could be relieved by the use of human coreceptor. The block could also be relieved by replacing HIV-1 with a SHIV containing HIV-1 Env with SIV core, indicating that non-Env portions of HIV-1 in combination with coreceptor can participate in postentry parts of the lifecycle of at least some HIV-1 isolates.

While blocking of fusion between virus and host cell membranes has been widely attributed to chemokines, there are findings that in certain cases chemokines can enhance HIV-1 infections (192, 246). As mentioned Kinter et al. found CC chemokine signaling resulted in increased association of CD4 and CXCR4 as well as higher infection rates by X4 HIV-1 isolates (154). A different study showed that high RANTES concentrations could enhance infection of macrophages with R5 HIV-1, perhaps by acting as a bridge to cell surface heparan sulfate (192, 246). Signaling events induced by HIV-1 Env may also be needed for entry. This was suggested by the finding that cytochalasin D, an inhibitor of actin filament polymerization, prevented entry and infection of PBMCs with X4 HIV-1 (145). Pretreatment of the PBMCs with cytochalasin D prevented cocapping of CXCR4 and HIV-1 Env and subsequent pseudopod formation. Similar findings with both R5 and X4 isolates were made with macrophages and various cell lines by Stanchev et al. (258).

A recent report by Alfano et al. suggests coreceptor signaling events may be important for entry in T-cells. Despite a number of reports that show pertussis toxin inhibits chemokine signaling but not HIV entry and infection, a recent study showed pertussis toxin did inhibit infection of primary T-cells, though it did not inhibit infection

of the T-cell line PM1 (7). Remarkably, they found that pertussis toxin or even just its nontoxic B (binding) oligomer, prevented entry of R5 strains, ADA and 92US660, but not X4 strains, LAI and 92UG21, while it prevented replication of both R5 and X4 strains in previously infected PBMCs. As G protein signaling was not impaired by the B oligomer, the authors conclude that G proteins did not mediate the inhibitory signaling event. However, this does not mean that the inhibitory signal was not through coreceptors. There is a finding that CCR5 can mediate signaling that is not dependent on G proteins (21). This is supported by unpublished data showing that cell-cell fusion was drastically inhibited by exposure of T cell line targets with tyrosine kinase inhibitors but not with pertussis toxin.

The nature of signaling by HIV-1 Env through coreceptor was investigated by a number of groups. Calcium flux induced by interaction of HIV-1 R5 gp120 with cell surface CCR5 has been reported by two groups (81, 280). Davis et al. found that protein tyrosine kinase Pyk2 was phosphorylated in CD4-pos/CXCR4-pos HL60 cells and CD4-pos/CCR5-pos DU6 cells following incubation with appropriate chemokines or with 293T cells transfected to express X4 and R5 HIV-1 Env, respectively (81). Antibody to coreceptor prevented Pyk2 phosphorylation.

Posttranslational modifications of coreceptors

Again, the use of antibodies or ligands is sometimes problematic for identification of important coreceptors for several reasons: first, some antibodies and natural ligands of known coreceptors do not block infection - they may bind to parts unnecessary for Env or

CD4 binding; second, many host cells express more than one coreceptor; and third, some chemokines can bind to multiple receptors, for example RANTES can bind to CCR3 as well as CCR5. Another difficulty that arises in the identification and evaluation of coreceptors is that there may be coreceptor isoforms that behave in various ways. Thus, for example, studies of coreceptor expression following transfection of quail fibroblasts or dog thymocytes, which are used in a large number of studies, or even the human astoglioma cells used in our studies, may be convenient, but may not accurately reflect how coreceptors are expressed in monocytic or lymphocytic host cells. GPCRs have been found to be differentially spliced or posttranslational modified (N and O—linked glycosylated, palmitoylated, phosphorylated, sulfated, and disulfide bonded) (261). Additionally, some are believed to form dimers and even heterodimers and to strongly associate with other surface molecules, such as CD4. Their associations with G-proteins and various signaling pathways also depend on cell type. Differentially spliced CXCR4 has been identified in HUVEC cells (129), and it has been suggested that a differentially spliced CXCR4 in macrophages does not function with X4 Envs, but does with R5X4 Envs.

Roles of N-linked glycosylations

The N11A mutation was second only to D187 among point mutations that allowed CXCR4 to act as a coreceptor for R5 HIV-1 Envs. It functioned well with six of six clade B R5 HIV-1 Envs in our cell-cell fusion assay. However, it did not function well with most non-B clade Envs tested. Only one mixed clade F/B Env (All of gp120 in

clade F and only about 200 base pairs of the 3 end of gp41 is clade B) and one clade A Env out of nine non-clade B R5 HIV-1 Envs was able to use N11A as a coreceptor. A T13A mutant was used to confirm that the expanded activity of CXCR4 achieved by the N11A mutant was due to the loss of glycosylation. The threonine two amino acids downstream from N11 is part of the consensus N-glycosylation sequence. Indeed, the T13A mutant had a similar phenotype as the N11A mutant.

The role of a putative glycosylation at N176 does not appear as significant as that at N11. Western analysis shows that if there is a glycosylation, its removal does not significantly shift the apparent molecular weight of CXCR4. Pseudovirus infection assay results with N176A show that it functions no better than wild-type CXCR4. While some activity was seen with N176A in cell-cell fusion assays with R5 HIV-1 Envs, these results were not always significantly higher than those with wild-type CXCR4. However, combining N11A with N176A resulted in greater cell-cell fusion and pseudovirus infection than was seen with N11A by itself.

Evidence presented here shows that N-linked glycosylations may have dramatic effects on CXCR4 s coreceptor activity, similar to those reported above for the D187A mutation of CXCR4. Both putative glycosylation sites of CXCR4, N11 and N176, also may play a role in chemokine binding. Mutation of either site caused a decrease by over 50% in the ability of CXCR4 to bind [^{125}I]SDF-1a, while the double mutant had less than 20% activity (296). The same study showed that changes in molecular weight as a result of mutation of glycosylation sites appear minimal by SDS-PAGE. This is in sharp contrast to our work, which showed that the N11A mutant appeared to result in a loss of 5

— 7 kD, and the work of Berson et al that showed a similar loss of size following endoglycosidase F digestion (33). This discrepancy is likely due to the unfortunate fact that the former study examined CXCR4 expression in insect cells. It is likely that the authors of that study chose insect cells due to the high expression levels achievable with baculovirus vectors in insect cells. Technical difficulties in observing coreceptors on SDS-PAG make baculovirus expression tempting. However, it is well known that glycosylation patterns in insect cells often vary dramatically compared to mammalian cell glycosylation patterns.

The large size of the sugar moieties of CXCR4, rather than unique structures or charges are likely the cause of the dramatic effects of glycosylation on CXCR4 coreceptor activity. Glycosylations do not appear to contain major antigenic determinants; all eight monoclonal antibodies against human CXCR4 that we tested reacted strongly by FACS analysis with deglyosylated CXCR4. However, if glycosylations on mouse CXCR4 are similar to those on human CXCR4, the sugars might not have been recognized as foreign.

Roles of disulfide bonds

There is one cysteine present on each of the four extracellular regions of CXCR4. The cysteines on ecl-1 and ecl-2 are conserved among most GPCRs, while those on the N-terminus and ecl-3 are conserved among all chemokine receptors. It has been suggested that chemokine receptors have a disulfide bond linking ecl-1 and ecl-2 and another linking the N-terminus and ecl-3. This fits with a view of the receptor as a barrel

structure with all extracellular regions in close proximity (88). This model is consistent with cryoelectron microscopic analysis of crystals of the seven transmembrane proteins, bacteriorhodopsin and rhodopsin (80, 127, 245, 270).

Our analysis of cysteine mutants supports this model of the three-dimensional structure of CXCR4. Cell-cell fusion data for mutations of cysteines from ecl-1, C109A, and ecl-2, C186A, indicate that these cysteines are required for coreceptor activity. FACS analysis reveal that these mutations prevent expression of CXCR4 on cell surfaces, probably due to misfolding. Neither conformation-dependent MAb directed at ecl-2 or a nonconformation-dependent antibody raised against an N-terminus peptide bound to cells expressing these mutants. However, a small but significant amount of surface expression was detected by both antibodies when both cysteines were mutated (C109A/C186A). A likely explanation is that mutation of one cysteine permits its disulfide bonding partner to form inappropriate disulfide bonds, resulting in misfolding of the molecule. Mutation of both cysteines prevents this form happening, but it alters the three-dimensional structure of the molecule, reducing its ability to get to the cell surface and function as a coreceptor. This supports the hypothesis that C109 and C186 are normally disulfide bonded.

We also presented evidence that the N-terminus cysteine of CXCR4 forms a disulfide bond with the cysteine in ecl-3. Mutation of these two cysteines, C28A and C274A, did not alter surface expression, as determined by FACS analysis with 4G10, a conformation-independent MAb. However, cell surface binding by 12G5, a conformation-dependent MAb to CXCR4, was reduced by over 2/3. A combination mutant, C28A/C274A, was only reduced by 50% for 12G5 binding. This is reminiscent of our findings with the ecl-

1 and ecl-2 cysteines; removal of one cysteine in a disulfide bonded pair causes more drastic misfolding than removal of both cysteines. Cell-cell fusion data also supports the hypothesis that C28A and C274A form a disulfide bond with each other. While C28A and C274A have reduced coreceptor activity compared to wild-type CXCR4, a double mutant had more activity than either single mutant. If the cysteines were not bound together then it would be expected that the double mutant would not have more activity than either of the single mutants.

Alanine scanning mutagenic analysis of the cysteines of CCR5 also indicate that the ecl-1 cysteine and the ecl-2 cysteine are required for coreceptor activity, and that mutation of the N-terminal cysteine and the ecl-3 cysteine result in partial loss of activity (122, 222). However surface expression of the ecl-1 and ecl-2 mutants of CCR5 is about 50% reduced compared to wild-type, while mutation of the same residues of CXCR4 prevents surface expression. It should be noted that these discrepancies may be due to differences in cell lines. However, the CCR5 studies and our CXCR4 both used human cells isolated from an astroglioma, though our cell line, U373, is spindle-shaped, consistent with astrocytoma cells, while the other cell line, U87, appear to be glioblastoma cells (1). Indeed, cell line differences may explain why an additional analysis of the disulfide bonds of CCR5 finds that mutation of any or even all of the cysteines of CCR5 does not completely knock out coreceptor function (39). The authors of this study used the human embryonic kidney 293T cell line, which constitutively express CXCR4. The possibility that CXCR4 might interact with CCR5, CD4, or Env makes this a non-optimal cell line to conduct studies of CCR5 function. In agreement

with other studies they did find that mutation of the ecl-1 and ecl-2 cysteines decreases coreceptor activity more than mutation of the other cysteines. Also in agreement with our findings with CXCR4, they found that double mutations of cysteines believed to be involved in disulfide bond formation may have may have resulted in more expression and activity than single cysteine mutations. In addition they found that all four cysteines are needed to support MIP-1§ binding, suggesting more stringent requirements for chemokine binding than coreceptor function. Interestingly, they also showed that culturing cells expressing cysteine mutants of CCR5 at 32^0C, as compared to 37^0C, resulted in higher surface expression and coreceptor activity.

Other posttranslational modifications

Another group used sodium chlorate, a sulfation inhibitor, to show [^{125}I]gp120 could not bind well to cells expressing unsulfated CCR5 are not able to bind R5 HIV-1 gp120, opening up the possibility that a cells ability to sulfate tyrosines and glycosylate asparagines may determine whether it is a suitable host for HIV-1 (113). Several groups have shown that various monoclonal antibodies to CCR5 and CXCR4 do not bind to certain cell types that are known to express the appropriate receptor. For example various MAbs to CXCR4 and CCR5 do not bind PBMCs. 12G5, a CXCR4 MAb inhibited infection of CXCR4$^+$ CD4$^+$ rhabdosarcoma cell line, but did not inhibit infection of T-cell lines (186). A CCR5 MAb known as 2D7 bound to a higher percentage of CCR5-pos cells than other CCR5 MAbs, suggesting that multiple isoforms

may be present on an individual cell type. Indeed different CCR5 bands were found by SDS-PAGE for CCR5 expressed by a single cell type, reflecting different stages of protein maturation or different isoforms.

Identification of coreceptor regions required for chemokine binding and signaling

As HIV has complex interactions with cell receptors, it is not surprising that chemokines also have multistep interactions with coreceptors. Mutagenesis has revealed that signaling by chemokines is generally mediated by certain chemokine residues, while binding is mediated by others (207). Chemokine receptors also have binding and activation domains. In the case of CCR5 chemokine (MIP-1[alpha], MIP-1§, and RANTES) binding is dependent on CCR5 s ecl-2, while signaling is dependent on the first 10 — 20 amino acids of the N-terminus and the last 10 — 15 amino acids of the C-terminus (288). The reverse appears true for CXCR4. Using point mutations and chimeras between CXCR4 and CCR2 it was found that the N-terminus of CXCR4 is important for SDF-1 binding, while ecl-2 is more important for signaling events (96). Surprisingly, deletion of the C-terminus did not prevent signaling. Results from another study indicate that all four extracellular domains of CXCR4 are important for chemokine binding (296). Polyclonal antibodies raised against peptides corresponding to any of the four extracellular domains of CXCR4 were able to prevent [^{125}I] SDF-1[alpha] binding. Each antibody had approximately the same inhibitory capability, except the one directed to ecl-1 was somewhat weaker.

Identification of coreceptor regions required for fusion and entry

Much as the ability to bind chemokine receptor is not sufficient for signaling events to take place, the ability of coreceptor to bind gp120 does not always correlate with the ability to support viral entry (22). Surprisingly, several CXCR4 mutants that do not bind soluble [^{125}I]gp120 in the presence of CD4 are still able to support X4 HIV-1 Env-mediated fusion (96). One region of coreceptor might be important for gp120 binding, while another region might be needed to induce the conformational changes in Env that are needed for membrane fusion events to occur. This idea is supported by findings of B. Lee et al. (169). Using point mutations of CCR5 and chimeric molecules containing various portions of CCR2b, CCR5, CCR1, CXCR2, and CXCR4 they were able to map the antigenic determinants of 18 CCR5 MAbs. They found that antibodies directed to the N-terminus of CCR5 blocked [^{125}I]gp120 binding to CCR5 better than antibodies to ecl-2, but antibodies to ecl-2 blocked HIV-1 infection (and chemokine binding) better than N-terminus antibodies. A possible explanation for this is that ecl-2 might be important for CD4 binding. Indeed, 2D7, a MAb to ecl-2, does prevent association of CCR5 with CD4 (290) and blocks HIV-1 infection. Several studies have implicated N-terminal residues as those most important for binding of gp120. Mutation of charged amino acids (39) and uncharged amino acids (113, 222) in the N-terminus of CCR5 prevent gp120 binding.

Interestingly, antibodies to the N-terminus of CCR5 did not work well in Western blots, suggesting the presence of complex secondary structure in the N-terminus of CCR5. This may help to explain the apparent discrepancy between findings that deletion

of a large portion (amino acids 4 — 36) of the N-terminus of CXCR4 does not completely eliminate coreceptor function (44), yet certain N-terminus point mutations, such as E15A and E32A, reduce activity by over 50% with multiple HIV-1 Envs (57). Disruption of the secondary structure may be more disruptive than elimination of the structure.

Data from many groups, including our own, show that multiple extracellular regions of CXCR4 and CCR5 are important for coreceptor activity. For CCR5 this is illustrated by work with chimeras between highly homologous but nonfunctional mouse CCR5 and functional human CCR5. Chimeras containing any of the first three extracellular domains of human CCR5 and three mouse extracellular domains had significant coreceptor activity with some but not all R5 HIV-1 Envs (233) and chimeras containing any three human domains with one mouse domain retained activity (19, 34, 212). An R5X4 HIV-1 Env, on the other hand, required at least three of four human extracellular domains, and they had to include ecl-2 and ecl-3 (34). Experiments with chimeras between human CCR5 and the highly homologous CCR2b also proved informative if somewhat contradictory. Any one domain of CCR2b could knockout CCR5 coreceptor function, but when the extracellular loops of CCR2b were combined with N-terminus of human CCR5 a functional chimera was formed (19, 237). Conclusions drawn from chimera studies are suspect because of the possibility that portions of the nonfunctional molecule may have unforeseen effects on portions of the functional molecule, but these studies indicate that at least three of the extracellular domains of CCR5 are important for coreceptor function, and there may be some functional redundancy within the molecule.

That multiple domains are important is not surprising given the structure of chemokine receptors. Their seven transmembrane alpha helices are believed to form a barrel shaped structures out of which the extracellular domains are brought together by disulfide bonds between ecl-1 and ecl-2 and between the N-terminus and ecl-3 (91). Data from our group and others supports this notion of the roles of extracellular cysteines in HIV-1 coreceptors (39, 58, 122, 222).

Alanine scanning mutagenesis confirmed that N-terminus amino acids are required for CCR5 coreceptor function. Mutation of acidic N-terminus amino acids at positions 2, 11, and 18 had significantly reduced activity (101) as did mutation of three of four N-terminus tyrosines, while mutation of the fourth tyrosine resulted in reduced activity for two R5X4 Envs, but not for two R5 Envs (112, 222). A semiquantitative analysis of alanine substitutions of charged residues in the extracellular regions of CCR5 found that D11 is the only crucial charged extracellular amino acid for CCR5 coreceptor activity (94). Genoud et al. mutated polar and nonpolar residues of CCR5 ecls and found reduced coreceptor activity with mutations in all three loops (122), again showing that amino acids in all extracellular regions play roles in coreceptor function.

For two reasons CCR5 has been the focus of more intense scrutiny than CXCR4: first, infectious strains generally require CCR5 for transmission, and second, due to the fact that CCR5 is not an essential protein, it is a good target for antiviral therapy. However, CXCR4 usage is often associated with progression to AIDS in HIV-1 infected individuals and, though transgenic knockout mice have shown that CXCR4 is essential for the developing mouse embryo, it is not known if CXCR4 is essential in adult humans.

Additionally, there are known cases of infections occurring in homozygous CCR5˜32 individuals, presumable by X4 strains of HIV-1 (36, 199, 266). Indeed, the discovery of several classes of CXCR4 antagonists (see Introduction) make CXCR4 a potential target for AIDS therapy.

We performed alanine scanning mutagenesis of extracellular charged residues of CXCR4 and found reduced coreceptor activity with glutamic acid mutations at positions 15 and 32 in the N-terminus, an aspartic acid mutation at position 97 in ecl-1, and with an arginine mutation at position 188 in ecl-2, again demonstrating that multiple domains are important for activity. Alanine scanning mutagenesis of the charged residues of ecl-2 by Brelot et al. confirmed the role of R188 and also showed that R183, Y184, and D193 are important for one or more HIV-1 Envs. Wang et al. also conducted alanine scanning mutagenesis of the charged residues of CXCR4 ecl-2 but found no mutations that drastically reduced coreceptor function (276). While one group used chimeras made between CXCR4 and nonfunctional CXCR2 to show that the N-terminus and ecl-1 are important for coreceptor activity (170), another group made similar chimeras and found that grafting ecl-2 from CXCR4 onto CXCR2 was all that was required by some strains for some degree of cell-cell fusion (178). However, the same study showed other domains were also important. For example grafting the N-terminus or ecl-3 of CXCR2 onto CXCR4 resulted in a significant loss of coreceptor function for all tested HIV-1 Envs. Experiments with chimeras between human and rat CXCR4 also showed that ecl-2 is important for an X4 and an R5X4 strain of HIV-1 (43). Rat CXCR4, however, might not have been a neutral chimera partner, as it will support infection by LAI but won t

support infection by NDK, both of which are X4 HIV-1 strains (44). The importance of ecl-1 and/or ecl-2 is also suggested by the finding that 12G5, an antibody mapped to both loops (178), blocks infection by some strains of HIV-1 (262).

Lu et al. were able to construct a univeral coreceptor by grafting the N-terminus of CCR5 onto CXCR4 (178). Though this chimera functioned with R5, X4 and R5X4 HIV-1 Envs it had significantly reduced activity as compared to wild-type corecptors for all tested Envs in a cell-cell fusion assay. The authors speculated that the N-terminus of CCR5 was important for coreceptor function, while the ecls of CXCR4 were important. However, in light of our more recent data showing that N11, C28, and R30 from the N-terminus of CXCR4 all interfere with R5 HIV-1 Envs, it is likely that results they saw were in part due to the removal of those residues. In conclusion, as with CCR5 it appears that multiple extracellular domains affect coreceptor function of CXCR4. It remains to be seen if different domains are responsible for gp120 binding, CD4 binding, and induction of conformational changes involved in fusion of host and viral membranes.

Chapter 7

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