# TWO MECHANISMS OF HOST CONTROL OF HUMAN IMMUNODEFICIENCY VIRUS TYPE 1 (HIV-1) INFECTION: ANALYSES OF HIV-1'S VIRAL INFECTIVITY FACTOR AND BIOCHEMICAL CHARACTERIZATION OF INHIBITION OF CHEMOKINE RECEPTOR SIGNALING BY VIRAL ENVELOPE gp120

by

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**A DISSERTATION**

Presented to the Department of Biochemistry and Molecular Biology
and the Oregon Health Sciences University
School of Medicine
in partial fulfillment of
the requirements for the degree of

Doctor of Philosophy

April 1999

School of Medicine
Oregon Health Sciences University

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CERTIFICATE OF APPROVAL

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# ACKNOWLEDGMENTS

I am indebted to Dr. David Kabat for providing the opportunity to work in his laboratory and for introducing me to the world of viruses. His creativity and knowledge never cease to amaze me. I thank him for his patience, guidance, and critical reading of this thesis. The past four years have been an amazing learning experience for me and I look forward to many more years of collaborating with him.

It is rare to work in a laboratory and to be able to call each member of it a friend. I was lucky enough to do that. My gratitude is extended to my friends and colleagues; Sue Kozak, Emily Platt, Chetankumar Tailor, Ali Nouri, Shawn Kuhmann, Mariana Marin, and Ethel Polonoff for providing a wonderful work environment. I thank all of them for their invaluable friendship, advice, and support throughout my graduate work.

I was very fortunate to collaborate with Dr. Michael Kavanaugh; I am grateful to him for teaching me how to use a single cell to characterize cell surface receptors. I would also like to thank the past and the present members of his laboratory: Jacques Wadiche, Alex Stein, Shirin Arastu, Jeff Hubbard, and Anastassios Tzingounis. They expanded my vocabulary, especially when oocytes were not expressing.

I owe all of this to my parents, Abdulmohammad Madani and Zahra

Ravanshid. Without their sacrifices, in particular in the past 20 years, I would not be here. They have taught me how to love, how to persevere, and how to have the courage to let go. I would like to thank my husband, Kirk Tanner, for his love and understanding, for his enthusiasm for science, and for always standing by me and teaching me never to become discouraged.

This thesis is dedicated to the loving memory of my grandfather, Ghasem Ali Madani, who tried to teach me to be a strong person. I am still learning.

## Abstract

Infection by human immunodeficiency virus type 1 (HIV-1) causes the depletion of $CD4^+$ T-cells and ultimately the demise of the immune system. The molecular mechanisms underlying these processes are unclear. HIV-1 infection is dependent on binding of the envelope glycoprotein 120 (gp120) to the cell surface receptors, CD4 and a chemokine receptor CXCR4, or CCR5. An auxiliary gene of HIV-1, the viral infectivity factor (Vif), is also necessary for production of infectious virions by some cell types. The studies that are described in this dissertation have focused on two mechanisms of viral infectivity; analyses of Vif function and characterization of HIV-1 coreceptors.

The *vif* gene of HIV-1 is neccessary for production of infectious virions in nonpermissive cells (e.g., macrophages and T-lymphocytes), but is not needed in permissive cell types (e.g., HeLa, COS, CEM). We investigated the viral and cellular specificities of Vif. We showed that despite their lack of infectivity, *vif*-deleted virions produced in nonpermissive cells had normal amounts of Gag proteins and only slight decreases in quantities of envelope glycoproteins. This lack of infectivity was not caused by a deficiency in envelope function since increasing the level of CD4 on the surface of target cells and pseudotyping with a murine leukemia virus (MLV) envelope did not rescue the *vif* defect. We also showed that Vif's effects are specific to HIV-1 since Vif did not enhance the infectivity of xenotropic BV2 virus.

Based on the cell type requirement for Vif, many investigators had hypothesized that either permissive cells contain a factor that functions like Vif, or that nonpermissive cells contain a viral inhibitor. We addressed this

issue by developing a complementation assay. The results of these studies showed that nonpermissive cells, the natural targets of HIV-1 infection, contain an endogenous inhibitor of HIV-1 replication that is counteracted by the viral Vif protein.

The signal transduction properties of HIV-1 coreceptors, CXCR4 and CCR5 were investigated by electrophysiological recordings. *Xenopus laevis* oocytes were coinjected with cRNAs for CD4, CXCR4 or CCR5, and a G protein-coupled inward rectifying $K^+$ channel (Kir 3.1). Kir 3.1-dependent currents were induced in response to chemokines, SDF-1$\alpha$ or MIP-1$\alpha$, in oocytes coexpressing Kir 3.1 with CXCR4 or CCR5, respectively. These currents were blocked by pertussis toxin, suggesting the involvement of inhibitory guanine nucleotide-binding proteins. In contrast to chemokines, perfusion with monomeric or oligomeric gp120 did not enhance currents in oocytes coexpressing CD4, chemokine receptor, and Kir 3.1. However, adsorption of gp120 resulted in a specific inhibition of chemokine receptor signaling responses to chemokines. This inhibition was direct, CD4-dependent, and tropism specific. These antagonisms were partially overcome at higher concentrations of chemokines suggesting a competitive mechanism. The inhibitory effects of gp120s on chemokine receptor signaling may be critical to HIV-1 pathogenesis.

# Chapter 1

## Introduction

### A. General Characteristics of Retroviruses

Retroviruses are RNA-containing enveloped viruses that are responsible for diverse disorders in animals and humans. These disorders range from severe immunodeficiencies, cancers, and wasting diseases, to benign tumors. Retroviruses contain single stranded RNA as their genome which is between 7-10 Kb in size and is surrounded by core capsid proteins (Figure 1 and 2). The core also contains reverse transcriptase which is a distinct feature of the retroviruses and distinguishes them from all other viruses. The core is enclosed by a lipid bilayer which the virus acquires from the host cell membrane. The lipid bilayer consists of glycoproteins that are critical for binding of the virus to specific cell surface molecules known as receptors. Based on their genetic and structural organization, retroviruses are divided into seven groups. Five of these groups are oncogenic viruses, and the other two are lentiviruses and spumaviruses.

Our understanding of retroviruses has come a long way since the first retrovirus, Rous sarcoma virus, was discovered in 1911 (103). However, we are still working toward understanding the molecular mechanisms of viral entry, reverse transcription, integration, replication and finally viral spread. Full understanding of these pathways will allow us to determine the ways in which retroviral infection and pathogenesis could be inhibited.

## B. Human Immunodeficiency Virus

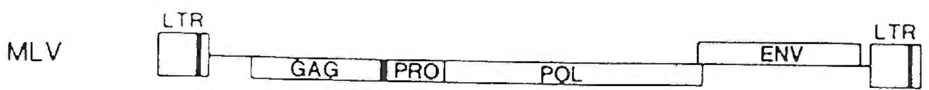
### I. Discovery

The disease we now call Acquired Immunodeficiency Syndrome (AIDS) was first recognized as an outbreak of opportunistic infections among homosexual men in 1981. In 1983 Luc Montagnier's group in France isolated the pathogenic agent that causes AIDS from the lymphocytes of AIDS patients (6). This agent became the third human retrovirus identified and is now known as the human immunodeficiency virus type 1 (HIV-1). The hallmark of HIV-1 infection is the rapid $CD4^+$ T cell depletion, which ultimately results in decreasing the ability of the immune system to fight any infection or disease. HIV-1 belongs to an extensive family of primate lentiviruses that also includes a second pathogen named HIV-2. While HIV-1 is the most widespread of the two, HIV-2 is the most prevalent in West Africa. Both viruses belong to a larger family of human and non-human primate lentiviruses that also includes simian immunodeficiency virus (SIV). Since the identification of HIV-1 in the mid 1980s the virus has continued to spread in both the developed as well as the third world countries. HIV-1 has now infected 30.6 million people globally, and strikes 16,000 people daily.

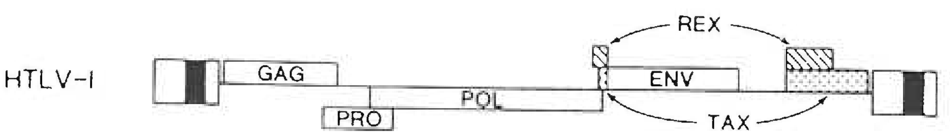
In 1995 two groups (59, 122) reported the effects of new inhibitors of HIV-1 protease and reverse transcriptase on plasma viremia in HIV-1 infected individuals. Even though viral loads in most patients who receive these drugs are dramatically decreased, there is a concern because the viral concentration in blood increases rapidly if the therapy is withdrawn. Identification of these inhibitors have advanced AIDS research dramatically. However, how the virus persists in the individual despite the presence of a vigorous immune

**Figure 1.1: The genomic organization of human retroviruses.** Schematic representation of proviral genome of known human retroviruses, human immunodeficiency virus type 1 (HIV-1), human T-cell leukemia virus type 1 (HTLV-1), and human foamy virus (HFV), compared to the genome of murine leukemia virus (MLV). All retroviruses have the three genes *gag*, *pol*, and *env*, whereas more complex retroviruses like HIV-1 and HTLV-1, contain auxiliary genes that are needed for efficient retroviral replication (figure modified from ref 119).

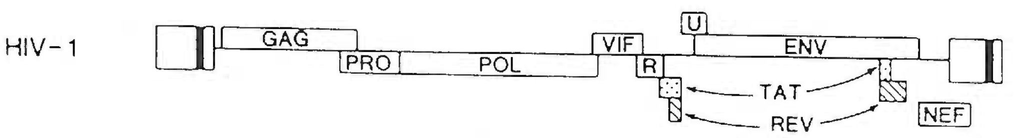
MLV



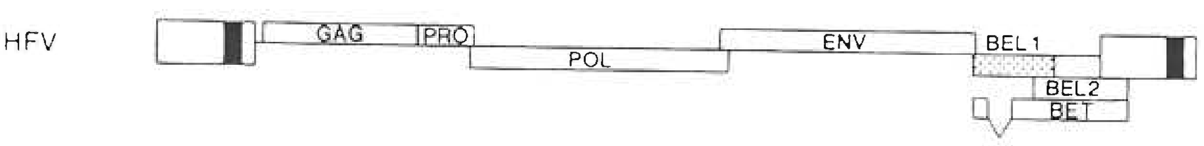

HTLV-I




HIV-1




HFV



response, as well as antiviral therapy, remains unclear. Presumably, there must be hidden reservoirs of virus in a dormant or semidormant state (44). In addition, one critical question that remains to be answered is how HIV-1 infection induces $CD4^+$ T cell depletion. In general, the lack of effectiveness of many viral inhibitors and the rapid emergence of drug-resistance in the viral population in patients has made it necessary to develop a more complete understanding of HIV-1's replication cycle and its mechanisms of infection.

The studies described here have shed light on two important aspects of HIV-1's life cycle; both concern the mechanisms of viral infectivity. The first deals with the function of a critical accessory gene product of HIV-1, Vif. The second concerns the important steps involved in viral entry, specifically how the virus uses its cellular receptors to enter the host cell. In order to focus on these two areas, the remainder of this introduction will be divided into two major parts. First, I will review the organization of HIV-1's genome and explain what is known about the gene products of this virus. Second, I will review the early steps of infection including virus binding to cell surface receptors and penetration through the plasma membrane. At the end of each section, I will summarize the goals of each project described in this thesis.

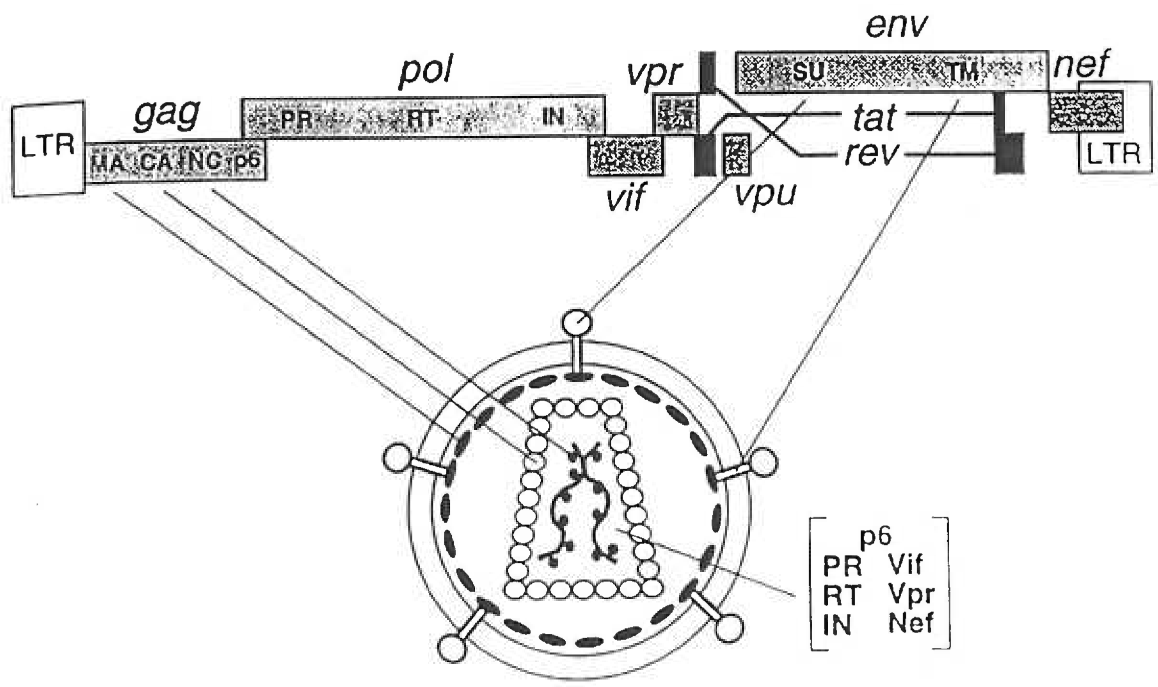
## 129. Genomic Organization

Primate lentiviruses have a closely related genetic organization which is an indication that they evolved from the same ancestral origins. Soon after HIV-1's genome was cloned, research determined that compared to other retroviruses, this genome exhibited an extensive genetic complexity (Figure 1).

The three genes, *gag*, *pol*, and *env* are common to all retroviruses (Figure 1). The four proteins that are produced from the *gag* gene; matrix (MA), capsid (CA), nucleocapsid (NC), and p6, are structural components that make up the core of the virus. The *env* gene encodes the envelope glycoprotein of the virus. The Env protein is processed by cellular enzymes to yield the surface glycoprotein gp120, and the transmembrane glycoprotein gp41; gp120 interacts with gp41 through noncovalent interactions. The "knobs" on the surface of HIV-1 are believed to be trimers of gp120-gp41. The enzymatic components of the virion are encoded by the *pol* gene and are protease (PR), reverse transcriptase (RT), and integrase (IN). In addition to *gag*, *pol*, and *env*, the HIV-1 genome contains six more open reading frames (Figure 1 and 2). The functions of these 6 genes were initially unclear; however, the fact that all 6 genes perform functions that are critical for HIV-1's efficient replication *in vivo* soon became evident. Two of these genes, *tat* and *rev*, are required for viral replication. The Tat protein is an RNA binding protein that serves as a potent transactivator of the long terminal repeat (LTR) (24). The Rev protein is produced by a fully spliced mRNA that is expressed shortly after infection of cells and serves to induce the efficient nuclear export of various incompletely spliced viral RNAs (83).

Three other auxiliary genes of HIV-1, *nef, vpr*, and *vpu* have unique functions that are critical to HIV-1's life cycle and hence its *in vivo* pathogenesis. Nef is a protein of 206 amino acids and functions in the down regulation of cell surface CD4 and major histocompatibility complex I (MHC I) proteins (84). The Vpr protein of HIV-1 is a late gene product that effects nuclear import and G2 cell cycle arrest (54, 99). HIV-1's *vpu* gene is

**Figure 1.2: Schematic representation of human immunodeficieny virus type 1 gene products in the viral particle.** The genome of HIV-1 consists of two copies of single stranded RNA that is between 9-10 kb in size. Reverse transcriptase, intergrase and nucleocapsid are associated with the RNA and the whole complex is surrounded by capsid proteins. The capsid proteins are surrounded by a lipid bilayer which contains the transmembrane envelope glycoprotein 41 (gp41) and the surface envelope glycoprotein 120 (gp120). Although the figure suggests that Vif, Vpr, and Nef occur in viral particles, recent evidence has implied that Vif is absent from highly purified HIV-1 virions (taken from ref 47a).



env
pol
vpr
nef
gag
LTR
PR
RT
IN
SU
TM
tat
rev
MA
CA
NC
p6
vif
vpu
LTR
p6
PR Vif
RT Vpr
IN Nef

unique to this virus. The $SIV_{CPZ}$ (CPZ, chimpanzee) also carries a closely related gene called *vpx*. The Vpu protein is an 81 amino acid integral membrane protein that is involved in the specific degradation of CD4 in the endoplasmic reticulum (124). Vpu has also been reported to enhance viral release (65), and serve as a cellular plasma membrane ion channel (74). In the following sections, I will describe the product of the final accessary gene of HIV-1, the viral infectivity factor (Vif).

## III. HIV-1 Vif

The Vif protein of HIV-1 has been classified an accessory gene (53, 125) since initial studies indicated that the product is dispensible for viral infection (45, 113, 116). In the last few years, however, several laboratories have reached the common conclusion that Vif is absolutely required for HIV-1 replication in T-lymphocytes and macrophages (1, 48, 121).

The *vif* gene of HIV-1 encodes a singly spliced mRNA. Vif is a *rev*-dependent late gene product that contains 192 amino acids and an apparent mass of 23 kD (113). The *vif* gene is highly conserved among all lentiviruses with the exception of equine infectious anemia virus (93). This conservation suggests that this gene is important in HIV-1's life cycle.

## IV. Function of Vif

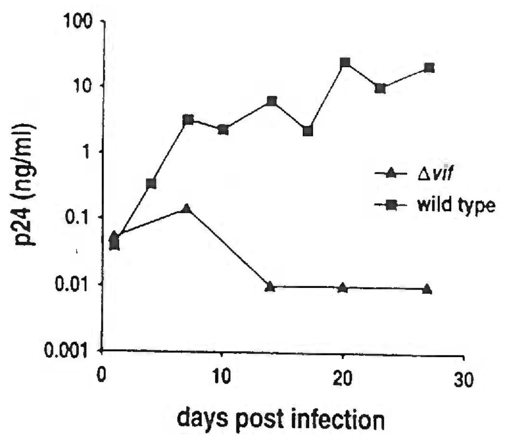
The Vif protein of HIV-1 significantly increases the infectivity of the virus (48, 109, 121). Several reports have shown that the requirement for Vif is strictly cell type dependent and is determined by the cells producing the virus rather than the cells that are the target of HIV-1 infection (48, 109, 121). The

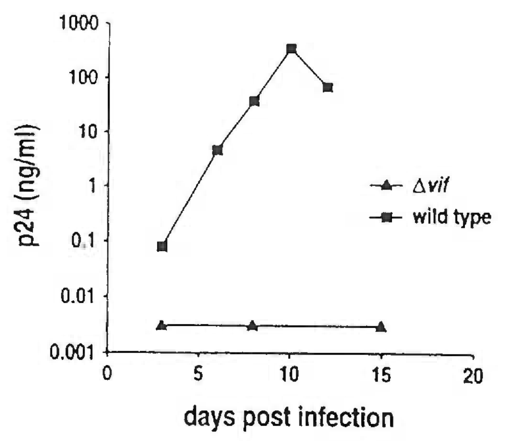
*vif*-deleted virions released from some cell types are attenuated and are 50-1000 fold less infectious compared to the wild-type virus (48, 109, 121). Because of this cell-type specificity, cells have been grouped into three different categories, nonpermissive (e.g., macrophages, peripheral blood lymphocytes, and H9 T-lymphocytes), semipermissive (e.g., CEM), and permissive (e.g., HeLa, Sup T1, COS) (Figure 3). This defect can be rescued by expressing *vif* in trans in the producer cells but not in the target cells (48, 109, 121). This cell type requirement suggests that either permissive cells express a factor that serves as a homologue of Vif, or that nonpermissive cells contain a viral inhibitor that is counteracted by Vif. In the absence of Vif this putative factor would inhibit viral assembly, or maturation, or both. As a consequence, the viral particles released from the cells would be noninfectious.

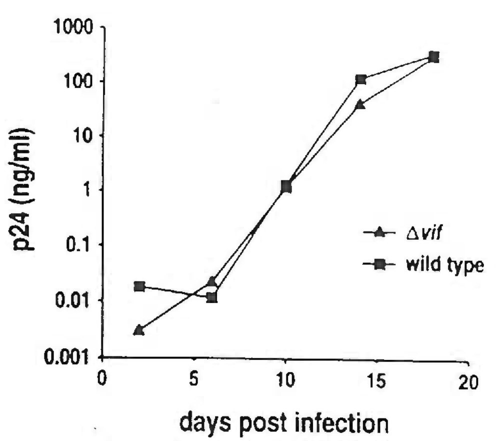
## V. The Effects of Vif on Viral Particles and Entry

To understand the mechanism of Vif function, early studies compared the morphologies and Gag and Env protein compositions of wild-type and *vif*-deleted HIV-1 particles released from nonpermissive cells. Several groups independently reported aberrant Gag and Env protein composition of *vif* mutant virions produced from nonpermissive cell lines (10, 105, 108). However, studies from other laboratories did not support these conclusions (12, 23, 121). Similarly, transmission electron microscopic studies initially suggested an abnormal morphology associated with the *vif*-deleted compared to the wild-type virions released from nonpermissive cell lines (10). However, a report by Ochsenbauer *et al.* (94) did not confirm this idea.

**Figure 1.3: The requirement for the *vif* gene of HIV-1 is cell type dependent.** Expression of *vif* is critical for HIV-1 replication (quantity of p24 Gag protein was used to determine HIV-1 replication ) in peripheral blood lymphocytes (A), and human T-leukemic H9 cells (B) but is not essential in human T-cell lines such as CEM-SS (C). (taken from ref 109).


A. PBL
100
10
1
0.1
0.01
0.001
p24 (ng/ml)
0
10
20
30
days post infection
Δvif
wild type


B. H9
1000
100
10
1
0.1
0.01
0.001
p24 (ng/ml)
0
5
10
15
20
days post infection
Δvif
wild type


C. CEM-SS
1000
100
10
1
0.1
0.01
0.001
p24 (ng/ml)
0
5
10
15
20
days post infection
Δvif
wild type

Association of Vif with the viral particle is also an area of controversy. While the results of sucrose density gradient studies have suggested that a small amount of the Vif protein may occur with the viral core (17, 63, 80) a recent report (32) using a more sensitive high velocity sedimentation technique has shown a virtual absence of Vif in the virus.

The fact that noninfectious *vif*-deleted virions are released from nonpermissive cells is intriguing and implies that these virions may be damaged or defective. To investigate this issue, von Schwedler *et al.* (121) produced wild-type and *vif*-deleted HIV-1 particles pseudotyped with envelope glycoproteins derived from amphotropic murine leukemia virus (MLV). Bypassing the regular entry mode of HIV-1 by using the MLV glycoproteins did not rescue the *vif* defect, suggesting that the abnormality in these virions affects a post-entry step of infection. Further studies showed that the *vif*-deleted virions derived from nonpermissive cells had a significant defect in a reverse transcription step of the viral life cycle (121). The same report showed that the amounts of reverse transcriptase, integrase, or protease did not differ in these *vif*-deleted virions. The quantities of viral RNA were similiar in the wild-type and *vif*-deleted HIV-1 released from nonpermissive cells. Since the presence of partial reverse transcripts has been detected in HIV-1 particles (120), the quantities of viral DNA in the wild-type and *vif*-deleted particles were compared but significant differences were not observed. These measurements indicated that reverse transcription can initiate normally in the *vif*-deleted particles. These studies suggest that the Vif protein of HIV-1 is needed to allow the efficient completion of reverse transcription and that the defect might lie at a post entry step of the viral life cycle.

In addition to the above study, other reports have also shown that infection by *vif*-deleted virions from nonpermissive cells results in undetectable (10, 23), or reduced levels (114) of reverse transcripts. However, Simon and Malim (109) have reported that normal levels of viral DNA are produced in cells infected by *vif*-deleted virus. However, viral DNA is rapidly degraded after infection suggesting that Vif is required for the sustained accumulation of reverse transcripts in the cells.

## VI. Subcellular Localization of Vif

Immunofluorescence studies using *vif* transfected COS-7 cells combined with subcellular fractionation of infected CEM cells showed that Vif is an intracellular protein that is present in the cytoplasm as well as the nucleus (49). Mutagenesis studies have shown the C-terminus of Vif anchors at membranes (49). This same region was also shown to be crucial for Vif's function, suggesting that association of Vif with cell membranes may be important for Vif's biologial function.

In addition to the above studies, Karczewski and Strebel (63) expressed *vif* in HeLa cells and found that a significant portion of the protein colocalized with the cytoskeletal fraction and resisted extraction with detergents or buffers that contained high concentration of salt. This association was confirmed by confocal microscopy, which demonstrated that Vif colocalized with vimentine and keratin filaments and collapsed the intermediate filament network.

The experiments described above determined the subcellular distribution of Vif using transfection of permissive cell lines such as HeLa or

COS; however, other reports examined Vif localization in infected nonpermissive cells. Experiments using H9 T-leukemic cell lines infected with HIV-1 showed that a substantial proportion of Vif colocalized with the viral Gag proteins (12, 109). Sedimentation studies showed that the majority of Vif became associated with the plasma membrane (109), suggesting that Vif is present at the site of assembly and maturation of HIV-1 virions.

## VII. Post-translational Modification of Vif

The Vif protein, like several other HIV-1 proteins (15, 52, 87), is phosphorylated. Work by Gabuzda and coworkers showed *in vivo* and *in vitro* phosphorylation of the Vif protein on Ser 144, Thr 155, and Thr 188 (49). These three phosphorylation sites are located in the highly conserved C-terminus of the protein and were insensitive to inhibitors of protein kinase C, protein kinase A, or protein kinase G, suggesting that these kinases are not involved in the phosphorylation of the indicated residues. The importance of Ser 144 to Vif function was demonstrated by showing that mutation of this residue to alanine resulted in a loss of Vif activity and greater than 90% inhibition of HIV-1 replication in CEM cells (49). A more recent study has identified two additional phosphorylation sites, one of the sites within the conserved C-terminus of Vif (128). In this report, Thr 96 and Ser 165 were shown to be specifically phosphorylated by the mitogen activated protein kinase (MAPK) family of kinases.

The functional significance of Vif phosphorylation is unclear. Phosphorylation may be needed for membrane association or Vif phosphorylation may induce a conformational change in the protein that is

needed for its activity. Further investigations are needed to characterize the role of Vif phosphorylation and the functional significance of these modifications.

## VIII. Species Specificity of Vif

With one exception (EIAV) all lentiviruses have a *vif* gene (93). In contrast, other retroviruses lack a *vif* gene. Nevertheless, recent studies have demonstrated HIV-1 Vif incorporation into MLV particles (17) enhances their infectivity (110). Simon *et al.* have shown that the Vif protein from either HIV-2 or $SIV_{MAC}$ (MAC, macaques) but not feline immunodeficiency virus and bovine immunodeficiency virus can serve the same function as HIV-1 Vif in human T-lymphocytes (112). This viral specificity was further investigated and was reported that HIV-1 Vif can confer infectivity on the $SIV_{AGM(TAN)}$(AGM, African green monkey; TAN, tantalus monkey) *vif*-deleted virus in human cells but not in monkey cells. Moreover the Vif protein of $SIV_{AGM(TAN)}$ cannot function in human cells (110). These data suggest that there is a species specific interaction between a cellular factor and the viral Vif protein that is needed for the efficient replication of HIV-1 in human cells.

## IX. The Goals of the Vif project

Several areas of controversy still exist regarding the function of the Vif protein of HIV-1, including association with viral particles; its subcellular localization; and effects on viral particle formation and reverse transcription, on processing of Gag proteins, and on incorporation of Env proteins into virions. In order to investigate these issues, we first developed a method to

efficiently produce large amounts of *vif*-deleted virus in nonpermissive cells. This production has been difficult because this virus does not replicate in these cells. We then used this technique to analyze the effect of Vif on production and processing of Gag and Env proteins in nonpermissive cells. We also used this method to analyze the viral specificities of Vif function. In addition, we analyzed the cellular specificities of Vif function in order to learn whether permissive cells have a protein that functionally replaces Vif or whether nonpermissive cells contain an inhibitor of HIV-1 replication that is counteracted by Vif. The studies that will be described in Chapter 1 and 2 of this thesis describe experiments that addressed the above questions.

## X. HIV-1 Life Cycle

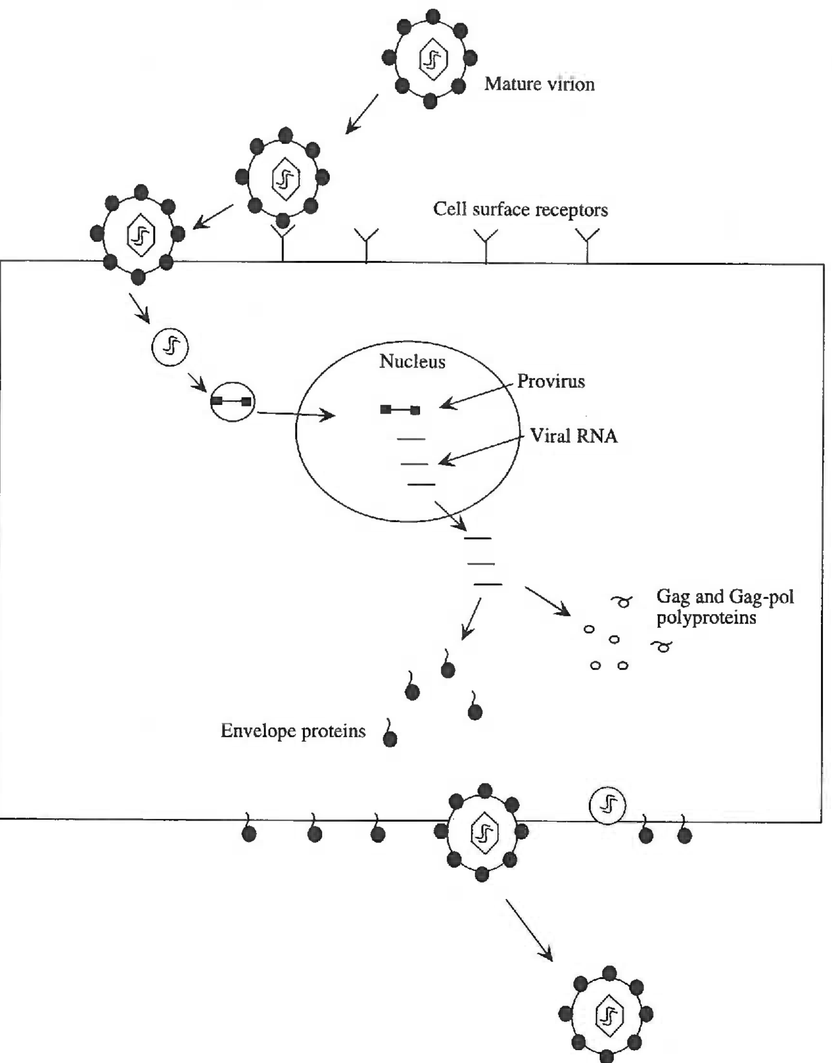
The HIV-1 life cycle can be divided into two stages. Stage one is mediated by virion proteins and includes viral binding and membrane fusion, reverse transcription and integration of the DNA provirus at a random site in a host cell chromosome. Stage two involves transcription of proviral DNA, translation of viral proteins and finally virus assembly and budding. Cellular proteins and enzymes are responsible for the second stage of the viral life cycle. These two stages of HIV-1's life cycle can be further subdivided into discrete steps (Figure 4). Step 1; Viral entry into the host cell, binding of the viral envelope to the cell surface receptors, followed by fusion of the viral membrane with the host cell membrane. Step 2; Reverse transcription of viral RNA form the DNA provirus. Step 3; Integration of the proviral DNA into the host cell genome. Step 4; Transcription of the integrated DNA and processing of the RNA. Step 5; Translation of viral RNA into proteins. Step 6; Assembly

and maturation of viral proteins and budding of virions from the infected cell. While segregating each of these steps into discrete pathways is easy, some of these processes may overlap. For example, proteolytic cleavages of Gag proteins are needed for viral infectivity and these stages of maturation occur in part after virions have budded from the host cells. For the purpose of the studies described in this thesis, I will focus on the molecular mechanism of viral entry.

## XI. HIV-1 Tropism

HIV-1 exhibits many genotypic and phenotypic variations. The ability of HIV-1 isolates to infect different types of cells is referred to as its tropism. Targets of HIV-1 infection include cells of the monocyte-macrophage lineage including microglial cells and antigen presenting dendritic cells as well as $CD4^+$ T lymphocytes. A variety of other cell types from many different tissues including heart, kidney, blood, brain, and testes have also been reported to be infected to varying degrees (78, 79). HIV-1 isolates have been divided into two major categories, macrophage tropic (M-tropic) and T-cell-tropic (T-tropic). These tropism designations are to some extent misnomers because all known HIV-1 isolates can infect CD4-positive T-lymphocytes. The T-tropic viruses are distinguished by their inability to replicate in macrophages in common culture conditions. Moreover, M-tropic isolates of HIV-1 cannot replicate in CD4-positive leukemic T cell lines, whereas most T-tropic isolates can. M-tropic isolates are predominant during the asymptomatic phase of the disease. T-tropic isolates often occur during the symptomatic phase of the

**Figure 1.4: A general overview of HIV-1 replication cycle.** Viral particles enter the host cells by binding to the cell surface receptors by fusing with the cell membrane, uncoating, reverse transcription, and integration. The viral DNA is transcribed by host proteins to RNA which is translated to viral proteins, followed by packaging of the viral proteins and budding at the cell surface.


Mature virion
Cell surface receptors
Nucleus
Provirus
Viral RNA
Gag and Gag-pol polyproteins
Envelope proteins

disease and their appearance usually correlates with a rapid decrease in $CD4^+$ T-cells and with ultimate demise of the immune system.

We now know that one of the most important factors determining tropism is how the viral Env glycoproteins interact with the cell surface receptors. The subunit of the Env protein that interacts with the cell surface receptors is the envelope glycoprotein 120 (gp120). gp120 has five conserved (C1-C5) and five variable (V1-V5) regions (90, 115). Binding to the cell surface receptor CD4 is considered to be dependent on the C3 and C4 regions of gp120 (68, 76). Studies using different domains of the virus' envelope have demonstrated that the V3 loop of gp120 is a critical determinant of viral tropism (18, 19). In addition, identification of the coreceptors for HIV-1 entry has provided evidence that different cellular membrane proteins contribute to the cellular tropism of HIV-1 (see below).

## XII. CD4 and HIV-1 Entry

Shortly after HIV-1 was discovered to be the agent causing AIDS, CD4 was identified as the high affinity receptor that interacts with the viral envelope gp120 (25). However, it soon became apparent that expression of human CD4 was insufficient for viral fusion and infection. HIV-1 binds to mouse cells that express human CD4, but membrane fusion does not ensue. Other work also supported the hypothesis that another human factor in addition to CD4 was needed for HIV-1 fusion with the host cell membrane (3, 21). CD4 expressing mouse-human hybrid cells were able to fuse with cells expressing the HIV-1 envelope glycoproteins, thereby resulting in formation of syncytia (35). These data suggested that human factors other than CD4

were needed for HIV-1 infection at the level of membrane fusion and viral entry.

Numerous molecules were proposed to be candidate cofactors. Most of these possible cofactors were suggested based on monoclonal antibody inhibition studies (16, 35, 57). While the majority of these candidates were shown to influence HIV-1 infection at the level of entry, they were not shown to be absolutely required for viral entry.

It took more than 10 years following the identification of CD4 as the high affinity receptor for HIV-1 for the true coreceptor to be discovered. Two critical findings resulted to this breakthrough. First, in December of 1995 Cocchi *et al.* (22) reported that certain soluble chemokines could inhibit HIV-1 infection, and second, in May of 1996 Burger and colleagues at the National Institute of Health functionally cloned the T-tropic HIV-1 coreceptor (42).

## XIII. HIV-1 Coreceptors

Identification of HIV-1 coreceptors in 1996 was a major breakthrough in AIDS research. A sensitive screening method was used to identify a human cDNA that enabled a non-human CD4 bearing cell to become infected by HIV-1 (42). Feng and coworkers used a recombinant vaccinia virus based transient expression system in which fusion between an envelope-expressing cell and a receptor-expressing cell would lead to activation of a reporter gene; they identified a single cDNA from a human HeLa cell cDNA library that converted a CD4 expressing mouse cell line into a cell line that could be infected by T-tropic HIV-1.

The cDNA encoded a protein of 352 amino acids that was a member of the superfamily of seven transmembrane G protein-coupled receptors. The protein had previously been cloned and identified by several groups and was known to belong to the family of chemokine receptors (41, 55, 62, 81). Since the natural ligand of the receptor had not yet been identified, the receptor had been described as an orphan receptor. However, within a few months of its identification as a coreceptor for T-tropic HIV-1, two groups identified its natural ligand to be the CXC chemokine stromal cell-derived factor 1 (SDF-1) (8, 92). Accordingly, SDF-1 was shown to inhibit infection by T-tropic and not M-tropic HIV-1. Because of its function in mediating fusion between the virus and the cell membrane, the newly identified HIV-1 coreceptor was named fusin. However, since fusin had closest homology to the CXC subgroup of chemokine receptors (33%) and since it was activated by the CXC chemokine SDF-1, fusin was renamed CXCR4.

## XIV. Chemokines and Chemokine Receptors

In the last few years, identification of chemokine receptors as the coreceptors for HIV-1 entry has significantly advanced research in the chemokine and chemokine receptor field. Currently there are at least 9 chemokine receptors and 50 chemokines that serve as their natural ligands. Chemokines are small proteins of 68-104 amino acids that were initially characterized by their ability to cause migration of lymphocytes to sites of inflammation and infection. Chemokines are absolutely essential for the host immune system to fight off pathogens and infections; they are produced and released from many cell types constitutively or as a result of an infection or

tissue injury. Based on their structural motif and chromosomal location they are grouped into α and β superfamilies. The α chemokines (CXC chemokines) have an amino acid separating the two cysteine residues that occur near the amino terminus, while in the β chemokines (CC-chemokines) the two cysteines are next to each other (61).

The chemokine receptors are subdivided into four categories, specific, shared, promiscuous, and viral. Specific receptors bind one chemokine; shared receptors bind more than one chemokine within either the CXC, or the CC group; while promiscuous receptors bind multiple chemokines within both the CXC and CC group of chemokines. In addition, chemokine receptor genes often occur in herpes viruses. The virus-encoded chemokine receptors bind multiple chemokines and have somehow been captured by the viral genome (89).

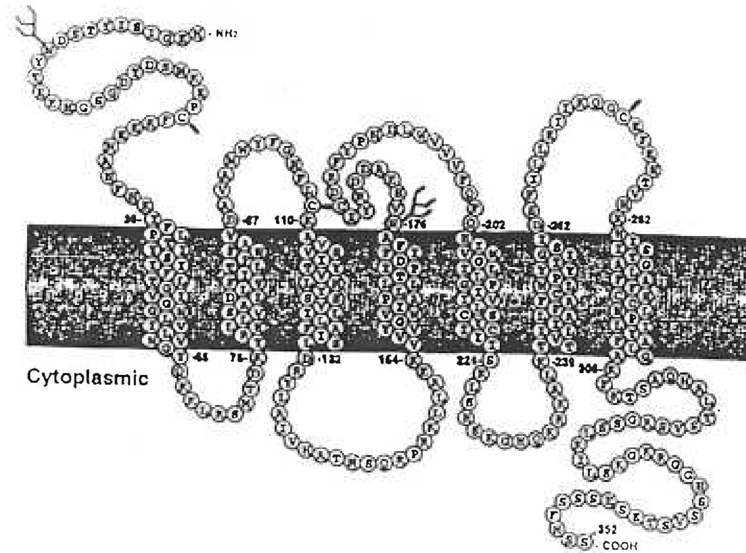
The majority of chemokine receptors within one group have approximately 65% identity in their amino acid sequences. Studies using chimeras have demonstrated that the N-terminus and some of the extracellular loops of these receptors are critical in their ligand specificities (61, 89).

## XV. Coreceptor Usage and HIV-1 Tropism

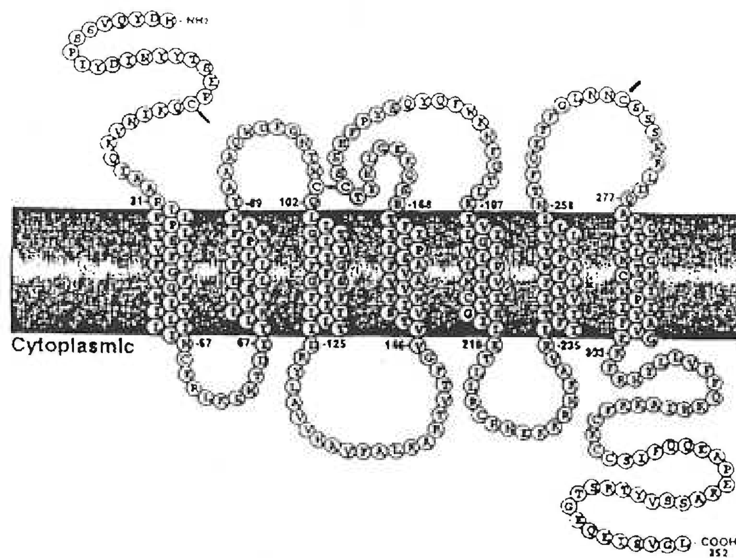
The chemokine receptor CXCR4 was shown to mediate fusion of T-tropic HIV-1 isolates but not M-tropic isolates with cell surfaces. However, the fact that the CC chemokines, RANTES, MIP-1α, and MIP-1β have suppressive activity for M-tropic strains of HIV-1 (22), led many investigators to hypothesize that the receptor for the above chemokines might serve as the

**Figure 1.5: The predicted membrane topology of chemokine receptors/HIV-1 cofactors, CXCR4 (A), and CCR5 (B).** HIV-1 fusion cofactors were identified to be 7 transmembrane G protein-couple chemokine receptors. The N-terminus as well as the extracellular loops of both coreceptors have been shown to be important for virus binding and fusion with the host cell membrane.

## A. CXCR4




## B. CCR5



fusion cofactor for M-tropic isolates. Within a few months, five different laboratories showed that the CC chemokine receptor CCR5 functions as a fusion cofactor for M-tropic isolates (2, 20, 30, 34, 36). Consistent with these ideas, CCR5 is activated by the three chemokines RANTES, MIP-1$\alpha$ and MIP-1$\beta$.

Chemokine receptors CCR3 and CCR2b were later identified to also serve as coreceptors for some M-tropic and dual tropic isolates of HIV-1 (20, 34). However, the efficiency of entry was shown to be less with CCR3 and CCR2b as compared to usage of CXCR4 and CCR5. In addition to serving as coreceptors for HIV-1 entry chemokine receptors also facilitate SIV infection. While CCR5 mediates infection by almost all SIV isolates, additional chemokine receptors can also be used by some SIV strains (85). Chemokine receptors also are necessary for HIV-2 fusions (31).

These breakthroughs in understanding of HIV-1 infections have had major implications for this field. For example, most primary HIV-1 isolates from patients can use CCR5 only or both CCR5 and CXCR4 for entry, whereas the T-tropic isolates use CXCR4 to enter the host cells. It is thought that disease progression is correlated with the coreceptor usage of HIV-1 in the patients. Early HIV-1 isolates following infection primarily use CCR5 to enter cells. However as the disease progresses, isolates are often able to use CXCR4 in addition to CCR5 and many are also capable of using CCR2b and CCR3. Because of these insights, M-tropic, T-tropic, and dual tropic strains of HIV-1 are now termed R5, X4, and R5X4, respectively, in order to indicate their coreceptor specificities (7).

A study by Paxon and colleagues using exposed uninfected individuals highlighted the importance of CCR5 for initial HIV-1 infection; they identified a population of Northern European individuals that have a 32 base pair deletion in their CCR5 gene that causes resistance to HIV-1 infection. These individuals were homozygous for a defective CCR5 gene. The deletion removes a segment in the central section of CCR5 protein (95) causing a frame shift mutation. This mutation ultimately results in premature termination, causing truncation of the last three transmembrane segments. The fact that M-tropic HIV-1 isolates were unable to infect $CD4^+$ T-lymphocytes from individuals homozygous for the Δ-32 CCR5 mutation strongly indicated the importance of this receptor in initiating infection. Therefore, R5 strains of HIV-1 are preferentially transmitted between humans. Although blood or semen samples may contain R5X4 or X4 viruses, it appears that only R5 strains are capable of establishing a new infection.

## XVI. Coreceptor, CD4, and Viral Envelope Complex

An important area of research concerns the regions critical for coreceptor binding to the Env-CD4 complex. Shortly after chemokine receptors were identified to be HIV-1 fusion cofactors, several laboratories identified sites that are important for gp120 binding and infection (37, 72, 96, 102, 104). These results suggested that the amino terminus of CCR5 and extracellular loops (ECL) 1 and 2 all contribute to infection. Site directed mutagenesis studies have highlighted specific residues in the amino terminus that are essential for gp120 binding and for viral infection. Wu *et al.* (127) and Siciliano *et al.* (107), have demonstrated the importance of ECL2 of

CCR5 in mediating HIV-1 infection. The amino terminal regions of CCR5 and CXCR4 that are important for HIV-1 infections are rich in tyrosines and acidic amino acids. Recently, Farzan and coworkers demonstrated that the tyrosines in these regions are sulfated, and that this sulfation contributes to gp120 binding and to HIV-1 infections (40).

An important discovery in 1998 was the X-ray crystal structure of the core of gp120 complexed with CD4 and a soluble neutralizing antibody (17b) that inhibits HIV-1 coreceptor interaction (73). Analyses of the crystal structure suggested that CD4 binds to a specific cavity of gp120. This contact buries similar surface areas from both proteins. The binding then results in a conformational change in gp120 that creates the binding site for the coreceptor, followed by binding of gp120 to the coreceptor and formation of a ternary complex. Biochemical studies have also suggested the formation of a ternary complex upon binding of HIV-1 envelope glycoprotein to CD4 and the coreceptors (58, 75, 118, 126). Following ternary complex formation, an additional conformational change ultimately results in exposure of gp41 domains and fusion of the viral and host cell membranes.

## XVII. Signal Transduction by Chemokine Receptors

The immune system fights infection by the migration and activation of leukocytes at the site of tissue damage. This immune response is achieved by binding of chemokines to their receptors, resulting in a downstream signal cascade that causes movement of immune cells to the site of infection. This process is referred to as chemotaxis. This sequence of events is accomplished by the immune cells sensing a small change in the normal concentration of

chemokines. Activation of chemokine receptors results in mobilization of intracellular $Ca^{+2}$ via activation of the inositol trisphosphate (IP3) pathway (9). *Bordetella pertussis* toxin (PTX) inhibits chemokine receptor signaling, suggesting that these receptors couple to the inhibitory guanine nucleotide-binding proteins (Gi) (91). While the majority of chemokine receptors couple to the Gi class, some β-chemokines also signal through the Gq class (70).

Activation of chemokine receptors also results in activation of small GTP binding proteins such as Ras, Rac and Rho that trigger processes such as membrane ruffling, pseudopod formation, rapid adhesion and cellular migration (117). These proteins have been shown to also stimulate the mitogen activated protein kinase and the Jun-N-terminal kinase (JNK) cascades. All these downstream signals cause intracellular $Ca^{+2}$ mobilization in leukocytes and movement of cells toward the sites of injury, infection, or inflammation (9).

## XVIII. The Goals of the Chemokine Receptor Signaling Project

Viral entry is one of the most important stages of the viral life cycle since it determines viral tropism as well as pathogenesis. Viral entry is also an attractive target for inhibition studies. Clearly, coreceptors play a major role in HIV-1 entry. While CD4 has been termed the major receptor for HIV-1's entry, the importance of coreceptors is becoming more clear since some HIV-1 isolates are able to infect CD4 negative cells (38, 56), but no known isolate enters the host cell without the aide of a chemokine receptor. The major aim of our studies that focused on viral entry was to gain a better understanding,

and to dissect the steps involved in viral envelope and chemokine receptor interaction.

Shortly after the HIV-1 coreceptors were identified to be members of the G protein-coupled receptor family, our goal was to determine whether binding of HIV-1 gp120 to the CD4 and the coreceptor would cause down stream signaling via activation of G proteins. Several investigators had already reported that binding of gp120 to CD4 causes association of a T cell specific tyrosine kinase p56-*Lck*, which then activates the Raf-mitogen-activated protein kinase and transcription factors including NF-κB in T-lymphocytes (13, 46, 97). Others reported that mutations in the regions of CXCR4 and CCR5 necessary for coupling to G proteins did not affect their abilities to serve as HIV-1 coreceptors (39, 50). These studies determined that coupling to G proteins was not essential for infection. However, we still did not know whether Env binding to the coreceptors would cause a conformational change in the receptor leading to G protein activation or conversely, preventing it. Because of the possible overlap and cross-talk between the gp120 activated p56-*Lck* and the G protein-coupled pathways in lymphocytes (11), we expressed the genes of interest in *Xenopus laevis* oocytes. *Xenopus* oocytes endogenously express a variety of G proteins, but they lack chemokine receptors, CD4, and in particular p56-*Lck*, enabling us to bypass the CD4 activation pathways. This system focused our studies on G protein activation via activation of coreceptors CXCR4 and CCR5. Chapter 3 of this thesis will describe in detail the approaches we took in determining the nature of interactions of gp120 with the coreceptors.

## XIX. Overall Goals of This Work

Both projects that comprise this thesis concern early steps in the HIV-1 infection pathway that precede integration of proviral DNA into a host cell chromosome. The major focus of the Kabat laboratory is to understand the viral envelope glycoproteins and their interactions with receptors and coreceptors on the host cell surfaces. Although the Vif project may seem only peripherally related to this issue, that was not known at the time my research was initiated. Indeed, it had been reported that *vif*-deleted HIV-1 released from nonpermissive cells contained a substantially reduced quantity of gp120-gp41 complexes in their envelope (9). Accordingly, our initial goal was to ascertain more conclusively whether a defect in gp120-gp41 function might be responsible for the phenotype of these *vif*-deleted virions. As we began to more definitively investigate this matter, I became fascinated by the complexity and importance of Vif and by the many opportunities it afforded for understanding the mechanisms of HIV-1 infection and pathogenesis. Moreover, I should also emphasize that the HIV-1 coreceptors had not been identified at the time I initiated my thesis research. The apparent diversity of my research therefore developed naturally as a consequence of progress in this field, including our own results. The work in this thesis is unified by my focus on key viral and cellular genes that control HIV-1 infectivity in specific cells.

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# Chapter 2

## Activity of the Vif Protein of Human Immunodeficiency Virus Type 1: Cellular and Viral Specificities

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Running Title: The HIV-1 Vif Protein

## Abstract

The *vif* gene of human immunodeficiency virus type 1 (HIV-1) is necessary for efficient production of infectious HIV-1 by cells classified as nonpermissive (e.g., lymphocytes, macrophages, and H9 leukemic T cells) but is irrelevant in permissive cells (e.g., HeLa or COS 7). Surprisingly, Simon *et al.* (34) recently reported that *vif* expression in nonpermissive cells dramatically enhances infectivity not only of HIV-1 but also of other enveloped viruses including murine leukemia virus (MLV). To address these issues, we first developed improved methods for producing substantial quantities of *vif*-deleted HIV-1 from healthy H9 cells. Despite their lack of infectivity, these virions had normal amounts of major core proteins and only slight decreases in quantities of envelope glycoproteins. Several lines of evidence, including pseudotyping with MLV envelope glycoproteins, suggested that this lack of infectivity was probably not caused by deficiency in envelope function. Furthermore, H9 cells that contained both *vif*-deleted HIV-1 and MLV simultaneously released noninfectious pseudotyped HIV-1 and fully infectious MLV, establishing that MLV envelope glycoproteins in these cells were functional. Accordingly, expression of *vif* in H9 cells had no effect on MLV infectivity. We conclude that the Vif protein of HIV-1 functions in a manner that is both cell-specific and substantially virus specific.

## Introduction

The Vif protein of HIV-1 is encoded by a regulatory gene that is critical for the viral replication (2, 10, 12, 14, 32, 39). This protein is highly expressed in infected cells, and is present in different locations including the plasma membrane, cytoskeleton and nuclear fractions, and in association with the viral Gag protein (5, 16, 17, 19, 30). The *vif* gene is conserved among all primate and non-primate lentiviruses with the exception of equine infectious anemia virus (24). Vif is a *rev*-dependent late gene product that contains 192 amino acids and an apparent Mr of 23,000 (36). While Vif's function is not yet known, early work has demonstrated that it increases infectivity of HIV-1 particles (12, 14, 32, 39). *vif* requirement is strictly cell type dependent, and it depends on the cells producing the virus rather than the target cells. Thus, cells have been categorized as permissive (e.g., HeLa, COS, Sup T1, Jurkat), semipermissive (e.g., CEM), or nonpermissive (e.g., peripheral blood lymphocytes, macrophages, H9 T-leukemic cells) (4, 12, 28, 39). *vif*-deleted virions released from nonpermissive cells are reported to be 50-1000 fold less infectious than wild-type virions made in the same producer cells (14, 39). This lack of infectivity can be rescued by supplying *vif* in trans in the producer cells but not in the target cells (14, 39), which suggests that Vif function might be critical during viral assembly, or maturation, or both. These results indicate that Vif is needed in the producer cells while it exerts its actions in the target cells. Based on this cell type specificity, many investigators had hypothesized

that either permissive cells contain an endogenous Vif-like factor, or that nonpermissive cells express a viral inhibitor that is counteracted by Vif (38). By using a complementation assay we and others recently reported that nonpermissive cells contain an inhibitor that prevents production of infectious *vif*-deleted HIV-1 (22, 31).

Although several groups previously found small amounts of Vif in virions (7, 19, 21), a recent study has reported an absence of this protein in highly purified virions (11). In addition, *vif*-deleted virions have aberrant morphology of their nucleocapsid core (4, 6), low levels of Env glycoproteins, and abnormalities in processing of Gag proteins (28, 29). Recent reports, however, have not detected any difference in quantities of virus-encoded proteins in these defective virions (6, 25).

Although the major defect in *vif*-deleted virions from nonpermissive cells is unknown, studies have shown that the block in their infectivity may precede reverse transcription of the genomic RNA (4, 39), or that the proviral DNA synthesis cannot be completed (10), or that the proviral DNA is synthesized but then rapidly degraded (32). This defect is apparently not caused by abnormalities in reverse transcriptase, or viral RNA (39).

Full infectivity of the well-studied oncogenic murine retroviruses depends only on expression of *gag*, *pol*, and *env*. Surprisingly however, a recent report has suggested that expression of HIV-1 *vif* increases the infectivity of MLV particles from nonpermissive cells (33). We further

examined this viral specificity by first developing an improved method to produce large quantities of *vif*-deleted HIV-1 from nonpermissive H9 cells. Our method involved coculturing a suspension culture of H9 cells with a monolayer of heavily infected permissive HeLa-CD4 cells. Virus released from the infected H9 cells was harvested quickly before severe cytopathology had occurred. We specifically analyzed the protein composition of *vif*-deleted and wild-type viral particles produced in H9 cells. This method was then adapted to study the effects of Vif on infectivity of xenotropic MLV particles.

## Materials and Methods

**Cells and reagents:** HeLa, COS 7, CCL-64, and H9 cells were from the American Type Culture Collection (ATCC, Rockville, MD). HeLa-CD4 cells expressing different quantities of cell surface CD4 were described previously (18). CCL64, HeLa, and HeLa-CD4 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS). COS-7 cells were maintained in DMEM containing high glucose with 10% FBS. H9 T-leukemic cells were maintained in RPMI medium with 10% FBS. The plasmids for wild type and *vif*-deleted NL4-3 provirus, HIV-$1_{HXBII}$ Vif antisera, HIV human immunoglobulin (HIVIG), and sheep HIV-1 IIIB gp120 antisera, contributed by Malcolm Martin, Ronald Desrosiers, Dana Gabuzda, Alfred Prince, and Michael Phelan, respectively, were obtained from the AIDS Research and Reference Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases, National Institute of Health (NIH). Xenotropic BV2 virus and goat anti-$p30^{Gag}$ sera were purchased from Quality Biotech Inc. (Camden, N.J.).

**Infection of H9 cells by xenotropic murine leukemia virus.** Replication competent xenotropic BV2 virus was used to infect CCL-64 cells pretreated with Polybrene (8 μg/mL; Sigma, St. Louis, Mo.) at 37 °C for 30 min. Chronically infected CCL-64 cells were then seeded at 3 x $10^5$ per 25 $cm^2$ flask. 24 h later, a suspension of H9 cells were added to the adherent CCL-64

cells. The newly infected H9 cells (H9/BV2) were removed to fresh cultures 24 h later. Medium containing xenotropic virus was collected from the H9/BV2 cells, filtered using a 0.45-μm-pore-size filter, and used for focal infectivity assays on HeLa cells. The H9/BV2 cells were assayed for viral proteins by Western immunoblot analyses (see below).

**Production of pseudotyped virions.** Production of wild-type or *vif*-deleted HIV-*gpt* virions from COS-7 cells was previously described (22, 26). Briefly, COS-7 cells were cotransfected with the DNAs of wild-type or *vif*-deleted pHIV-*gpt* and pSVIII*env* using the DEAE-dextran transfection protocol in the presence of chloroquine (3). 24 h after transfection, a suspension of H9/BV2 cells was cocultured with the virus producing COS-7 cells. 48 h later, the HIV-*gpt* infected H9/BV2 cells were removed, washed extensively, and separated into fresh cultures and selected with medium (26) containing mycophenolic acid (MPA) (20 μg/mL; Sigma) for 15-21 days. Selected cells were seeded at 2 $\times 10^6$ cells in a 25 $cm^2$ tissue culture flask and 24 h later, pseudotyped virus from the cells was harvested and used for focal infectivity assays (see below). This virus was also used to infect HeLa cells (5 mL of cell free virus per 25 $cm^2$ tissue culture flask) pretreated with Polybrene at 37 °C for 30 min. After 48 h, the infected HeLa cells were then seeded in 100-mm culture dishes in the presence of MPA (40 μg/mL); the resistant colonies were fixed and stained 15-21 days later.

**Determination of viral infectivity:** The infectivity of HIV-1 and xenotropic virions on HeLa-CD4 and HeLa cells, respectively, was measured using a focal infectivity assay (9). Briefly, $5 \times 10^3$ cells were seeded in a 1 $cm^2$ well of a 48-well tissue culture dish. 24 h later, cells were pretreated with DEAE/dextran (8 μg/mL; Sigma) for HIV-1 infection, or with Polybrene for MLV infection, at 37 °C for 20-30 min . The cells were washed with serum free medium, incubated with 0.1 mL of virus for 4-6 h, replenished with DMEM containing 10%FBS, and were grown for 72 hrs. The cells were fixed in 95% ethanol, and HIV-1 infected foci were detected by incubating the cells with HIVIG, peroxidase-conjugated goat anti-human immunoglobulin G, and a substrate solution of 3-amino-9-ethycarbazole (Sigma). Xenotropic infected foci were visualized by using goat anti-$p30^{Gag}$ of Roucher virus followed by incubation with peroxidase-conjugated rabbit anti-goat immunoglobulin G (9).

**Purification of viral particles:** Medium from H9 cells infected with replication competent wild-type or *vif*-deleted NL4-3 virus was collected, filtered using a 0.45-$\mu$m-pore-size filter, layered over a 25% sucrose cushion in phosphate buffered saline (PBS), and centrifuged for 2 h at 100,000 x *g*. The viral pellet was lysed in sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis sample buffer (62.5 mM Tris-HCl [pH 6.8], 10% glycerol, 2% SDS, 0.1% bromophenol blue, 10% 2-mercaptoethanol) then loaded onto a

10% polyacrylamide gel in the presence of 0.1% SDS and subjected to electrophoresis.

**Western immunoblot analyses of production of viral proteins:** H9/BV2 cellular extracts were obtained by washing the cells in PBS followed by lysing the cells in SDS-polyacrylamide gel sample buffer. The samples were boiled, equal amounts loaded onto a 10% SDS-polyacrylamide gel, and subjected to electrophoresis. The proteins were transferred to nitrocellulose membranes which were used for immunoblotting. Viral proteins were detected by incubating the membranes with either HIVIG, HIV-$1_{HXBII}$ Vif antiserum, sheep anti-gp120 serum, or MLV $p30^{Gag}$ antiserum from goat. The antisera were diluted at a 1:1000 dilution in 5% milk-0.1% Tween 20-Tris buffered saline (Bio-Rad laboratories; Hercules, Calif.). These incubations were followed by incubating with protein A-conjugated horseradish peroxidase (HRP) (Bio-Rad) at a 1:10,000 dilution for HIVIG and Vif antiserum, or protein G-conjugated HRP (Bio-Rad) at a 1:5000 dilution for sheep gp120 antisera and $p30^{Gag}$ antiserum. Antibody binding was then detected with a phototope-HRP Western blot detection kit (New England Biolabs, Mass).

## Results

**Development of a method to rapidly and efficiently infect nonpermissive H9 cells with wild-type and *vif*-deleted HIV-1.** In order to produce large amounts of *vif*-deleted virus from nonpermissive H9 cells we devised a method that relied on first transfecting a molecular clone of the laboratory adapted NL4-3 strain of HIV-1 or *vif*-deleted mutant into HeLa cells (Fig. 1; step 1). The wild-type and *vif*-deleted virions were harvested from the culture media at 48-72 h post-transfection, and were amplified by infection of HeLa-CD4 cells (Fig. 1; step 2). In preliminary studies we found that wild-type and *vif*-deleted virus were both produced in much higher yields by clones of permissive HeLa-CD4 cells that express only low rather than large levels of CD4. For example, the virions released by the H1-Q clone of HeLa-CD4 cells had reproducible titers that were approximately 5-10 times higher than virions released from the H1-J clone (data not shown). H1-Q and H1-J cells contain approximately 4 x $10^4$ and 4 x $10^5$ moclecules of CD4 per cell, respectively (Kozak and Kabat, unpublished data). These low titers are expected since CD4 forms intracellular complexes with HIV-1 envelope glycoproteins, thereby inhibiting the production of infectious virions (8, 20).

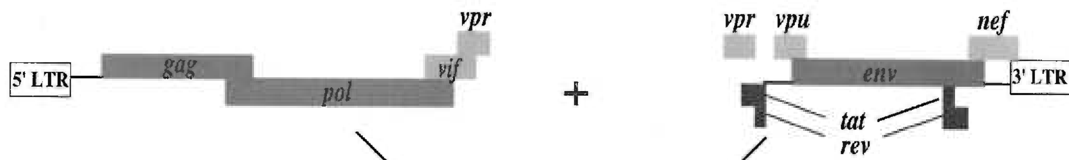
Optimal production of *vif*-deleted HIV-1 virions by nonpermissive cells such as H9 leukemic T-cells requires massive infection using virus from permissive cells. We cocultured suspensions of nonpermissive H9 cells for 24-48 h with monolayers of the infected H1-Q cells (Fig. 1; step 3). The H9 cells

became infected rapidly as determined by synthesis of viral proteins as well as by production of infectious wild-type virions (see below). The suspension of H9 cells were removed from the H1-Q cells and were washed extensively to remove any residual virus. Control assays of media from the different washes determined that contaminating virus from the cocultures were absent and could not account for any infectious virus that was subsequently found in the media (data not shown). Following the extensive washes, the H9 cells were then transferred to new culture dishes for 6 h to allow adherance of any contaminating H1-Q cells. The suspension of H9 cells were then removed and incubated in fresh medium and new culture flasks for 24 h to allow virion production (Fig. 1; step 4). After that time, the production of virions declined dramatically and the cells began to aggregate and to show other degenerative changes. This result is consistent with previous evidence that cytopathic changes including apoptosis occur in H9 cells after contact with other cells that express HIV-1 envelope glycoproteins (23).

**Analyses of infected H9 cells and released viral particles.** We analyzed the infected H9 cells for synthesis and processing of viral proteins and for the release of viral particles (Fig. 2). The H9 cells that had been infected by wild-type or *vif*-deleted HIV-1 produced similar amounts of viral $p55^{Gag}$ and $p24^{Gag}$

**Figure 2.1: Schematic diagram of a procedure to chronically infect nonpermissive H9 cells with wild-type or *vif*-deleted NL4-3 HIV-1.** Two halves of wild-type or *vif*-deleted NL4-3 HIV-1 proviral DNA were used to transfect HeLa cells (15) (step 1). 48-72 h following transfection, medium containing viral particles was collected, filtered and used to infect HeLa-CD4 cells (Clone H1-Q) pretreated with DEAE-dextran (step 2). This infection procedure was repeated until the cells were confluent at which point the virus was collected and used to infect uninfected H1-Q cells. Viral titers were monitored by focal infectivity assays, and when the titers were detected to be high, a suspension of H9 cells were added to infected HeLa-CD4 cells (step 3). The cocultures were grown for 48 hrs. The H9 cells were removed from the H1-Q cells, washed extensively, and cultured separately for 24 hrs (step 4). The medium containing virus was collected from the infected H9 cells, filtered, and used for further analyses.

# Flow Chart of HeLa-CD4 Infection




**Step 1**

**Transfect HeLa cells**

↓ 2-3 days

**Collect Media Containing Virions**

**Step 2**

↓

**Infect HeLa-CD4**

↓ 10-15 days

**Step 3**

**H9 suspension cells co-cultured with infected HeLa-CD4 cells**

↓ 2 days

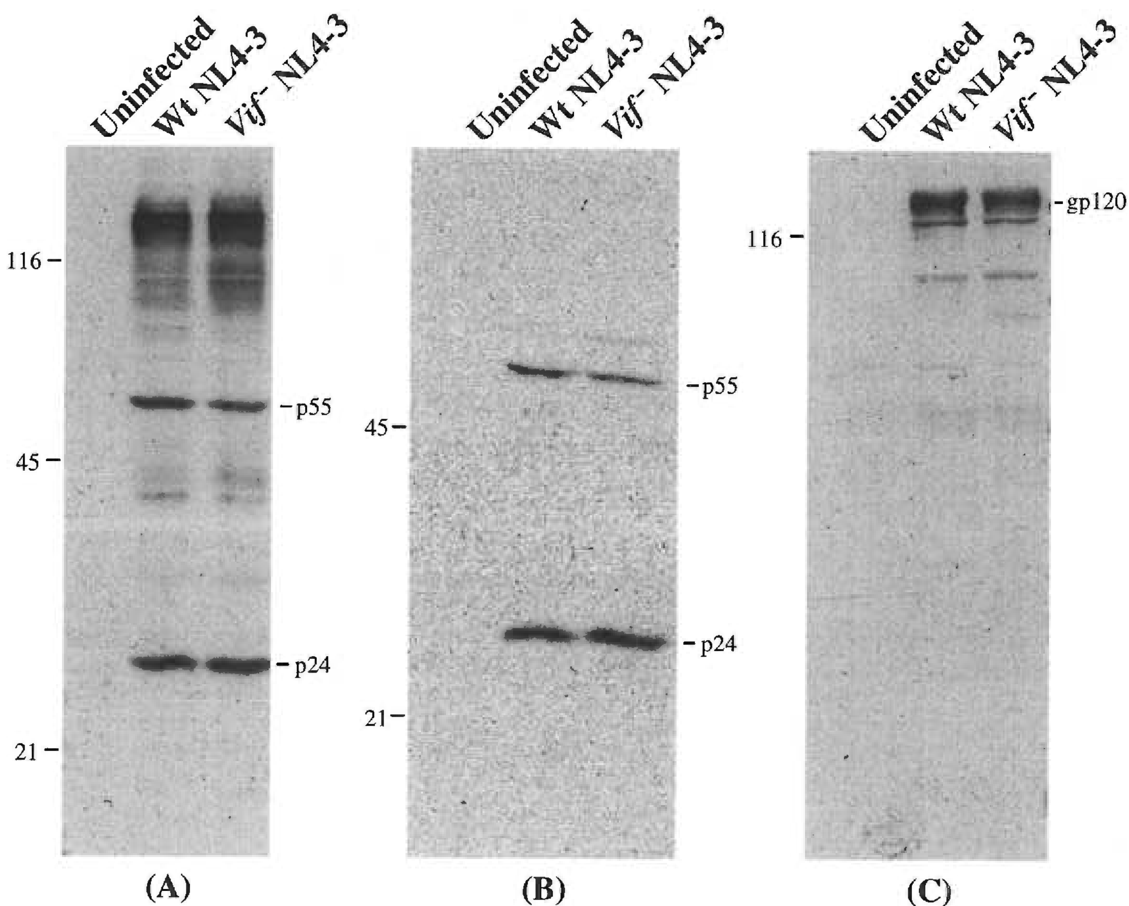
**Step 4**

**Remove and wash the infected H9 cells and culture separately**

↓ 24 h

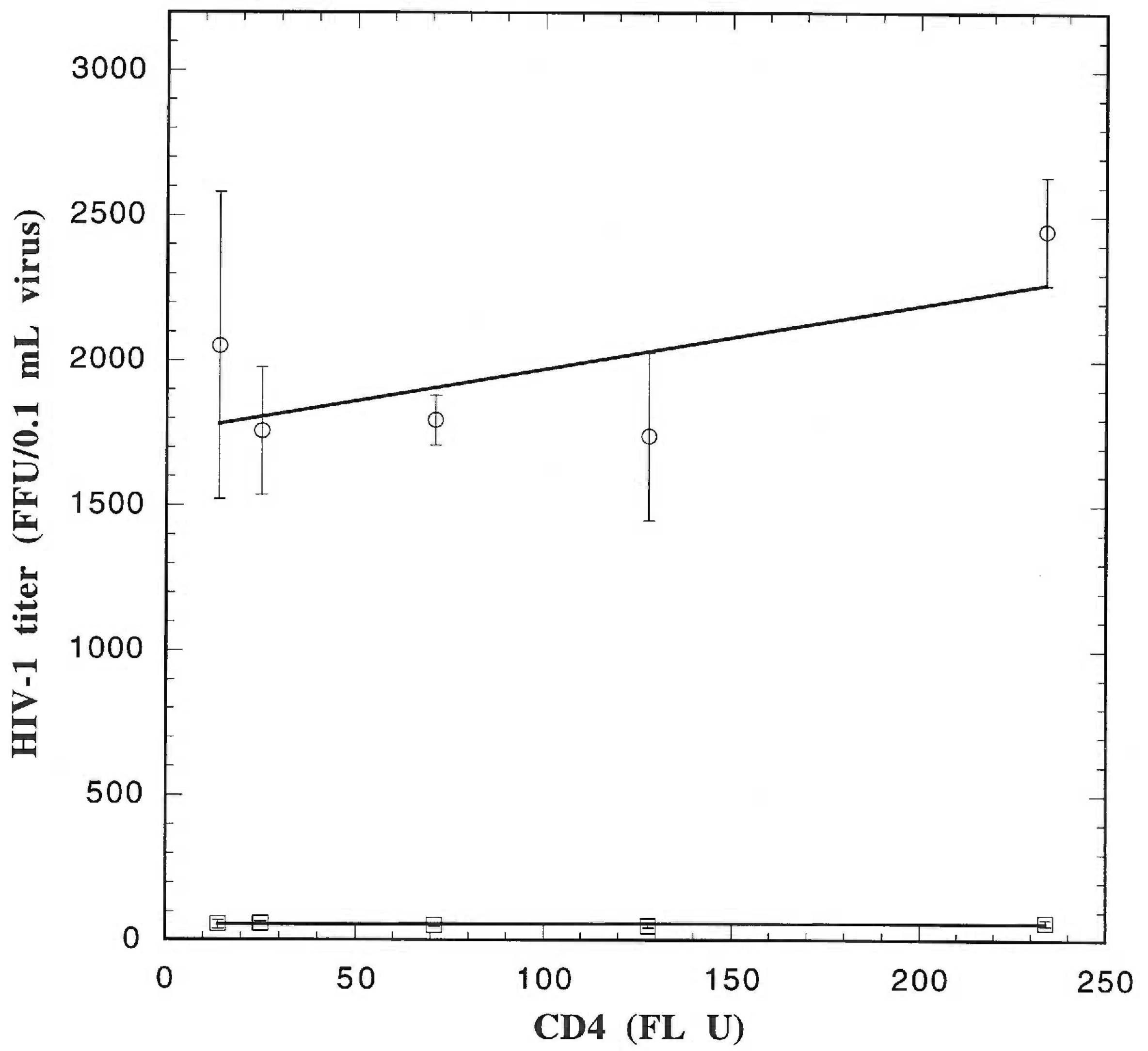
**Collect media containing virus for FIA and Western immunoblot analyses**

**Figure 2.2: Western immunoblot analyses of viral proteins from infected H9 cells and virion particles.** At 72 hrs after the start of coculture (see fig. 1), cell extracts of infected H9 cells (A), or virion particles produced from H9 cells (B-C) were prepared for Western immunoblot analyses. HIV-1 proteins electrotransferred to nitrocellulose membranes were detected with HIVIG (A-B), or sheep gp120 antiserum (C), and chemiluminescence reagent. Mrs are indicated on the left, in thousands.



Uninfected
Wt NL4-3
Vif⁻ NL4-3
116
45
21
p55
p24
(A)
Uninfected
Wt NL4-3
Vif⁻ NL4-3
45
21
p55
p24
(B)
Uninfected
Wt NL4-3
Vif⁻ NL4-3
116
gp120
(C)

**Figure 2.3: Infectivity of wild-type and *vif*-deleted HIV-1 on HeLa-CD4 cells.** The viruses produced from nonpermissive H9 cells infected with wild-type or *vif*-deleted NL4-3 strain of HIV-1 were used to infect HeLa-CD4 clones expressing different amounts of cell surface CD4, and were analyzed by the focal infectivity assay. The HeLa-CD4 clones and their relative CD4 fluorescent units (FL U) are : H1-R, 14; H1-Q, 25; H1-K, 71; H1-P, 128; and H1-J, 234. The infectivity for the *vif*-deleted NL4-3 virions ranged from 25-50 FFU per 0.1 mL of virus. Each point is the mean of 3 independent experiments and the error bars represent standard errors of the means.


HIV-1 titer (FFU/0.1 mL virus)
3000
2500
2000
1500
1000
500
0
0
50
100
150
200
250
CD4 (FL U)

proteins (Fig. 2, left panel). Virions pelleted from the cultures of H9 cells also contained similar levels of p24 and other Gag proteins (Fig. 2, middle panel). However, in a majority of our experiments the quantity of the gp120 envelope glycoprotein was slightly lower in *vif*-deleted virions compared to the wild-type virions from H9 cells (Fig. 2, right panel), consistent with an earlier report (39).

We then assayed the virus produced from the infected H9 cells by the focal infectivity assay (9). We used a panel of HeLa-CD4 cell clones that express different quantities of CD4 (18). This assay is able to differentiate between HIV-1 isolates that have different affinities for CD4 (27). Figure 3 shows the average of three independent focal infectivity assays for wild-type and *vif*-deleted virus recovered from nonpermissive H9 cells. The titers of *vif*-deleted virus are approximately 50 times lower than the titers of wild-type virus. The fact that the low infectivity of *vif*-deleted virus was independent of cell surface CD4 quantities on the target cells suggests that the *vif* defect is probably not caused by a deficiency in gp120 content or in virus binding to CD4.

**Expression of HIV-1 Vif does not increase the infectivity of xenotropic MLV particles produced in H9 cells.** In order to examine the effects of Vif on infectivity of MLV particles we used the above coculturing approach to infect H9 cells with xenotropic (BV2) virus. A suspension of H9 cells was

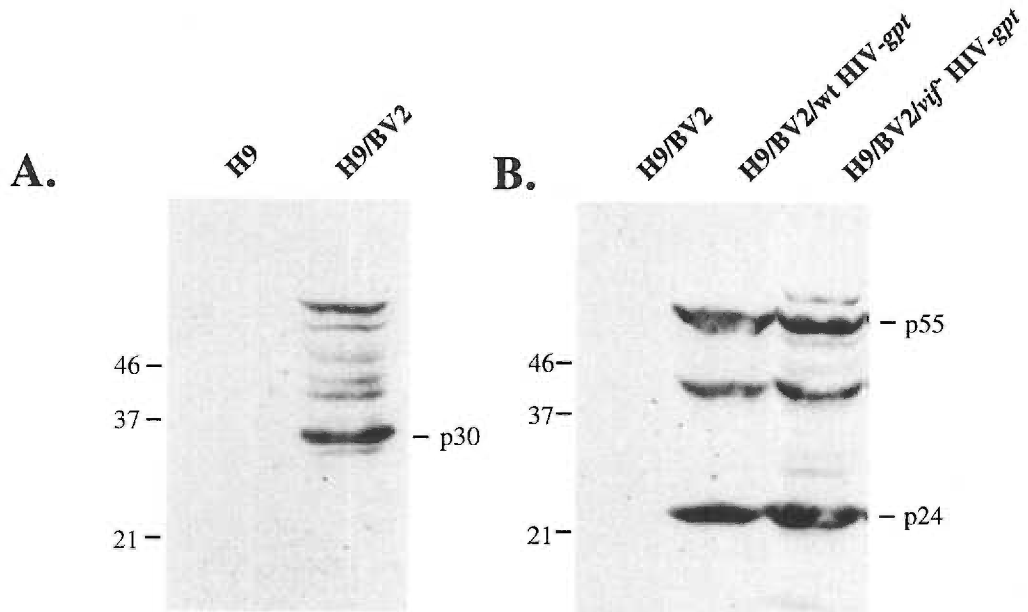
cocultured with adherent CCL64 mink lung fibroblasts that chronically produce replication competent BV2 virus. H9 cells were removed from CCL64 cells 24 h later, washed, and cultured separately for 48-72 hrs. Western immunoblot analyses showed that the H9 cells expressed $p30^{Gag}$, indicating that they were infected with BV2 virus (Fig. 4A).

To examine the effects of Vif on infectivity of MLV particles, we cocultured the xenotropic infected H9 cells (H9/BV2) with transfected COS-7 cells producing wild-type or *vif*-deleted HIV-*gpt* virus (22). pHIV-*gpt* encodes the HIV-1 provirus HXBII with the bacterial *gpt* gene for MPA resistance substituted for the viral *env* gene (26). The H9/BV2 cells were superinfected with either wild-type or *vif*-deleted HIV-*gpt* virus. The infected H9/BV2 cells were removed into fresh cultures and selected with MPA for 15-21 days. This procedure resulted in recovery of the cells that had been infected with the HIV-*gpt* viruses. Western immunoblot analyses confimed that the H9/BV2 cells were infected to the same extent by the wild-type and *vif*-deleted HIV-*gpt* (Fig. 4B). Following selection, media containing virus were collected from the H9/BV2 cells that had been superinfected with wild-type or *vif*-deleted HIV-*gpt* viruses and from the control unselected H9/BV2 cells. The virions harvested from these cells were filtered through 0.45 $\mu$m filters and were assayed by the focal infectivity assay using HeLa cells. The foci of infection were stained for MLV proteins using specific antibodies and immunoperoxidase methods (9). Figure 4C is a representative of two

independent experiments and shows that the titers of BV2 virus produced by H9 cells in the absence and presence of Vif were the same. Moreover, the presence of HIV-*gpt* proviruses in the cells had no effect on production of BV2 virus as indicated by the H9/BV2 control data.

The infected H9/BV2 cells described above produce xenotropic MLV particles as well as HIV-*gpt* virions pseudotyped with a xenotropic envelope. Consequently, we were able to assay the HIV-*gpt*(X-MLV) pseudotype virions by selecting the infected HeLa cells for colony formation in the presence of 40 $\mu$g/mL MPA. The resistant colonies were fixed and stained 15-21 days later. The results of four independent expriments showed that the pseudotyped virus that contained wild-type HIV-*gpt* provirus was much more infectious than the *vif*-deleted HIV-*gpt* viral particles (Table 1). In control experiments, in which medium from H9 cells infected with either wild-type or *vif*-deleted HIV-*gpt* virus was used to infect HeLa cells, no colonies were detected (data not shown). Thus, the infectious HIV-*gpt* viruses assayed in Table 1 were pseudotyped with the X-MLV envelope. These results confirmed the evidence in figure 3C that functional X-MLV envelope glycoproteins were made in the H9 cells that contained the HIV-*gpt* proviruses. Most importantly, our results show that infectious X-MLV BV2 virus was produced independently of Vif in H9 cells (see Fig. 3C), whereas the same cells released infectious HIV-*gpt* (X-MLV) pseudotype virions only in the presence of Vif (Table 1).

**Figure 2.4: Expression of HIV-1 *vif* does not enhance the infectivity of xenotropic MLV particles.** H9 cells infected with xenotropic virus were super-infected with wild-type or *vif*-deleted HIV-*gpt* virus. Expression of BV2 $p30^{Gag}$ (A), or HIV-1 viral proteins (B), in infected H9 cells was monitored by Western immunoblot analyses. Mrs are indicated on the left, in thousands. (C) Medium containing viral particles was collected from the infected cells, used to infect HeLa cells, and were analyzed by a focal infectivity assay using $p30^{Gag}$ antiserum. The graph shows the BV2 titer of a representative experiment.




C.

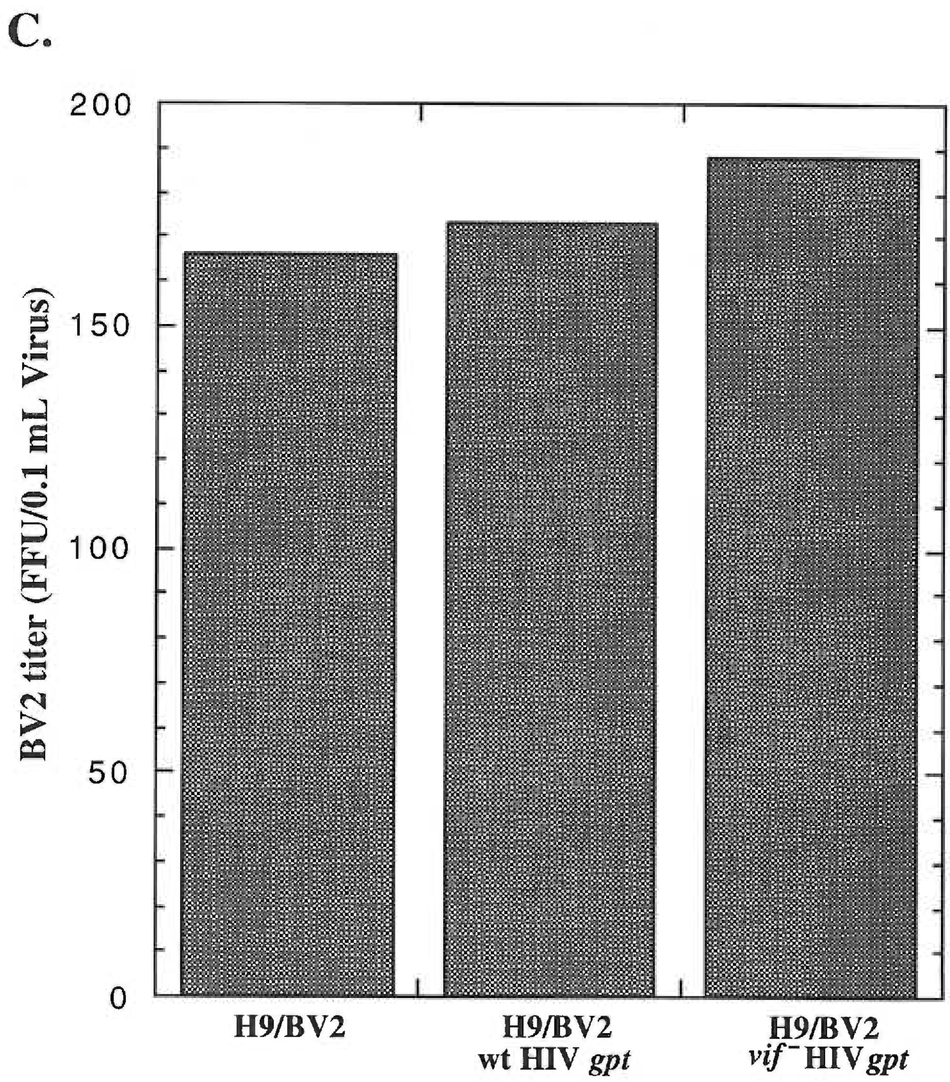


**Table 1. Differences between the infectivities of wild-type and *vif*-deleted HIV-*gpt*/BV2 pseudotyped virions produced from H9 cells**

| Experiment | HIV-*gpt*/BV2-Env pseudotyped viral titer (no. of colonies/dish)[a] | |
|---|---|---|
| | Wild-type | *vif*-deleted |
| 1 | 28 | 0 |
| 2 | 113 | 0 |
| 3 | 23 | 0 |
| 4 | 58 | 0 |

[a] H9 cells were superinfected with xenotropic BV2 virus and wild-type or *vif*-deleted HIV-*gpt* virus. The cells were then selected with medium containing mycophenolic acid for 2-3 weeks. 2 x $10^6$ H9 cells were seeded in a 25 $cm^2$ tissue culture flask. 24 h later virions were collected and used to infect HeLa cells. Infected HeLa cells were selected with medium containing mycophenolic acid and resistant colonies were counted 15-21 days later (see Methods).

**Discussion**

Our results differ in several aspects from earlier reports (4, 28, 29, 34). First, we find that wild-type and *vif*-deleted virions from H9 cells have similar quantities of the $p24^{Gag}$ protein and that *vif*-deleted virions only have slight reductions in quantities of envelope gp120. These differences may derive from our experimental approaches. For example, we were able to efficiently and rapidly infect nonpermissive H9 cells by coculturing them for 24-48 h with chronically infected HeLa-CD4 (clone H1-Q) cells (Fig. 1 and 2). We then harvested virus from nonpermissive H9 cells prior to development of significant cytopathology. Therefore the virions used in our assays were from healthy H9 cells and contained minimal amounts of immature particles released from damaged cells. In other conditions including more prolonged infections, the nonpermissive cells can exhibit cytopathic changes or production of cytokines or interferons that could secondarily alter synthesis or processing of virion proteins (1). In contrast to other reports (4, 28, 29), our results indicate that *vif*-deleted particles from H9 cells have a low infectivity that is not caused by an absence of p24 or a severe reduction in gp120-gp41 content (Fig. 2).

An important result of our investigation was that expression of *vif* in nonpermissive cells did not enhance the infectivity of MLV particles. This observation is in contrast to a recent report that Vif increased the infectivity of amphotropic MLV produced from nonpermissive cells (34). This difference

might also derive from our experimental approaches. Their study was based on analyses of amphotropic MLV produced from nonpermissive HUT78 cell lines. They monitored viral spread by measuring the activity of reverse transcriptase for 10-15 days. In contrast, we directly measured the effects of Vif on infectivity of BV2 virus by using the quantitative focal infectivity assay (Fig. 4C). Moreover, we produced wild-type or *vif*-deleted HIV-*gpt* viruses pseudotyped with a xenotropic MLV envelope in H9 cells that simultaneously produced infectious xenotropic MLV (BV2). Control assays showed that the H9 cells were equally infected by these viruses (Fig. 4A, B). In addition, our study benefited from a critical internal control. After the H9/BV2 cells were superinfected with wild-type or *vif*-deleted HIV*gpt*, they were selected using media containing MPA. This selection significantly enriched the X-MLV-infected cells that contained the HIV-*gpt* proviruses. We showed that the xenotropic envelope was fully functional since the wild-type HIV-*gpt* (BV2) pseudotype virus and the xenotropic MLV particles were both fully infectious. However, the *vif*-deleted HIV-*gpt* (BV2) virions were not infectious (Table 1). These results indicate that the *vif* defect blocks a post-entry step of infection, in agreement with an earlier report (39). In our assay conditions, Vif increased the infectivity of HIV-1 particles dramatically. However, Vif did not enhance the infectivity of xenotropic virus produced from the same nonpermissive H9 cells (Table 1). These results suggest that Vif functions in a manner that is more virus-specific than previously proposed (34).

Our study is in agreement with an earlier report that the infectivity of *vif*-deleted virus from H9 cells may be defective at a postpenetration step of infection (39). Our data showed that the low infectivity of the *vif*-deleted virions was not enhanced by increasing the CD4 content of target cells (Fig. 3). Moreover, the *vif* defect was not rescued when HIV-1 virions were pseudotyped with MLV envelope glycoproteins (Table 1).

Determining the function of Vif has been elusive. However, based on our findings as well as other reports (39), Vif's importance for a step(s) of infection that follows virus binding and internalization into the cells is evident. Viral core abnormalities in *vif*-deleted virions produced by nonpermissive cells have been reported earlier (4, 6). Vif has also been shown to be essential for proviral DNA synthesis or stability in the infected cells (32, 37, 39). Whether Vif exerts its effects on these steps directly or indirectly is yet to be conclusively determined. Several groups have reported that a small amount of Vif is incorporated into HIV-1 virions (13, 19, 21) as well as MLV particles (7). Since Vif is highly expressed in the infected cells and is partially localized at the plasma membrane, its incorporation into virions may be non-specific. Moreover, a recent report (11) has shown a virtual absence of Vif in the virion particle. Based on these findings, it seems likely that Vif functions indirectly and that its presence is critical in the producer cells but Vif exerts its effects in the target cells.

The *vif* gene is present in all members of the primate and nonprimate (with the exception of EIAV) lentiviruses (24). However, members of the oncovirus and spumavirus families of retroviruses lack this gene. The Vif protein of HIVs and SIVs share between 26 to 29% amino acid sequence identity. Simon *et al.* reported that the Vif protein of HIV-2 and $SIV_{MAC}$(SIV, simian immunodeficiency virus; MAC, Macaque monkey) could functionally substitute for HIV-1 Vif in nonpermissive human cells (35), whereas the Vif protein of $SIV_{AGM/TAN}$(AGM, African green monkey; TAN, Tantalus monkey), $SIV_{AGM/SAB}$(SAB, Sabaeus monkey), and $SIV_{SYK}$(SYK, Sykes monkey) could not (34). In the same study, Simon and coworkers reported that Vif increases the infectivity of amphotropic MLV released from nonpermissive HUT78 cells; they concluded that Vif creates an environment that nonspecifically enhances the production of diverse infectious viruses. In a recent study we and others reported the existence of an endogenous HIV-1 inhibitor in nonpermissive cells that is counteracted by Vif (22, 31). In this report we show that Vif's effects are specific to HIV-1. If Vif were to enhance the infectivity of diverse enveloped viruses, such as the MLV xenotropic virus, this would imply that the endogenous factor present in nonpermissive cells nonspecifically decreases the infectivity of a broad range of virions. This idea would be difficult to reconcile with other evidence that many viruses including murine leukemia viruses that lack a *vif* gene are able to productively infect T-

lymphocytes. Additional studies will be needed to fully understand the cell-specific and virus-specific effects of Vif.

**Acknowledgements**

This research was supported by NIH grant (CA67358). We are grateful to our colleagues Emily Platt, Susan Kozak, Shawn Kuhmann, Ali Nouri, Chetankumar Tailor, and Mariana Marin for their encouragement and helpful suggestions.

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# Chapter 3

## An Endogenous Inhibitor of Human Immunodeficiency Virus in Human Lymphocytes is Overcome by the Viral Vif Protein


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## Abstract

The *vif* gene of human immunodeficiency virus type 1(HIV-1) encodes a basic Mr 23,000 protein that is necessary for production of infectious virions by nonpermissive cells (human lymphocytes and macrophages) but not by permissive cells such as HeLa-CD4. Previous studies had proposed that permissive cells may contain an unidentified factor that functions like the viral Vif protein. To test this hypothesis, we produced pseudotyped wild-type and *vif*-deleted HIV-*gpt* virions (these contain the HIV-1 genome with the bacterial mycophenolic acid resistance gene *gpt* in place of the viral *env* gene) in permissive cells, and we used them to generate nonpermissive H9 leukemic T-cells that express these proviruses. We then fused these H9 cells with permissive HeLa cells that express the HIV-1 envelope glycoprotein gp120-gp41; and we asked whether the heterokaryons would release infectious HIV-*gpt* virions. The results clearly showed that the *vif*-deleted virions released by the heterokaryons were noninfectious, whereas the wild-type virions were highly infectious. This observation strongly suggests that nonpermissive cells, the natural targets of HIV-1, contain a potent endogenous inhibitor of HIV-1 replication that is overcome by Vif.

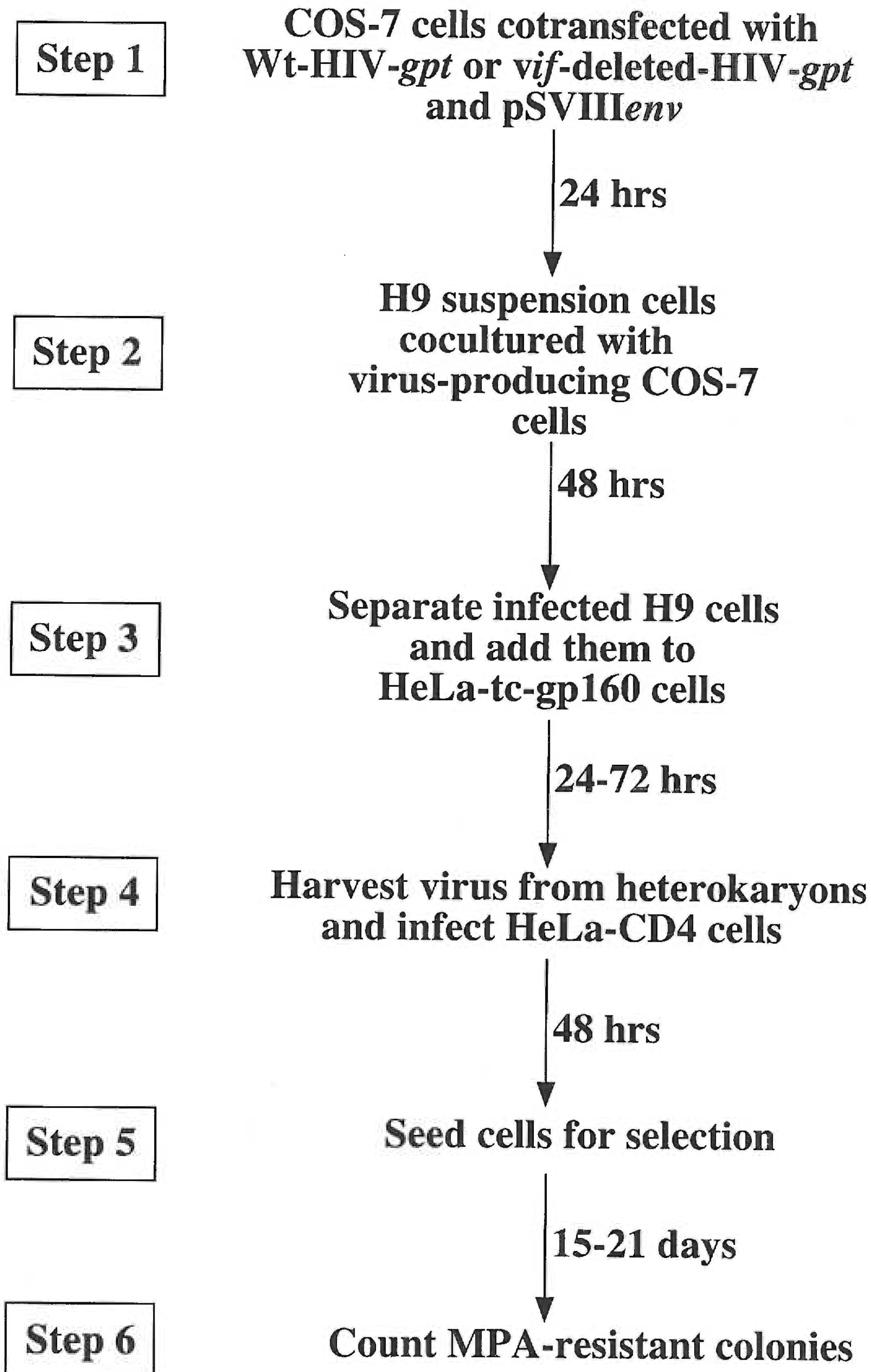
The *vif* gene of human immunodeficiency virus type 1 (HIV-1) encodes a highly basic Mr 23,000 protein that collapses intermediate filaments (14), locates in both cytosolic and nuclear sites (11, 14), is present in small amounts in virions (16), and is highly conserved among lentiviruses (20). Vif protein is not essential for HIV-1 replication in permissive cells such as HeLa-CD4 or in semipermissive cells such as SupT1 (9, 24). However, Vif is necessary for production of infectious virions by cells that are natural targets for infection including CD4-positive T-lymphocytes and macrophages and by the H9 line of leukemic T-cells (2, 9, 28). Nonpermissive cells are susceptible to infection by *vif*-mutant virus made in permissive cells, but the result is production of only weakly infectious virions that cannot spread in the cultures. Recent studies have shown that the defectiveness of *vif*-mutant virus made in nonpermissive cells is not caused by gross abnormalities in protein composition (4, 8) and cannot be overcome by assaying the virus in permissive cells or in nonpermissive cells that contain Vif protein (7). The defective virus enters target cells and begins reverse transcription, but the resulting proviral DNA is prematurely degraded (3, 7, 28). Thus, the defectiveness of *vif*-mutant HIV-1 conditionally depends on the cellular source of the virus rather than on the cells used to analyze infectivity.

The above results strongly suggest that the Vif protein performs a critical function in cells that produce HIV-1 virions. However, little is known about this critical Vif function. Previous work had hypothesized that permissive cells may contain a cellular protein that functions like Vif, thereby enabling active replication of *vif*-mutant HIV-1 (26, 27). Alternatively, nonpermissive cells might contain an inhibitor of HIV-1 replication that is counteracted by Vif. These distinct hypotheses have not previously been investigated.

We have addressed this issue by analyzing infectivities of wild-type and *vif*-mutant virions produced by heterokaryons formed by fusion of nonpermissive H9 leukemic T-cells with fully permissive HeLa cells. Presumably, if permissive cells contain a protein that functions like Vif, the *vif*-mutant virions released from the heterokaryons would be infectious. Alternatively, if nonpermissive cells contain an inhibitor that is counteracted by Vif, the *vif*-mutant virions made by the heterokaryons would be inactive.

A schematic outline of this genetic complementation experiment is shown in Fig 1. The strategy involved production of wild-type and *vif*-deleted HIV-*gpt* virions. pHIV-*gpt* encodes the HIV-1 provirus HXBII with the bacterial *gpt* gene for mycophenolic acid resistance substituted for the viral *env* gene (21). This plasmid was modified to construct pHIV-*vif*-deleted-*gpt* by

**Figure 3.1: Schematic of the *vif* complementation assay.** In Step 1, COS-7 cells at 80% confluency in a 100 mm culture dish were cotransfected with 5 μg wild type pHIV-*gpt* or pHIV*vif*-deleted-*gpt* and 5 μg pSVIII*env* . In step 2, suspensions of nonpermissive H9 leukemic T cells were added to the virus-producing COS-7 monolayers for 48 hrs. In step 3, the H9 cells were separated from the monolayer and were cocultured with induced HeLa-tc-gp160 cells. This method resulted in spontaneous cell fusion to produce heterokaryons, and virus was harvested from the culture media after 24, 48, and 72 hrs. In step 4, virus was filtered (0.45 μm pore size) and then used to infect HeLa-CD4 (H1-J clone) cells. In step 5, infected HeLa-CD4 cells were seeded in 100 mm culture dish and selected with medium containing mycophenolic acid (40 μg/mL). Mycophenolic acid-resistant colonies were fixed, stained, and counted after 15-21 days of selection (22).

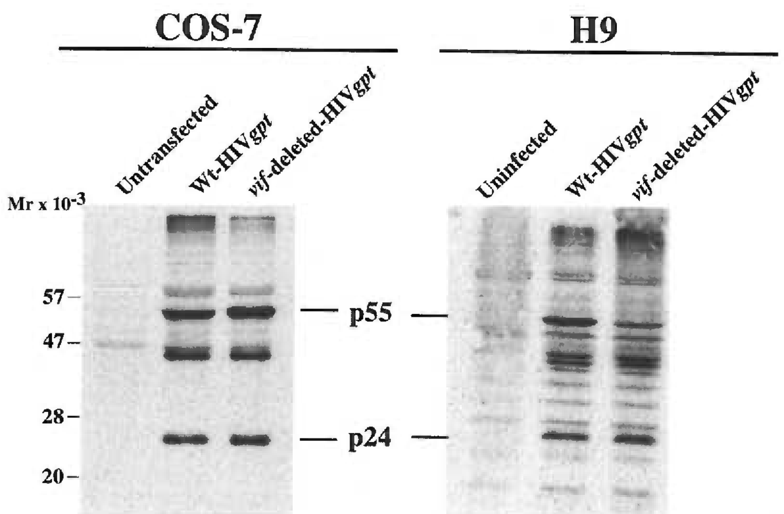

Step 1
COS-7 cells cotransfected with
Wt-HIV-*gpt* or *vif*-deleted-HIV-*gpt*
and pSVIII*env*
24 hrs
Step 2
H9 suspension cells
cocultured with
virus-producing COS-7
cells
48 hrs
Step 3
Separate infected H9 cells
and add them to
HeLa-tc-gp160 cells
24-72 hrs
Step 4
Harvest virus from heterokaryons
and infect HeLa-CD4 cells
48 hrs
Step 5
Seed cells for selection
15-21 days
Step 6
Count MPA-resistant colonies

digesting with SalI and NdeI restriction endonucleases (New England Biolabs, Beverly, MA.), blunting with the Klenow fragment of DNA polymerase I (Life Technologies, Grand Island, NY), and incubating with T4 DNA ligase (Life Technologies, Grand Island, NY) to allow self ligation. This deleted a large portion of the *vif* gene and also removed a portion of the *vpr* gene. However, since the nonessential accessory genes *vpr*, *vpu*, and *nef* are all defective in the HXBII molecular clone of HIV-1 (with *vpr* having a mutation in the initiation codon (19), this deletion did not have any effect on viral production (see below). Step 1 involved cotransfection of fully permissive COS-7 cells with pSVIII-*env*, encoding HXBII envelope, plus either pHIV-*gpt* or pHIV-*vif*-deleted-*gpt* using the DEAE/dextran with chloroquine transfection protocol (1). COS-7 cells were propagated as described previously (22). As shown by Western blotting, the transfected COS-7 cells expressed HIV-1 Gag proteins to equal extents, indicating that deletion of *vif* did not inhibit expression of the HIV-*gpt* provirus (see Fig 2). Moreover, these transfected COS-7 cells released equal titers of the wild-type and *vif*-deleted HIV-*gpt* viruses. For example, in a representative experiment when 4 mL of cell free media from wild-type and vif-deleted HIV-*gpt* transfected COS-7 cells were used to infect HeLa-CD4 cells, we obtained titers of 3250 and 3050 colonies

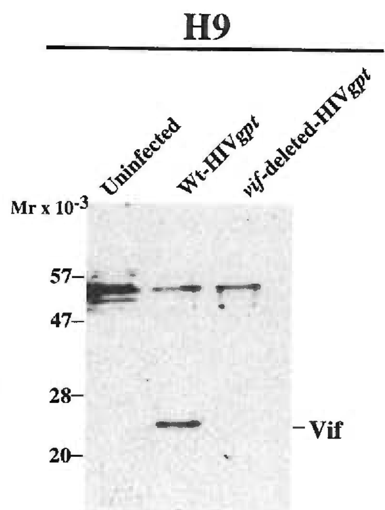
**Figure 3.2: Western immunoblot analyses of viral protein production in transfected COS-7 and infected H9 cells.** H9 cells were cocultured with transfected virus-producing COS-7 cells as described in figure 1. At 72 hrs post-transfection of COS-7 cells (5 x $10^6$ cells per 100 mm culture dish) and 48 hrs postinfection of H9 cells (1 x $10^6$ cells), extracts of control and transfected or infected cells were obtained by washing the cells in phosphate buffered saline (Life Technologies, Grand Island, NY) followed by cells lysis in SDS-PAGE sample buffer (62.5 mM Tris-HCl, pH 6.8, 10% glycerol, 2% sodium dodecyl sulfate, 0.1% bromophenol blue, 10% 2-mercaptoethanol). The samples were then boiled and equal amounts were loaded onto 10% polyacrylamide gels in the presence of 0.1% sodium dodecyl sulfate and subjected to electrophoresis. The proteins were then electrotransferred onto nitrocellulose membranes and used for immunoblotting (21). Viral proteins were detected by incubating the membranes with HIV-IG antiserum (A) (obtained through NIH AIDS Research and Reference Reagent Program, donated by Alfred Prince), or with HIV-$1_{HXB2}$ Vif antiserum (B) (obtained through NIH AIDS Research and Reference Reagent Program, donated by Dana Gabuzda) at 1:1000 dilution in 5% milk, 0.1% tween-20, and Tris-buffered saline (Bio-Rad Laboratories, Hercules, Ca.) followed by protein A-conjugated horseradish peroxidase at 1:10,000 dilution (Bio-Rad,Ca.).

Antibody binding was then detected using phototope-HRP Western blot detection kit (New England Biolabs, Beverly MA.).

A.




B.



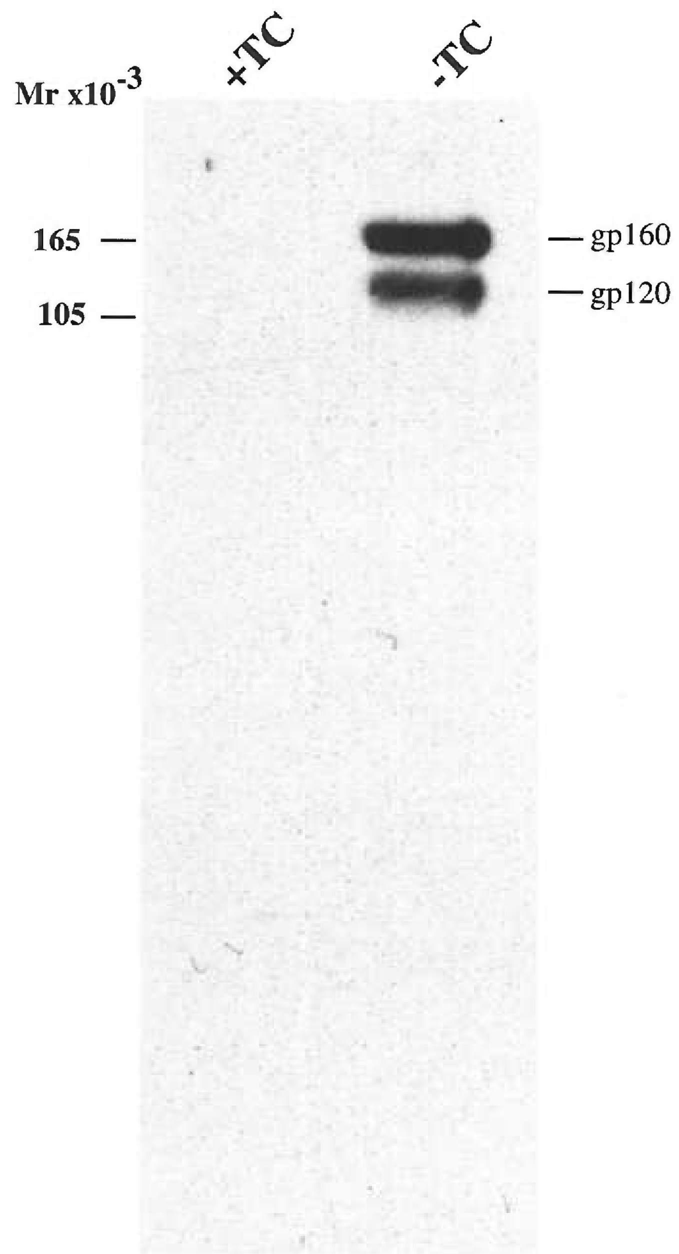
per 100 mm diameter culture dish for wild type and *vif*-deleted HIV-*gpt*, respectively. Therefore the COS-7 cells are fully permissive. In step 2, the virus-producing monolayers of adherent COS-7 cells were cocultured with a suspension of nonpermissive H9 T cells; 48 hrs later, the newly infected H9 cells were removed into fresh cultures. The resulting suspensions of H9 cells appeared morphologically homogeneous and did not produce any infectious HIV-*gpt* virions, indicating that they were not significantly contaminated with the virus-producing COS-7 cells. Other control studies substantiated this conclusion (results not shown). As illustrated by Western blotting (Figure 2A), the H9 cells that had been infected with these wild-type and *vif*-deleted HIV-*gpt* virions reproducibly expressed similar amounts of the HIV-1 p55 and p24 Gag core proteins. These results suggested that the infections in step 2 occured with similar efficiencies and that processing of the core p55 Gag polyproteins in nonpermissive H9 cells was not significantly altered by the *vif*-deletion. As also shown by western blotting (Figure 2B), the $M_r$ 23,000 Vif protein was present in the H9 cells that had been infected with wild-type HIV-*gpt* virus, but was absent from the cells that had been infected with the *vif*-deleted-HIV-*gpt* virus. In Step 3, the infected H9 cells were mixed with a clone of tetracycline inducible HeLa-gp160 cells that expressed the HIV-1 IIIB *env* gene. These cells were generated by transfecting HeLa-tTA (12) cells with pUHD10-

3(*gp160/rev)* using the calcium phosphate transfection method (1). The pUHD10-3(*gp160/rev)* was constructed by cloning a XbaI 2.9 Kbp gp160/rev fragment from pGCneo/gp160-rev (E. Platt and D. Kabat unpublished) into the same site of the tetracycline responsive plasmid pUHD10-3. Fig 3 presents evidence that our clone of HeLa-tc-gp160 cells expressed abundant gp120-gp41 Env glycoproteins when cells were induced by removal of tetracycline from the medium. We also used the methods described above for COS-7 cells and confirmed that this clone of HeLa-tc-gp160 cells was fully permissive for HIV-1 production. Moreover, the induced cells formed abundant syncytia when they were cocultured in step 3 with the infected H9 cells (Figure 4). Staining of the rinsed monolayers after this coculturing of the HeLa-tc-gp160 cells with the suspension of CD4-positive H9 HIV-*gpt* or H9 *vif*-deleted HIV-*gpt* cells showed equal numbers of syncytia. Thus, in one experiment these two monolayers contained 29 $\pm$ 3% and 27 $\pm$ 4% of the total nuclei in syncytia, respectively. Because uninfected H9 cells could also form syncytia with induced HeLa-tc-gp160 cells, we isolated stable populations of H9 HIV-*gpt* and H9 *vif*-deleted HIV-gpt cells by selection with mycophenolic acid. In control experiments we found that these selected cells and the control uninfected H9 cells all formed syncytia with HeLa-tc-gp160 cells to equal extents. Moreover, the H9 cells that stably expressed the HIV-*gpt* proviruses

contained the same quantities of cell surface CD4 as the uninfected H9 cells as determined by binding a monoclonal antibody to CD4 followed by [$^{125}$I]protein A (13). Consequently, there was not an effect of *vif* expression on cellular quantities of CD4 or on the ability of H9 cells to fuse with the induced HeLa-tc-gp160 cells. Because *vpu* and *nef* genes can down-modulate CD4 expression (5), their absence from the HXBII-derived pHIV-*gpt* provirus (19) was presumably a positive factor in enhancing syncytia formation in step 3. Interestingly, many of the syncytia contained giant nuclei or nuclei of widely divergent sizes. Presumably, these are generated after heterokayons proceed through aberrant mitoses and the nuclear membranes then reform around groups of intermixed chromosomes (18). This morphological feature suggests that the nuclear contents within the syncytia had substantially intermixed by 24-48 hrs after initiation of step 3.

In steps 4-6, virus produced by the heterokaryons was used to infect HeLa-CD4 cells (clone H1-J) (13) that were pretreated with polybrene (8μg/ml; Sigma, St. Loius, Mo.) for 30 min at 37 °C. The infected cells were seeded 48 hrs later in the presence of 40 μg/mL mycophenolic acid and the resistant colonies were fixed and stained 15-21 days later. We have obtained identical results in nine independent experiments including the four representative studies summarized in Table 1. In all cases, the heterokaryons

**Figure 3.3. Western immunoblot analysis showing induced expression of gp160 upon removal of tetracycline from HeLa-tc-gp160 cells.** HeLa-tc-gp160 cells were seeded in a 25 $cm^2$ tissue culture flask and grown in the absence or presence of tetracycline (0.5 μg/mL; Sigma St. Louis, Mo.). Total cell extracts were collected 48 hrs later. Protein concentrations were measured using the Bio-Rad Bradford assay (Bio-Rad Laboratories, Hercules, Ca.) and 20 μg of protein was analyzed by electrophoresis and immunoblotting as described in Figure 2. The blot was developed using 1:1000 dilution of sheep anti-gp120 antiserum (obtained through NIH AIDS Research and Reference Reagent Program, donated by Michael Phelan) followed by protein G-conjugated horseradish peroxidase at 1:5000 dilution (Bio-Rad, Ca.), and detection as described in Fig 2.



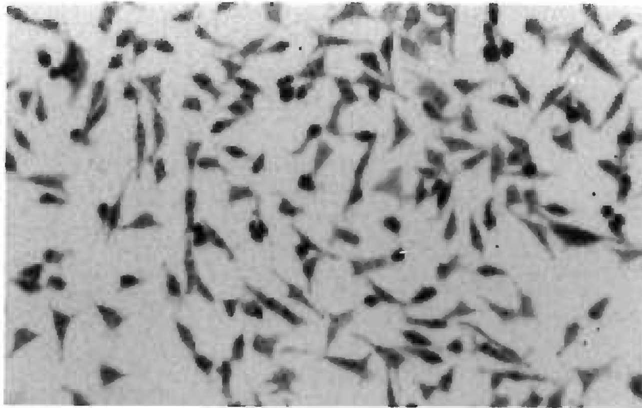
Mr x10-3
+TC
-TC
165
105
gp160
gp120

that contained the wild-type HIV-*gpt* provirus produced much more infectious virus than the heterokaryons that contained the *vif*-deleted HIV-*gpt* provirus. Indeed, the latter titers were zero in several experiments and the average titers of wild-type virus in our nine independent experiments were approximately 20 times higher than the average titers of *vif*-deleted virus. This result was obtained regardless of whether the infectious virions were harvested 24, 48, or 72 hrs after mixing the HIV-*gpt* expressing H9 cells with the HeLa-tc-gp160 cells.

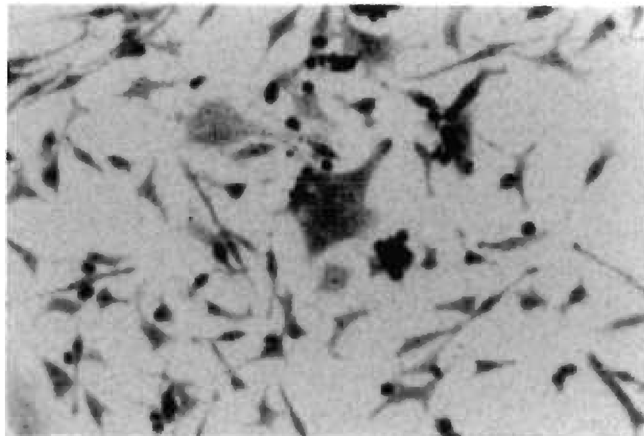
We also attempted to perform this genetic complementation study in the reverse manner by expressing the HIV-*gpt* provirus in HeLa-CD4 cells and the HXB2 *env* gene in H9 cells. Unfortunately, the H9 cells could be transfected only inefficiently, and we were unable to obtain significant numbers of syncytia in step 3 or of rescued infectious virus in step 4. Even if more efficient transfection of the H9 cells could be achieved, it is likely that the resulting cells with gp120-gp41 complexes would fuse not only with HeLa-CD4 cells but also with other CD4-positive H9 cells in the cultures. Apoptotic changes within the transfected H9 cultures would also have been expected (17). For these reasons, we concluded that the simple inverse protocol was not technically feasible.

**Figure 3.4. Formation of syncytia by coculturing HeLa-tc-gp160 and infected H9-42 (*vif*-deleted HIV-*gpt*) cells.** Panel A: HeLa-tc-gp160 cells grown in the presence of tetracycline do not synthesize gp160 or fuse with the H9-42 cells. HeLa-tc-gp160 cells ($3 \times 10^5$) were seeded in a 25 $cm^2$ tissue culture flask and grown for 2 days in medium containing 0.5 μg/ml tetracycline (Sigma, St. Louis, Mo) before H9 cells ($4 \times 10^6$) were added. The cocultures were then grown for 48 hrs. Cells were observed under light microscopy after removing the medium and the unabsorbed cells, rinsing the adherent cells with phosphate buffered saline (Life Technologies, Grand Island, NY), fixing with cold 100% methanol and staining with 0.1% toluidene blue in 30% ethanol. Panel B: H9 cells infected with *vif*-deleted HIV-*gpt* were added to HeLa-tc-gp160 cells that were grown in the absence of tetracycline. Heterokaryons were allowed to form for 48 hrs. The cells were then fixed and stained as in panel A. The small round darkly stained cells in panels A and B are H9 cells that adhered to the monolayers and were not removed by rinsing. Indistinguishable results were obtained with the H9 cells that expressed wild-type HIV-*gpt* (see text). Magnification for panels A and B is 800X.

A.



B.



The simplest interpretation of these results is that nonpermissive H9 cells contain a potent inhibitor of infectious HIV-1 production and that this inhibitor is counteracted by the viral-encoded Vif protein. The alternative hypothesis, that the permissive HeLa cells contain a permissivity factor that replaces the requirement for Vif, appears to be inconsistent with our results. Even if this putative factor were slowly acting or incapable of repairing aberrant HIV-1 components that had been synthesized in the H9 cells prior to heterokaryon formation, we would have expected to see a relatively enhanced titer of *vif*-deleted-HIV-*gpt* virus by 48-72 hrs after initiating the heterokaryon formation. However, the heterokaryons that contained the *vif*-deleted-HIV-*gpt* provirus did not slowly begin to produce infectious virus. Alternatively, the possibility exists that a putative permissivity factor of HeLa cells might become inactivated in heterokaryons. However, this inactivation seems very unlikely because its function was not observed even at the initial stages of heterokaryon formation (see Table 1).

Although the heterokaryons that express wild-type HIV-*gpt* release many more infectious virions than the heterokaryons that express *vif*-deleted HIV-*gpt*, the titers in these experiments were also quite low (see Table 1). Indeed, these titers were generally only approximately 1-2% as high as the titers released in step 1 by the initially transfected COS-7 cells. This

Table 1. Differences between the infectivities of wild-type and *vif*-delelted HIV-*gpt* virions produced from heterokaryons assayed on HeLa-CD4 cells

| Experiment # | Time of viral harvest [a] (hrs) | HIV-*gpt* infection [b] (no of colonies /dish) | |
|---|---|---|---|
| | | Wild-type | *vif*-deleted |
| 1 [c] | 24 | 20 | 0 |
| | 48 | 13 | 0 |
| | 72 | 4 | 0 |
| 2 [d] | 24 | 36 | 7 |
| | 48 | 10 | 0 |
| | 72 | 2 | 0 |
| 3 [e] | 24 | 41 | 7 |
| | 48 | 16 | 3 |
| | 72 | 10 | 3 |
| 4 | 48 | 110 | 0 |
| | 48 | 39 | 0 |
| | 48 | 34 | 0 |

[a] Heterokaryons were formed by coculturing H9 with HeLa-tc-gp160 cells as described in Figure 1. Virus harvested at 24, 48, and 72 hrs after coculturing the cells was used to infect HeLa-CD4 cells.

[b] HeLa-CD4 cells were infected with wild-type or vif-deleted HIV-gpt virus produced from heterokaryons. Infected cells were selected with medium containing mycophenolic acid and selected colonies were counted 15-21 days later.

[c,d,e] In these experiments H942 cells that are expressing high levels of CD4 were used to coculture with HeLa-tc-gp160 cells. The H9-42 cells were made by transducing H9 cells with pSFF-CD4 retroviral vectors as described previously (13), and were generously donated by Dr. Emily J. Platt.

quantitative difference is reasonable in part because COS-7 cells express transfected plasmids that have the SV40 origin very efficiently. Moreover, only small fractions of the H9 nuclei appeared to enter the heterokaryons in step 3, and the latter may produce virions inefficiently. In addition, the H9 and HeLa-tc-gp160 cells aggregated in the cocultures and many aggregates lifted into the culture medium. Previous work has shown that CD4-positive lymphoid cells undergo degenerative changes including apoptosis in cultures with cells that express HIV-1 envelope glycoprotein (17). These morphological changes occurred equally in the cocultures that contained the wild-type and *vif*-deleted HIV-*gpt* proviruses. For these same reasons, however, even trace amounts of H9 cell contamination with virus or transfected COS-7 cells in step 3 might distort the eventual results. Because the COS-7 cells are fully permissive as documented above, this factor would have contributed equally to the titers of the wild-type and *vif*-deleted viruses, and could not have reproducibly given the results that we observed.

Based on these considerations, we conclude that nonpermissive human T-lymphocytes contain an endogenous and potent inhibitor of HIV-1 production that is overcome by the viral-encoded Vif protein, and we infer that this inhibitor may also occur in other nonpermissive cells including macrophages. It is ironic that this inhibitor is apparently present only in these

natural targets for HIV-1 infection, and that it is absent in other human cells. Thus, we propose that CD4-positive T-lymphocytes and macrophages may have an endogenous intracellular capacity to block HIV-1 replication and to cure the disease, but this potent inhibitor is held in check by Vif. This conclusion is consistent with a recent report that the Vif proteins of HIV-1 and simian immunodeficiency virus from African green monkeys may be active only in CD4-positive T-lymphocytes from the respective species (25). Moreover, these Vif proteins act in a cell-specific rather than in a virus-specific manner. These results imply that Vif neutralizes the inhibitory activity of a cellular factor that occurs in lymphocytes, in agreement with our results. Several viral proteins function to thwart the host immune system (6, 10, 23), to block apoptosis, or to extend cellular lifespans (15), but we are unaware of another viral protein that blocks an intracellular inhibitor of infectious virus production. Further work will be needed to determine whether this inhibitor damages the virions during assembly in the producer cells or whether it is incorporated into the virions to block their infectivity in the target cells. We are currently attempting to identify an endogenous inhibitor in nonpermissive human cells. The implication that Vif counteracts a potent inhibitor of HIV-1 replication suggests that it would be an exceptionally promising target for drug development.

**Acknowledgements**

This research was supported by NIH grant (CA67358). N.M. is partially supported by an NIH predoctoral fellowship in Molecular Hematology and Oncology (T32HL07781). We thank Hermann Bujard (Universitat Heidelberg, Germany) for the generous gift of pUHD10-3. We are especially grateful to our colleague Emily Platt for generously donating the H9-42 cells that were used in several experiments, and for providing advice for construction of *vif*-deleted pHIV-*gpt*, and to Susan Kozak, Shawn Kuhmann, Ali Nouri, David Johnson, and Chetankumar Tailor for their encouragement and helpful suggestions.

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# Chapter 4

## gp120 Envelope Glycoproteins of Human Immunodeficiency Viruses Competitively Antagonize Signaling by Coreceptors CXCR4 and CCR5

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Running Title: Chemokine Receptors/HIV-1 Coreceptors
Classification: Biochemistry

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**Abstract**

Signal transductions by the dual function CXCR4 and CCR5 chemokine receptors/human immunodeficiency virus type 1 (HIV-1) coreceptors were electrophysiologically monitored in *Xenopus laevis* oocytes that also coexpressed the viral receptor CD4 and a G protein-coupled inward rectifying $K^+$-channel (Kir 3.1). Large Kir 3.1-dependent currents generated in response to the corresponding chemokines (SDF-1$\alpha$ for CXCR4; MIP-1$\alpha$, MIP-1$\beta$ and RANTES for CCR5) were blocked by pertussis toxin, suggesting involvement of $G_i$ proteins. Prolonged exposures to chemokines caused substantial but incomplete desensitization of responses with time constants of 5-7 minutes; and recovery time constants of 12-19 minutes. CXCR4 and CCR5 exhibited heterologous desensitization in this oocyte system, suggesting possible inhibition of a common downstream step in their signaling pathways. In contrast to chemokines, perfusion with monomeric or oligomeric gp120 preparations derived from several isolates of HIV-1 did not activate signaling by CXCR4 or CCR5 regardless of CD4 coexpression. However, adsorption of gp120 from a T-cell-tropic virus resulted in CD4-dependent antagonism of CXCR4 response to SDF-1$\alpha$, whereas gp120 from macrophage-tropic viruses caused CD4-dependent antagonism of CCR5 response to MIP-1$\alpha$. These antagonisms could be partially overcome by high concentrations of chemokines and were specific for coreceptors of the corresponding HIV-1

isolates, suggesting that they resulted from direct interactions of gp120-CD4 complexes with coreceptors and that they did not involve the desensitization pathway. These results indicate that monomeric or oligomeric gp120s specifically antagonize CXCR4 and CCR5 signaling in response to chemokines, but do not exclude the possibility that gp120s might also function as weak agonists in some cells. The gp120-mediated disruption of CXCR4 and CCR5 signaling may contribute to AIDS pathogenesis.

## Introduction

Fusion of the viral membrane with the cell surface membrane during infection by human immunodeficiency virus type 1 (HIV-1) involves collaboration between the CD4 receptor and a coreceptor in the host cell membrane (1, 7, 14, 17, 20, 23). The coreceptors are seven transmembrane G protein-coupled receptors for proinflammatory chemokines (1, 7, 14, 17, 20, 23). The major coreceptor for macrophage-tropic (M-tropic) HIV-1 isolates is CCR5 which is activated by the chemokines MIP-1α, MIP-1β, and RANTES, whereas the major coreceptor for T-cell-tropic HIV-1 isolates is CXCR4 which is activated by SDF-1 (1, 7, 14, 17, 20, 23). Individuals homozygous for a defective CCR5 Δ32 allele are resistant to infection, suggesting that M-tropic viruses dependent on CCR5 are responsible for initial viral transmission (44, 52). In contrast, viruses that use CXCR4 or other alternative coreceptors accumulate late in disease during the demise of the immune system (10, 54). Recent evidence has suggested that the gp120 envelope glycoprotein of HIV-1 forms a ternary complex with CD4 and coreceptors, and that the interaction with CD4 causes a conformational change in gp120 that facilitates subsequent interaction with the coreceptor (40, 60, 62). Binding of gp120-CD4 complexes to coreceptors reduces their affinity for [$^{125}$I]chemokines (30, 31, 46, 60, 62).

The roles of chemokine receptor signaling in HIV-1 infection remain uncertain. Several mutations in CCR5 and CXCR4 that prevent G protein activation do not perturb their coreceptor activities (16, 21, 22, 27), suggesting

that virus-induced activation of these chemokine receptors cannot be essential for infection. On the other hand, evidence reported during preparation of this manuscript indicated that gp120 derived from several M-tropic isolates of HIV-1 could induce CCR5-mediated $Ca^{+2}$ mobilization and chemotaxis in a proportion of activated CD4-positive human T-lymphocytes (61). However, similar glycoprotein preparations from other immunodeficiency viruses that also use CCR5 failed to activate signaling. Moreover, gp120 preparations from T-cell-tropic viruses did not activate signaling by CXCR4. These results imply that gp120 shed from nests of cells infected with some M-tropic HIV-1 might chemoattract uninfected CD4 and CCR5-positive cells or enhance their susceptibilities to infection or to apoptotic stimuli. Another recent report indicated that gp120s from various T-cell-tropic or M-tropic HIV-1 isolates did not induce detectable $Ca^{+2}$ mobilization in activated CD4-positive T-lymphocytes, despite the substantial responses of these same cells to chemokines (12). However, this group detected significant gp120-mediated increases in tyrosine phosphorylation of Pyk2 tyrosine kinase in these cells and they presented evidence that this response was dependent on the chemokine receptors CXCR4 and CCR5. The implications of these recent observations are more thoroughly discussed below (see Discussion).

A potential complication in this field derives from evidence that gp120 binding to CD4 can activate the associated *Lck* tyrosine kinase, with resultant

activation of the Raf-MAP kinase pathway and of transcription factors including NF-κB (5, 6, 24, 49), and with an enhancement in the susceptibility to infection of certain cells (58, 59). Signal transduction by chemokine receptors has also been reported to activate MAP kinases and src-family tyrosine kinases (3, 13, 15, 19, 37, 45). Because of the likelihood of substantial overlap and cross-talk between the gp120-activated *Lck* and chemokine receptor signaling pathways, which are presumably initiated in a common multiprotein assemblage, a thorough understanding of gp120 signaling will ultimately require controlled studies using cells that lack either or both of these pathways. In addition, the assays such as $Ca^{+2}$ mobilization that have been commonly used to study chemokine receptor signaling generally appear to have high backgrounds and poor signal-to-noise ratios. Many factors other than G proteins including tyrosine kinases can induce $Ca^{+2}$ mobilization with resultant Pyk2 activation (13, 26, 41) or chemotaxis (2). As one approach toward analyzing these issues, we developed a sensitive system in which chemokine receptor signaling in *Xenopus* oocytes is coupled to a G protein-activated inward-rectifying $K^+$-channel (Kir 3.1) (11, 39). Because Kir 3.1 is a high conductivity channel that is promiscuously activated by Gβγ subunits (8, 9, 43, 51, 57), we reasoned that this channel might generate large currents in response to activation of CXCR4 or CCR5. *Xenopus* oocytes lack CD4, *Lck* and chemokine receptors but contain many heterotrimeric G proteins

and have been widely used to study chemokine receptors and other G protein-coupled receptors (18, 35, 53). Our results suggest that this system may be exceptionally useful for quantitatively evaluating signal transduction by CXCR4 and CCR5. In this oocyte system, highly purified gp120 preparations did not activate CXCR4 or CCR5 in a CD4-dependent or independent manner. Rather, gp120 from a T-cell-tropic HIV-1 isolate caused a CD4 dependent inhibition of CXCR4 activation by SDF-1α. Similarly, gp120 from an M-tropic HIV-1 isolate caused CD4-dependent inhibition of CCR5 signaling.

## Results

### Expression and analyses of biologically active proteins in *Xenopus* oocytes

This investigation depended on expression of proteins in *Xenopus* oocytes and use of purified chemokines and gp120s that were biologically active. As shown in Figure 1A, our T-cell tropic gp120 IIIB preparation was highly purified and did not appear to be significantly contaminated with chemokines or other proteins that contained amino groups able to react with the [$^{125}$I]Bolton-Hunter reagent. Similarly, the SDF-1α appeared to be homogeneous. Figure 1C shows the kinetics of binding different concentrations of [$^{125}$I]gp120 IIIB onto HeLa-CD4 cells (clone H1-J) at 37 $^{o}$C. Negligible binding occurred onto control HeLa cells. Moreover, almost identical binding kinetics occurred at 25 $^{o}$C (results not shown). To estimate the binding affinity of this [$^{125}$I]gp120 preparation, we performed competition experiments (n=2) using increasing concentrations of unlabeled gp120 IIIB (see Figure 1D). Analysis of this data suggested a $K_D$ for the binding of approximately 43 nM. Similar degrees of purity and binding activity were obtained with the M-tropic gp120s used in this investigation, although a higher background of binding onto control HeLa cells was observed with the JR-FL gp120 preparation (results not shown). Figure 2 shows protein immunoblot analyses demonstrating expression of CD4 (panel A) and CCR5 (panel B) in membranes from oocytes that had been injected previously with the

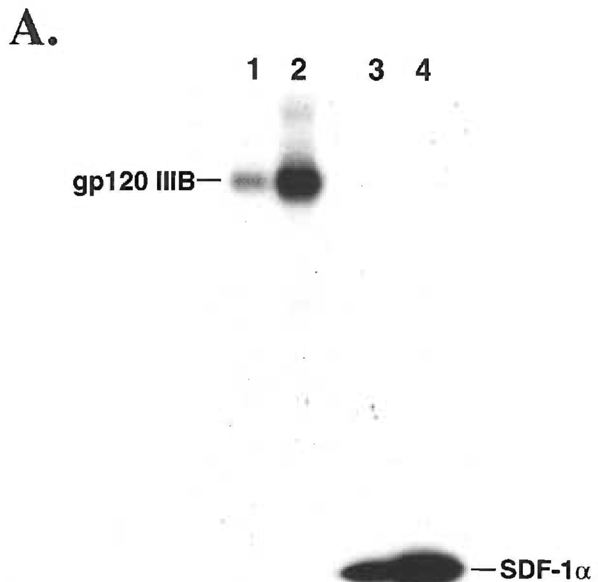
corresponding cRNAs. The CD4 was present on oocyte surfaces as indicated by binding of [$^{125}$I]gp120 IIIB from the medium (Figure 1B). Evidence for functional expression of CD4, CCR5, CXCR4 and Kir 3.1 on oocyte surface membranes was also obtained by two-electrode voltage clamp analyses (see below).

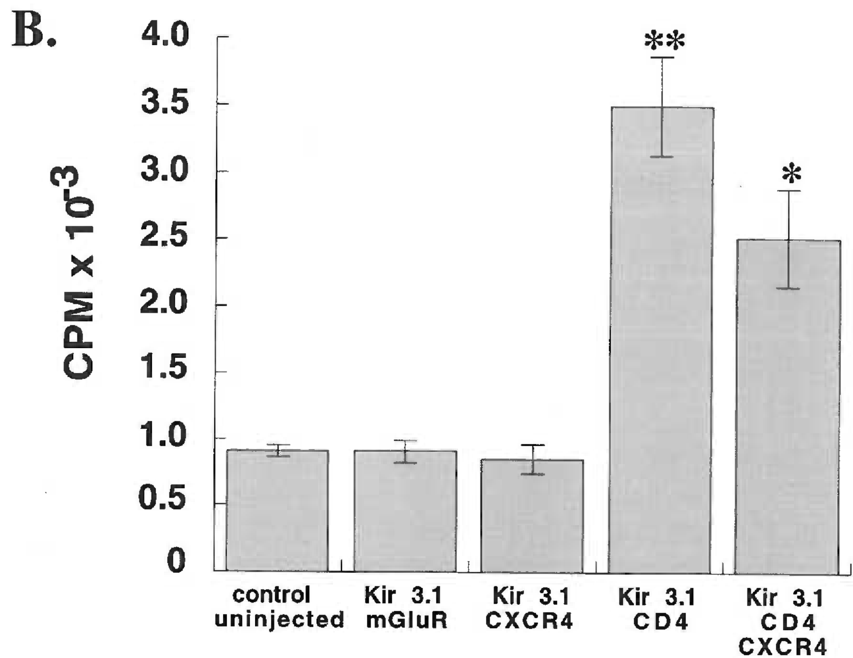
**Chemokine-induced activation of Kir 3.1 in *Xenopus* oocytes**

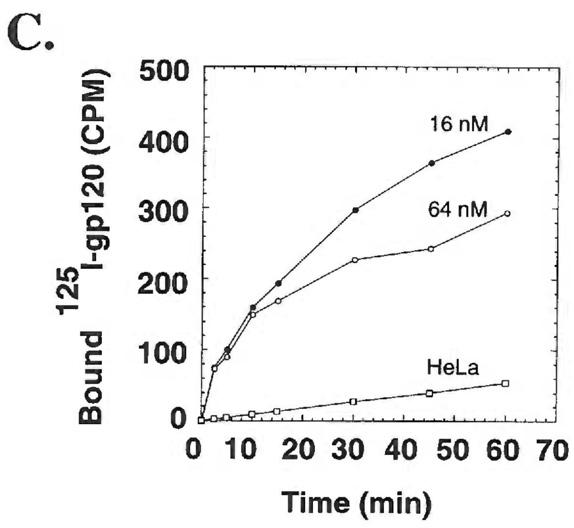
Capped cRNAs for chemokine receptors and Kir 3.1 were coinjected into oocytes, and voltage clamp current recordings were obtained 2-3 days after injection. Oocytes were clamped at -30 mV in high $K^+$-Ringer, and varying concentrations of receptor agonists were applied by bath perfusion. Since glutamate is known to activate Kir 3.1 through binding to the G protein-coupled metabotropic glutamate receptor mGluR 2 (53), we used this receptor as a control. Figure 3A shows the concentration dependence of inward currents induced by L-Glu. This response saturated at glutamate concentrations of 50-100 μM with half maximal stimulation at 5.96 ± 1.43 μM (n=4). As shown in figure 3B, the inward currents induced by SDF-1α in oocytes expressing CXCR4 were also concentration-dependent. Saturation was reached between 10-30 nM SDF-1α with a half-maximal stimulation at 2.84 ± 0.57 nM (n=3). Similarly, MIP-1α activated CCR5 (Figure 3C) with

**Figure 4.1. Characterization of $[^{125}I]$gp120 IIIB binding to *Xenopus* oocytes and HeLa cells.** A. Analysis of purity of HIV-1 gp120 IIIB. $[^{125}I]$Bolton-Hunter reagent was used to label HIV-1 gp120 IIIB and SDF-1α. $[^{125}I]$gp120 (lanes 1-2) and $[^{125}I]$SDF-1α (lanes 3-4) were then analyzed by electrophoresis in the presence of 0.1% sodium dodecylsulfate in a 10% polyacrylamide gel followed by autoradiography. Lane 1, $[^{125}I]$gp120 (30,000 cpm); lane 2 $[^{125}I]$gp120 (150,000 cpm); lane 3 $[^{125}I]$SDF-1α (60,000 cpm); lane 4 $[^{125}I]$SDF-1α (300,000 cpm). B. $[^{125}I]$gp120 IIIB binding to *Xenopus* oocytes. $[^{125}I]$gp120 IIIB ($1.2 \times 10^6$ cpm/mL) was used for binding to one batch of oocytes that coexpressed combinations of Kir 3.1 with mGluR 2, CD4 and CXCR4 as well as to uninjected control oocytes. The binding to the oocytes that expressed CD4 was significantly greater than to control oocytes (**$p<0.0001$ and *$p<0.0024$ by the Student's unpaired *t* test). Each column represents mean ± SEM for 5 determinations. C. $[^{125}I]$gp120 IIIB binding to HeLa-CD4 (clone H1-J) and to control HeLa cells. Cell cultures were incubated at 37 $^{o}$C with $[^{125}I]$gp120 IIIB for various times in the presence of unlabelled gp120 IIIB at a concentration of 16 nM (solid circle) or 64 nM (open circle). Non-specific background was determined by incubating HeLa cells lacking CD4 with the same amount of $[^{125}I]$gp120 IIIB in the absence of unlabelled gp120 IIIB (open squares). D. Competitive

binding and Scatchard analysis of $[^{125}I]$gp120 IIIB binding onto HeLa-CD4 cell clone H1-J. H1-J and HeLa cells were incubated at 37 $^{o}C$ for 1 hr with $[^{125}I]$gp120 IIIB in the presence of competing unlabelled gp120 at the concentrations shown. The Scatchard plots (inset) from two assays indicated an average $K_D$ of 43 nM for this labelled gp120 and the presence of $4.5 \times 10^5$ accessible binding sites per HeLa-CD4 cell (B is bound CPM, F is free CPM).


A.
1
2
3
4
gp120 IIIB—
—SDF-1α


B.
CPM x 10-3
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0
**
*
control uninjected
Kir 3.1 mGluR
Kir 3.1 CXCR4
Kir 3.1 CD4
Kir 3.1 CD4 CXCR4


C.
Bound 125I-gp120 (CPM)
500
400
300
200
100
0
16 nM
64 nM
HeLa
0 10 20 30 40 50 60 70
Time (min)

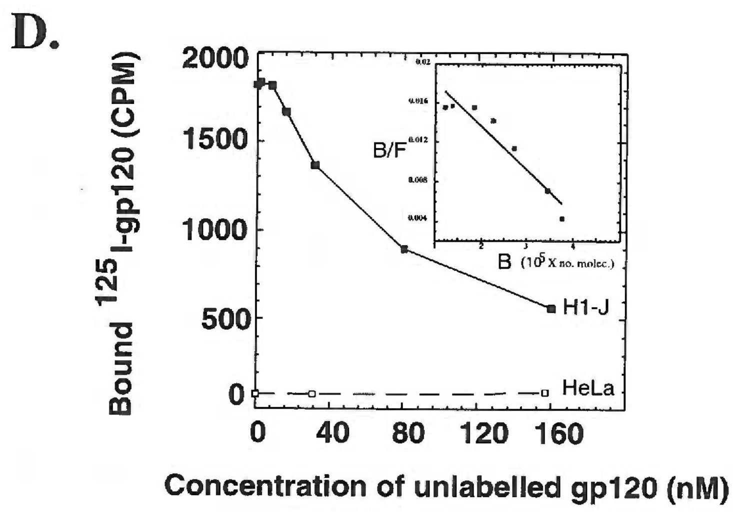
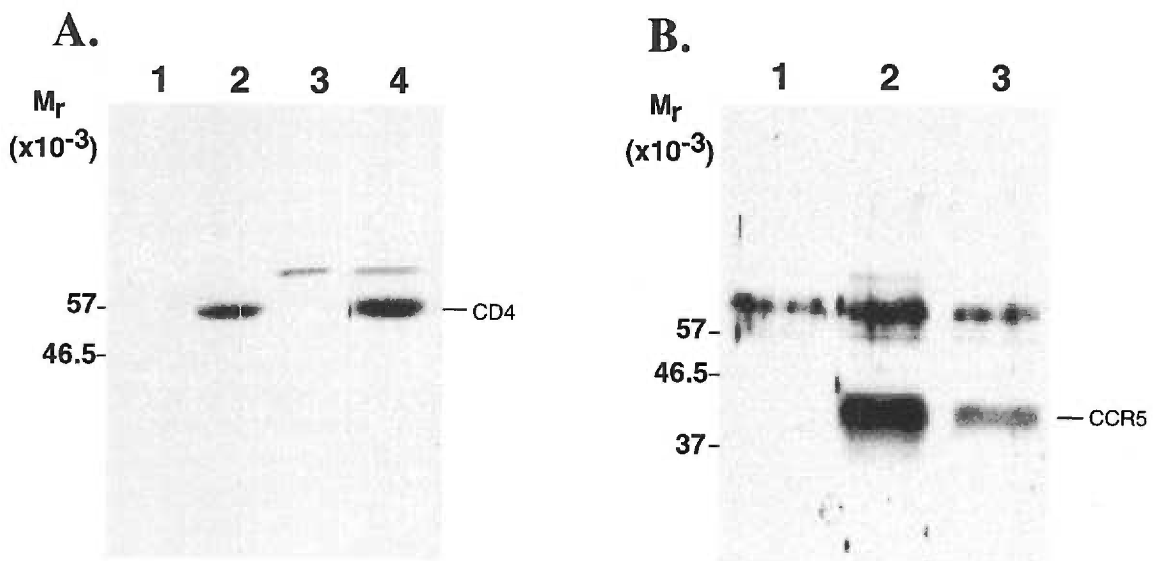

D.
Bound 125I-gp120 (CPM)
2000
1500
1000
500
0
B/F
B (10⁵ X no. molec.)
H1-J
HeLa
0 40 80 120 160
Concentration of unlabelled gp120 (nM)

**Figure 4.2. Western immunoblot evidence for expression of CD4 and CCR5 in oocyte membranes.** A. Immunoblot detection of CD4 in HeLa-CD4 cells and in membranes from single oocytes. Lane 1: control HeLa cells; lane 2: HeLa-CD4 cells (clone H1-J) cells. Lanes 3-4 are membranes collected from a control uninjected oocyte and from an oocyte injected with CD4 cRNA, respectively. The proteins were separated by electrophoresis, transferred to nitrocellulose and blotted with polyclonal CD4 antibody. B. Immunoblot detection of CCR5 in *Xenopus* oocyte membranes. Lane 1: membranes from uninjected control oocytes; membranes in lanes 2 and 3 were from oocytes injected with two different dilutions of CCR5 cRNA. The nitrocellulose membrane was immunoblotted with rabbit antiserum to CCR5.


A.
1
2
3
4
$M_r$
($x10^{-3}$)
57-
46.5-
CD4
B.
1
2
3
$M_r$
($x10^{-3}$)
57-
46.5-
37-
CCR5

half-maximal activation at 0.94 $\pm$0.04 nM MIP-1$\alpha$ (n=3).

Application of RANTES also induced currents in oocytes that coexpressed CCR5 and Kir 3.1 (data not shown). In addition, the CCR5 agonist MIP-1$\beta$ activated currents in oocytes expressing this receptor with a half maximal stimulation at 0.33 $\pm$ 0.04 nM (data not shown). The activities of these agonists were specific, as concentrations of 10 nM MIP-1$\alpha$ did not induce currents in oocytes coexpressing Kir 3.1 and CXCR4 (n=2). Conversely, 10 nM SDF-1$\alpha$ did not induce currents in oocytes that coexpressed Kir 3.1 and CCR5 (n=7). Control uninjected oocytes and oocytes lacking chemokine receptors or Kir 3.1 din not show currents upon application of the ligands (data not shown).

To verify that the currents induced by chemokine application were the result of specific activation of the G-protein dependent $K^+$ channel Kir 3.1, we characterized the properties of these currents. Figure 4A-C shows the voltage dependence of currents induced by different concentrations of L-Glu, SDF-1$\alpha$ and MIP-1$\alpha$, respectively. As previously shown for Kir 3.1, the currents showed a strong inward rectification at all agonist concentrations (18). In addition, the reversal potential of currents induced by 3 nM SDF-1$\alpha$ changed by 54.2 mV per 10 fold change in extracellular $K^+$, close to the prediction of the Nernst equation for a potassium-selective conductance (Figure 4D). We

**Figure 4.3. (left) Activation of currents in oocytes coexpressing Kir 3.1 with mGluR 2 (A), CXCR4 (B), and CCR5 (C).** Inward currents were seen in response to superfusion of ligands for duration indicated by bar above trace. Holding potential was -30 mV and recording solutions were $K^+$-Ringer containing 100 mM KCl. (right) Concentration-dependence of currents. Data were fitted to a rectangular hyperbola, yielding the following $EC_{50}$ values for each ligand: L-glutamate, 5.9 ± 1.4 (n=4); SDF-1α, 2.8 ± 0.6 nM (n=3); MIP-1α, 0.9 ± 0.04 (n=3)

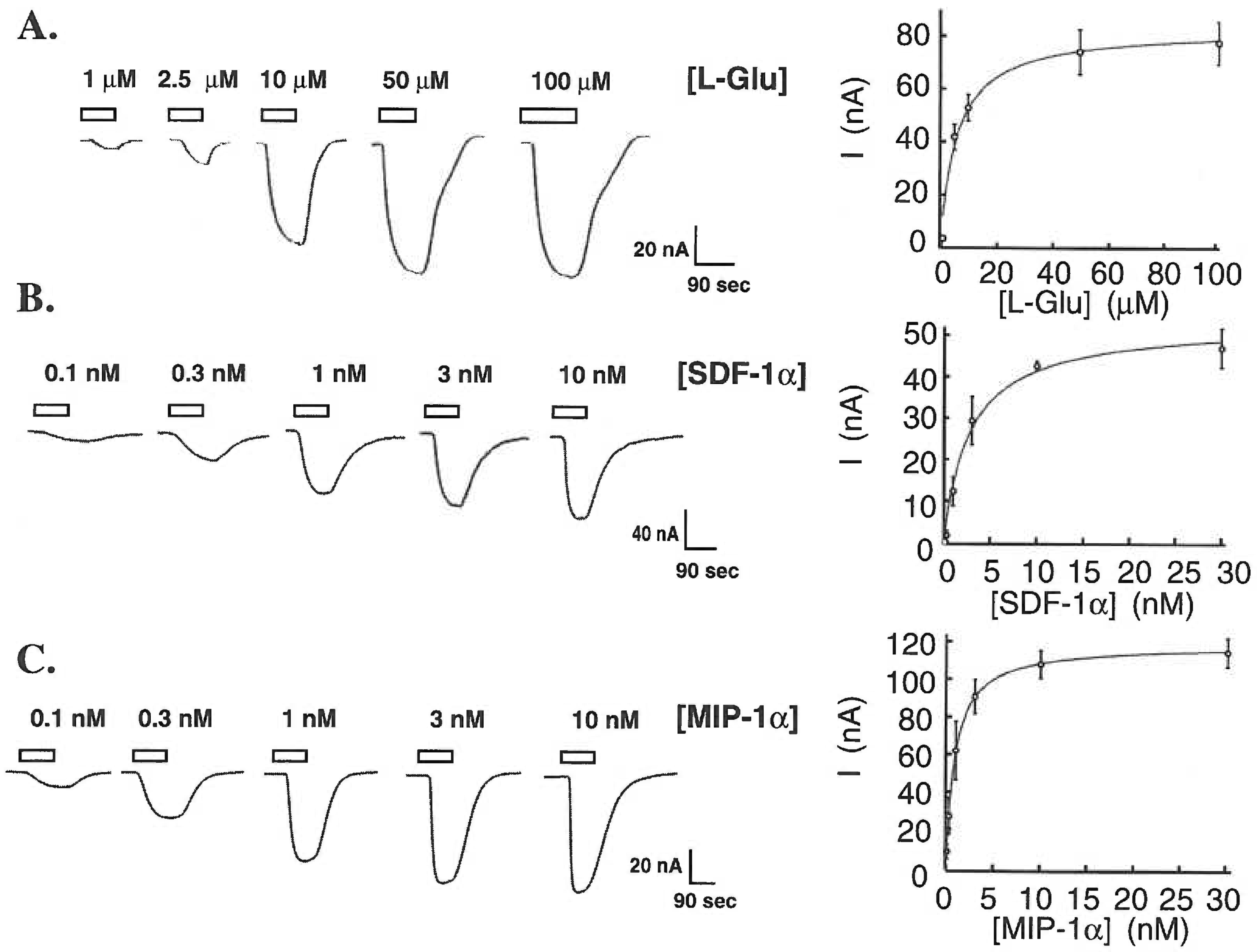
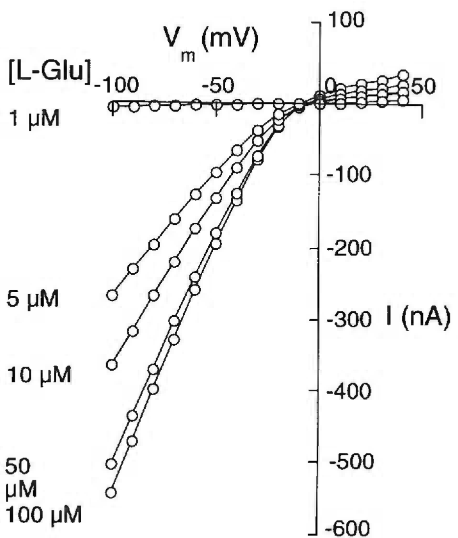
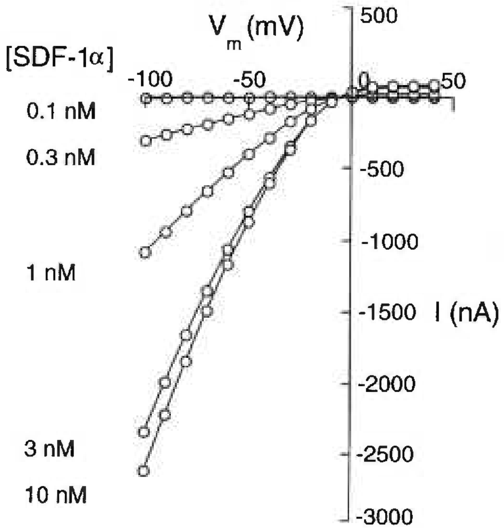
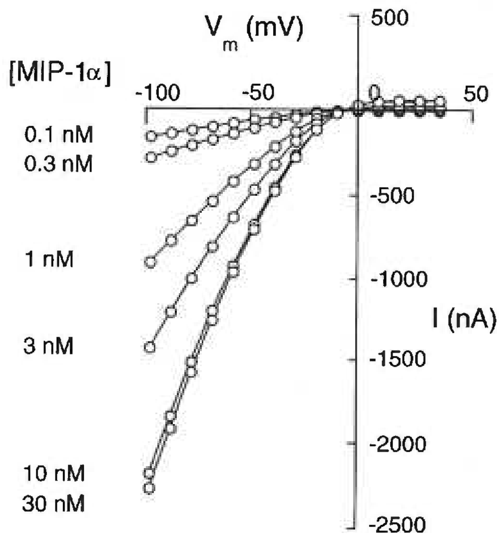

A.
1 μM
2.5 μM
10 μM
50 μM
100 μM
[L-Glu]
20 nA
90 sec
I (nA)
80
60
40
20
0
0 20 40 60 80 100
[L-Glu] (μM)
B.
0.1 nM
0.3 nM
1 nM
3 nM
10 nM
[SDF-1α]
40 nA
90 sec
I (nA)
50
40
30
20
10
0
0 5 10 15 20 25 30
[SDF-1α] (nM)
C.
0.1 nM
0.3 nM
1 nM
3 nM
10 nM
[MIP-1α]
20 nA
90 sec
I (nA)
120
100
80
60
40
20
0
0 5 10 15 20 25 30
[MIP-1α] (nM)

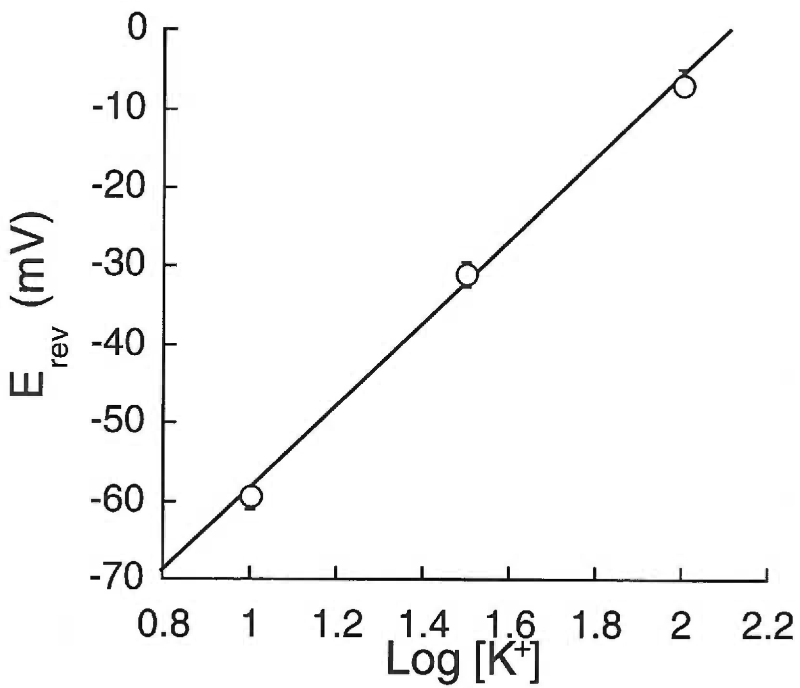
**Figure 4.4. Characterization of ligand-induced currents.** (A-C) Voltage-dependence of currents induced by superfusion of indicated concentrations of ligand in $K^+$-Ringer onto three representative oocytes coexpressing Kir 3.1 with either mGluR2 (A), CXCR4 (B), or CCR5 (C). D. Reversal potential of currents produced by 3 nM SDF-1$\alpha$ in oocytes coexpressing Kir 3.1 and CXCR4 is dependent on external $K^+$ concentrations. Line shown is least squares fit (54mV/decade) through points representing mean ± SEM (n=3). E. Inhibition of CXCR4/Kir 3.1 mediated currents by pertussis toxin (PTX) pretreatment (1 μg/ml, 48 hrs) or $BaCl_2$ (100 μM). Each point represents mean ± SEM, n=4.


A.
[L-Glu]
$V_m$ (mV)
-100
-50
0
50
100
-100
-200
-300
-400
-500
-600
I (nA)
1 µM
5 µM
10 µM
50 µM
100 µM

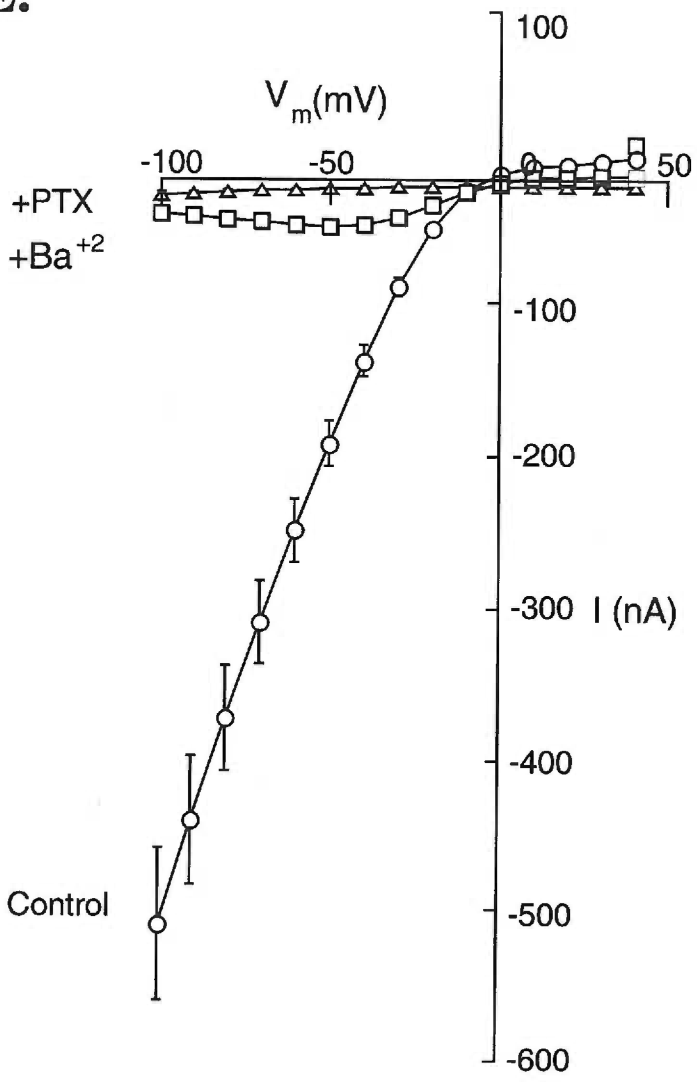

B.
[SDF-1α]
$V_m$ (mV)
-100
-50
0
50
500
-500
-1000
-1500
-2000
-2500
-3000
I (nA)
0.1 nM
0.3 nM
1 nM
3 nM
10 nM


C.
[MIP-1α]
$V_m$ (mV)
-100
-50
0
50
500
-500
-1000
-1500
-2000
-2500
I (nA)
0.1 nM
0.3 nM
1 nM
3 nM
10 nM
30 nM

D.




E.



also tested the ability of the potassium channel blocker $Ba^{+2}$ to block the currents induced by SDF-1α interaction with CXCR4. Addition of 100 μM $BaCl_2$ voltage-dependently inhibited SDF-1α activation of Kir 3.1 (Figure 4E; n=4). In addition, we found that pertussis toxin (1μg/ml, 48 hr) largely abolished 1 nM SDF-1α activation of CXCR4 (Figure 4E; n=4). These data show that the currents induced by chemokine application resulted from activation of Kir 3.1 by specific G protein-receptor signaling pathways in the oocyte.

**Desensitization of chemokine receptor signaling**

Figure 5A shows a further analysis of the currents induced by 10 μM L-Glu in oocytes that coexpressed mGluR 2 and Kir 3.1. Glutamate-dependent currents desensitized during prolonged agonist application with a time constant of 7.5 ± 1.2 min (n=5), to a steady-state value representing 44.3 ± 5.1% of the initial response. Figure 5B shows a similar response of the CCR5 receptor to a prolonged application of 10 nM MIP-1α. The response decayed with a time constant of 5.3 ± 0.4 min (n=11) to 46.7 ± 6.0 % of the initial response. Similarly, prolonged application of 10 nM SDF-1α resulted in attenuation of the CXCR4 response to 23.2 ± 2.7 % of the initial value with a time constant of 6.1 ± 0.6 min (n=10) (Figure 5C). Following washout of agonist, the responses recovered slowly from desensitization (Figure 5). The time constant for

recovery of CXCR4 following steady-state desensitization induced by SDF-1α was 12.8 ± 1.7 minutes, while the time constant for CCR5 recovery after desensitization with MIP-1α was approximately 18.7 ± 4.4 minutes.

Cross desensitization in oocytes coexpressing CXCR4 and CCR5 together with Kir 3.1 was also apparent. Figure 5C shows that brief non-desensitizing applications of MIP-1α induced similar current amplitudes. Following a prolonged exposure to SDF-1α that resulted in desensitization of the CXCR4 response, the CCR5 response to MIP-1α was also desensitized. This cross-desensitization by SDF-1α in oocytes coexpressing CXCR4, CCR5 and Kir 3.1, resulted in inhibition of CCR5 responses to MIP-1α by 50.2 ± 5.9% (n=6). Conversely, desensitization with MIP-1α inhibited SDF-1α activation of CXCR4 by 66.0 ± 9.5% (n=4). Similarly, desensitization with SDF-1α in oocytes that coexpressed CXCR4, mGluR 2 and Kir 3.1 inhibited L-Glu activation of mGluR 2 by 23.8 ± 5.5% (n=4), while desensitization with L-Glu inhibited SDF-1α activation of CXCR4 by 84 ± 5.9% (n=3). This heterologous desensitization suggests the involvement of effectors downstream of receptor activation including G-proteins, ion channels, or both.

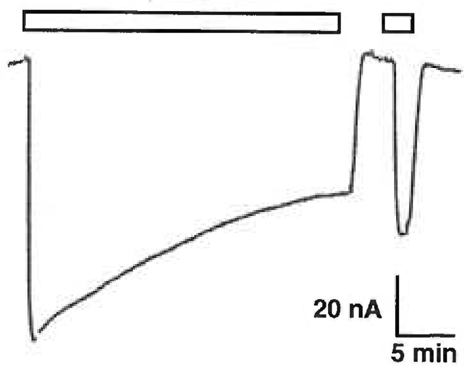
**Effects of viral gp120 on signal transduction by CXCR4 and CCR5**

To determine whether HIV-1 gp120 induces a signal upon binding to CD4 and the coreceptor, we tested highly purified T-cell tropic as well as M-tropic HIV-

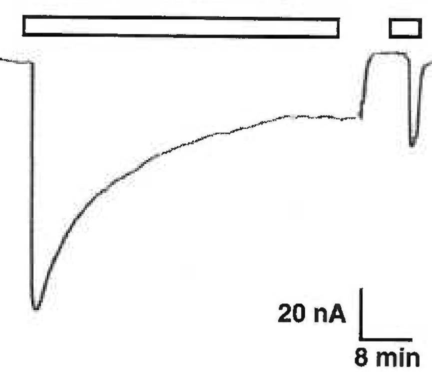
1 gp120 in the potassium channel activation assay. Currents were not generated in response to perfusion of HIV-1 gp120 IIIB (16nM) onto voltage-clamped oocytes coexpressing CD4, CXCR4, and Kir 3.1. Figure 6A shows currents induced upon application of 3 nM SDF-1$\alpha$ before and after application of gp120 IIIB. A total of 39 oocytes from 14 batches coexpressing CD4, CXCR4, and Kir 3.1 all failed to respond to application of gp120 IIIB. Moreover, the currents induced by SDF-1$\alpha$ did not decrease after short applications (30-90 seconds) of gp120 IIIB. Similarly, M-tropic gp120 from the JR-FL isolate of HIV-1 was perfused onto oocytes that coexpressed CD4, CCR5, and Kir 3.1. An inward current was induced upon application of 10 nM MIP-1$\alpha$, while M-tropic gp120 (20 nM) did not elicit any currents in the same oocyte, and brief applications of JR-FL gp120 did not decrease subsequent MIP-1$\alpha$ activation of CCR5 (Figure 6B). Identical results were obtained in 12 oocytes from 3 different batches coexpressing CD4, CCR5, and Kir 3.1. In addition, we found that M-tropic Ba-L gp120 also failed to induce signals in oocytes that had been coinjected with cRNAs for CD4, CCR5, and Kir 3.1 (n=3) (data not shown). Superfusion of gp120 onto voltage-clamped oocytes for longer times (10-20 min) was similarly ineffective at inducing currents in oocytes that were coinjected with cRNAs that encode CD4, respective coreceptors and Kir 3.1 (data not shown).

**Figure 4.5. Desensitization of chemokine and glutamate receptors.** A. Decay of inward current during continued application of 10 μM L-Glu (indicated by bar above trace) and partial recovery following washout of agonist in a representative oocyte coexpressing mGluR 2 and Kir 3.1. B. Similar homologous desensitization of CCR5 chemokine receptor response during long exposure to MIP-1α. C. Heterologous desensitization of CCR5 response by prolonged CXCR4 activation in oocytes coinjected with CXCR4, CCR5, and Kir 3.1 cRNA.

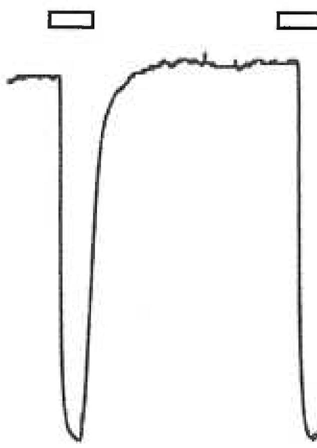
A.

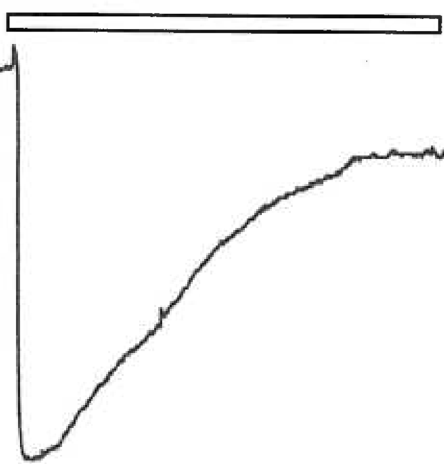



B.




C.



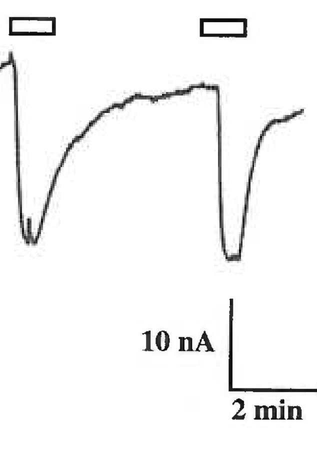
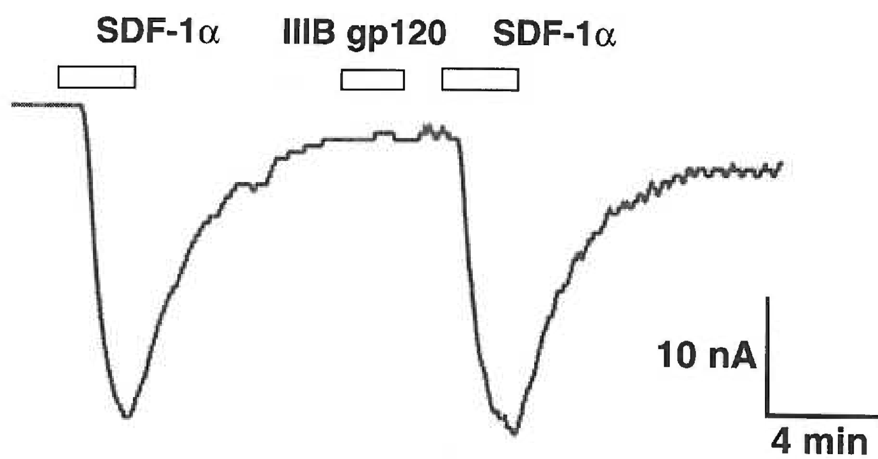
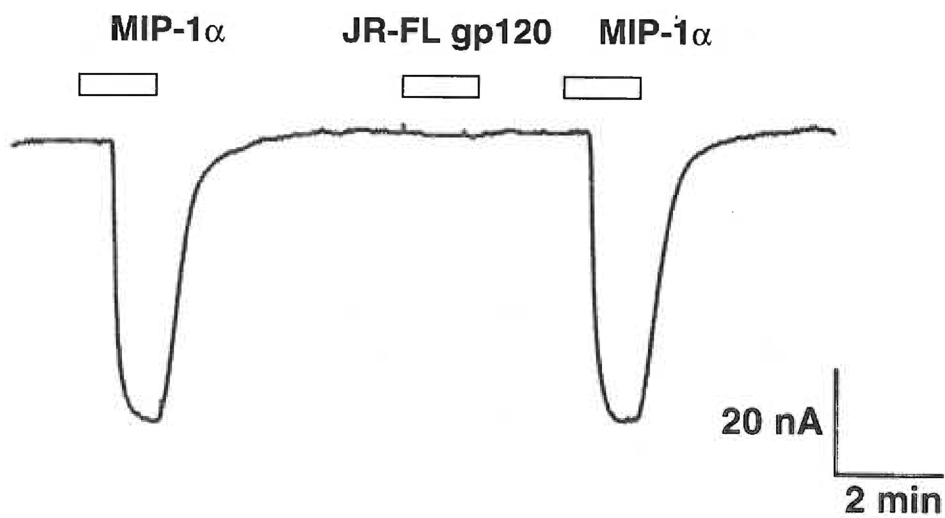



**Figure 4.6. HIV-1 gp120 IIIB and JR-FL do not induce currents in oocytes coexpressing CD4 and Kir 3.1 with their respective coreceptors.** A. An inward current was produced by application of 3 nM SDF-1α to a representative oocyte coexpressing CD4, CXCR4, and Kir 3.1. Application of 16 nM gp120 IIIB did not induce a current and also did not reduce the subsequent SDF-1α activation of CXCR4. B. Similar experiment showing lack of effect of JR-FL gp120 in a representative oocyte coexpressing CD4, CCR5, and Kir 3.1. An inward current was observed during application of 10 nM MIP-1α, but application of 20 nM JR-FL gp120 did not induce a current or reduce the magnitude of the subsequent response to MIP-1α. Holding membrane potential for both experiments were –30 mV.

A.



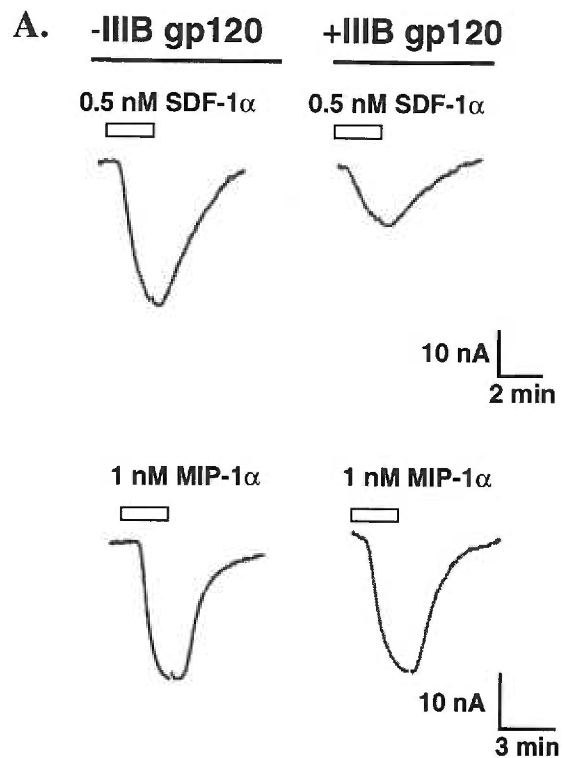

B.



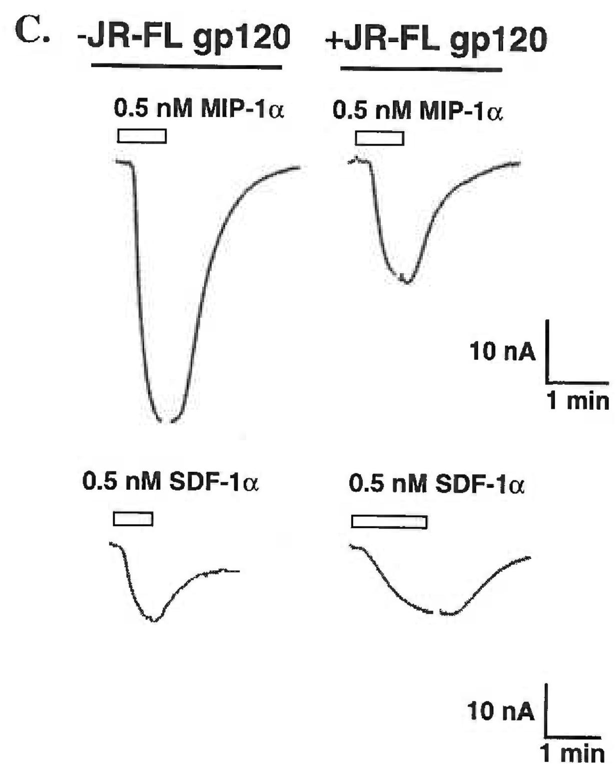
We then examined the effects of prolonged preincubations with high concentrations of T-cell-tropic as well as M-tropic gp120s on chemokine receptor signaling. Saturating concentrations of highly purified gp120 IIIB were incubated for 1-2 hrs with oocytes that coexpressed CD4, CXCR4, CCR5 and Kir 3.1. The oocytes were then voltage clamped and the membrane conductance was measured. Similar to previous results with short applications, preadsorption of gp120 IIIB did not activate a significant baseline $K^+$-current [slope conductances at -95 mV were $6.3 \pm 0.5$ (n=20), and $6.9 \pm 0.3$ (n=19) for control and gp120 treated oocytes, respectively]. However, this preincubation with T-cell tropic gp120 IIIB significantly decreased SDF-1$\alpha$ induced currents compared with untreated control oocytes (Figure 7A, top left panel), whereas this pretreatment did not affect MIP-1$\alpha$ induced currents (Figure 7A, lower tracings). Similarly, oocytes that were preincubated for 2 hrs with a saturating concentration of M-tropic JR-FL gp120 had significantly decreased responses to MIP-1$\alpha$ compared to the untreated control oocytes (figure 7C, top right panel), whereas the magnitude of SDF-1$\alpha$ induced currents in the same oocytes did not change (figure 7C, lower tracings). The results from a number of separate experiments are represented in figure 7, panels B and D. These results suggest that gp120s from T-cell-tropic and M-tropic HIV-1 specifically interact with their corresponding coreceptors and interfere with their activation by chemokines.

**Figure 4.7. Prolonged incubation with T-cell or macrophage tropic HIV-1 gp120s specifically inhibits activation of CXCR4 or CCR5, respectively.** A. Current records for two representative oocytes coexpressing CXCR4, CCR5, CD4, and Kir 3.1. Oocytes were incubated with 0.4 μM gp120 IIIB for 1-2 hrs, then voltage clamped at -30 mV and superfused with 0.5 nM SDF-1α. The SDF-1α-induced currents in untreated oocytes were larger than in oocytes that had been preincubated with gp120 IIIB (summarized in B), while gp120 IIIB adsorption didn't have significant effect on 1 nM MIP-1α activation of CCR5 in the same oocyte (bottom traces). B. Summary of currents induced by application of 0.1 nM SDF-1α activation of CXCR4 with and without incubation with gp120 IIIB. Each column represents mean ± SEM for 29-30 determinations (*$p<0.02$). C. *Xenopus* oocytes coexpressing CXCR4, CCR5, CD4, and Kir 3.1 were incubated with 0.3 μM JR-FL gp120. gp120 incubation significantly reduced 0.5 nM MIP-1α activation while the magnitude of SDF-1α activation of CXCR4 was not significantly affected compared to the untreated oocytes. D. Summary of currents measured during application of 0.5 nM MIP-1α activation of CCR5 in presence and absence of incubation with JR-FL gp160. Each column represents mean ± SEM for 4 determinations (*<0.02). Gaps in current records correspond to times when voltage pulse protocols were applied.

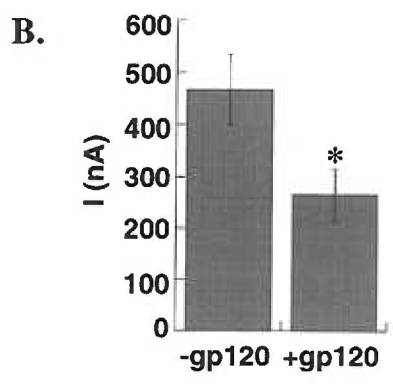
A.




C.




B.




D.

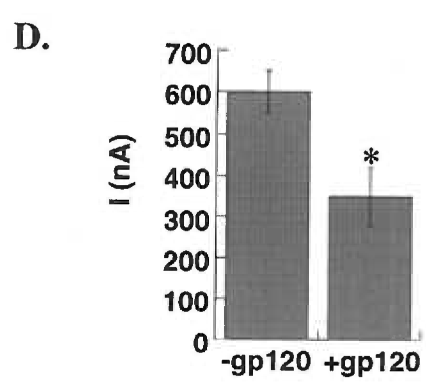
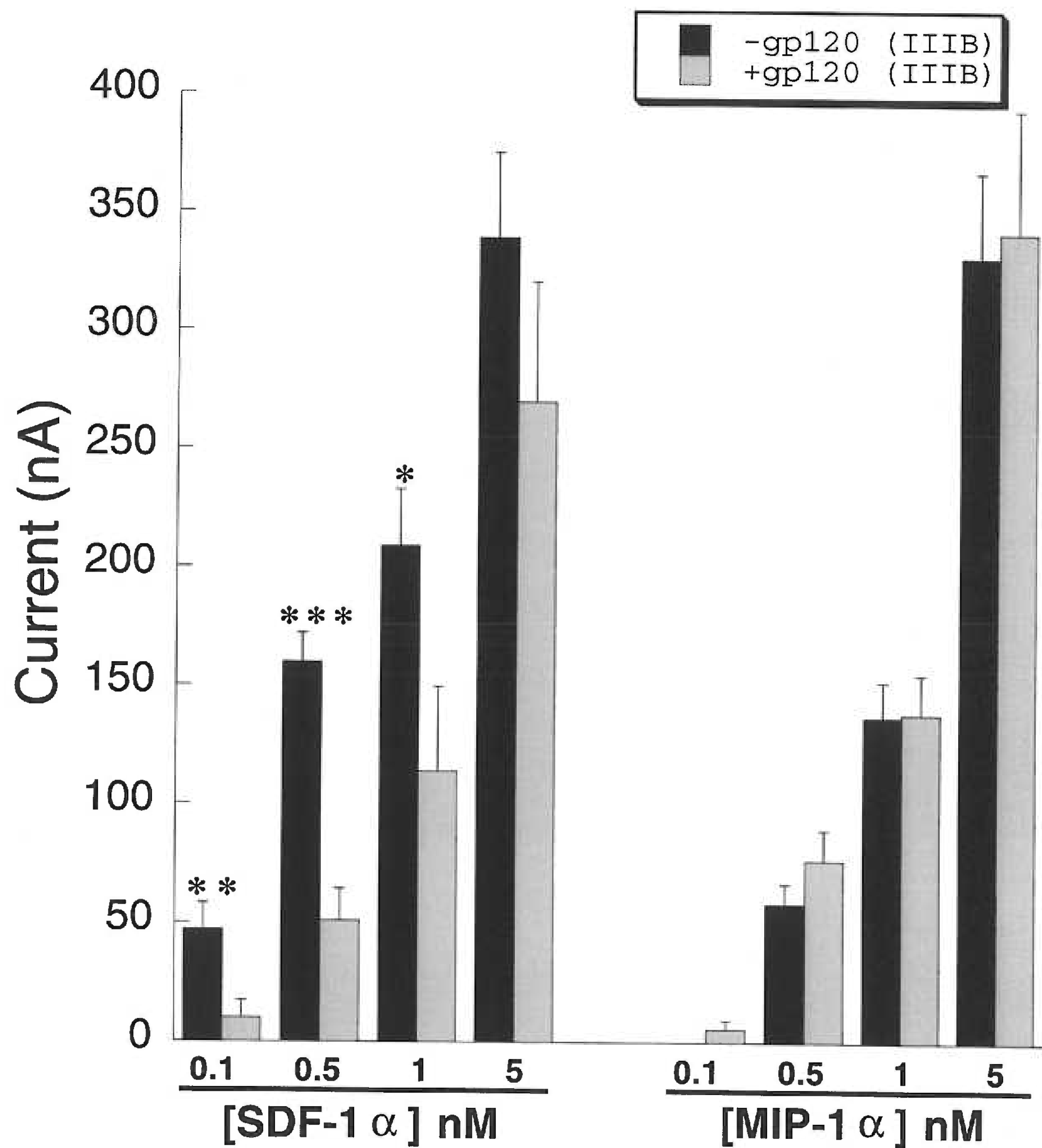
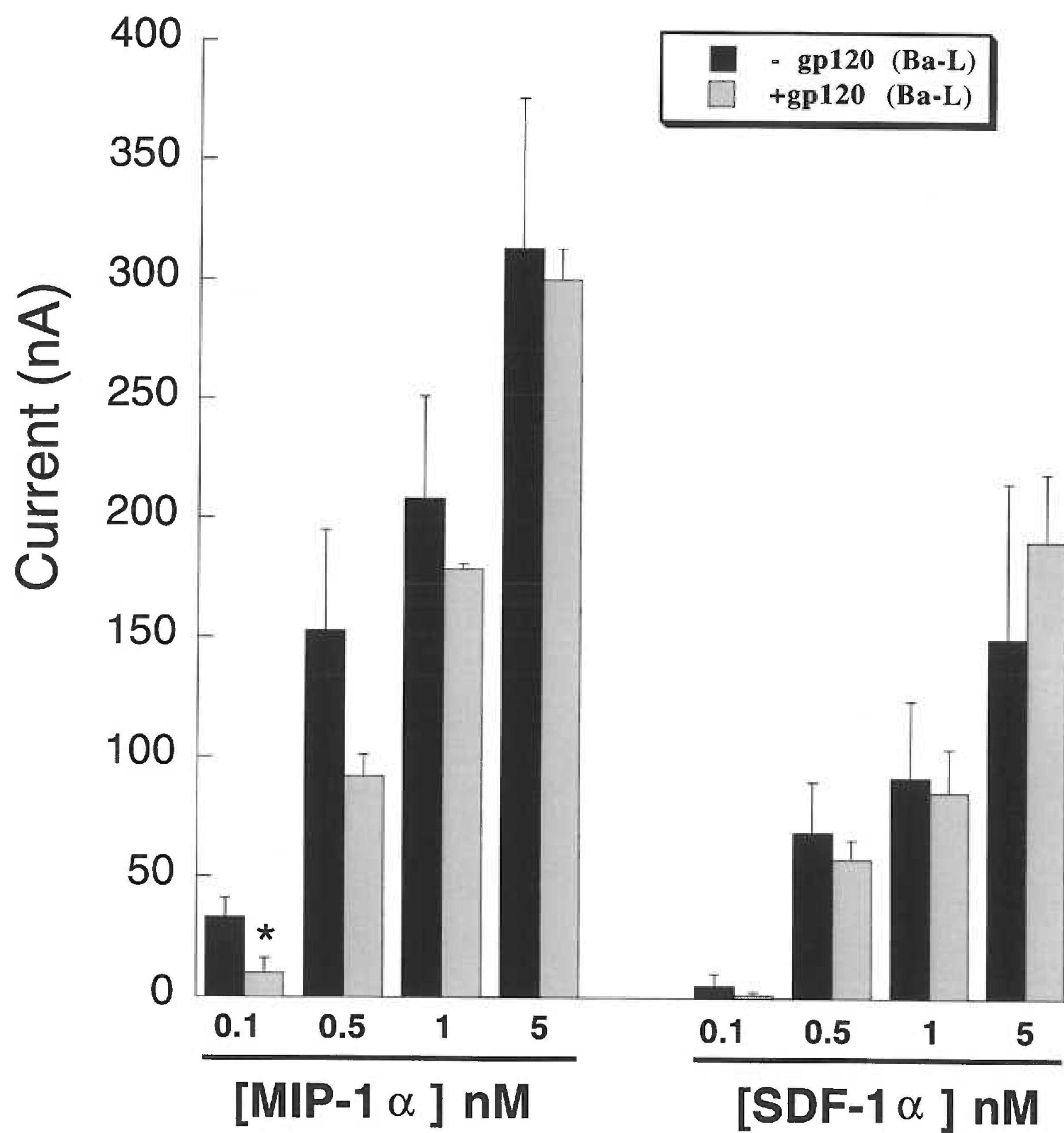


**Fig. 4.8. Extensive adsorption of monomeric T-cell-tropic or oligomeric M-tropic gp120s specifically and competitively antagonize CXCR4 and CCR5 responses to chemokines in a tropism specific manner.** All oocytes coexpressed CXCR4, CCR5, CD4, and Kir 3.1. Oocytes were preincubated with either 0.4 μM gp120 IIIB, or with 0.3 μM oligomeric gp120 BaL for 1-2 hrs prior to voltage clamping and superfusing with SDF-1α and MIP-1α at the concentrations indicated. A. Monomeric gp120 IIIB inhibition of CXCR4 activation by SDF-1α over a range of concentrations, without any effect on CCR5 responses to different concentrations of MIP-1α in the same oocytes. Measurements were recorded at -80 mV during voltage pulses (***$p<0.0002$; **$p<0.0012$; *$p<0.05$; each column represents mean ± SEM; n=5-6). B. Monomeric gp120 Ba-L inhibition of CCR5 responses to different concentrations of MIP-1α, without any significant effect on CXCR4 responses to different concentrations of SDF-1α. Measurements were recorded at -80 mV during voltage pulses (*$p<0.08$; each column represents mean ± SEM; n=3).

A.



B.



The ability of gp120s to inhibit chemokine receptor activation by their natural ligand was analyzed over a wide range of SDF-1α or MIP-1α concentrations (0.1 nM-5 nM). As shown in figure 8A, extensive preadsorption with gp120 IIIB significantly decreased SDF-1α activation of CXCR4 at all concentrations compared to the control oocytes. However, this inhibition of signaling was proportionately greater at lower concentrations of SDF-1α. Specifically, the inhibition was 80% at 0.1 nM SDF-1α, and approximately 20% at 5 nM SDF-1α. In contrast, extensive pre-adsorption of gp120 IIIB didn't have any significant effect on CCR5 responses to MIP-1α in these same oocytes. These results confirm the specificity of the gp120 IIIB inhibition and suggest that a competitive mechanism may be involved in the inhibition of CXCR4 activation by SDF-1α. A similar analysis was also done using monomeric gp120 from M-tropic Ba-L isolates, with nearly identical results (see Figure 8B). In this case, the inhibition was specific for CCR5 responses to MIP-1α and was greatest when the concentration of MIP-1α was low. The Ba-L gp120 had no significant effect on CXCR4 signaling responses to SDF-1α in these same oocytes.

## Discussion

### Chemokine receptor signaling

We have expressed the chemokine receptors CXCR4 and CCR5 with the G-protein coupled potassium channel Kir 3.1 in *Xenopus* oocytes in order to monitor signal transduction by these receptors and to examine the effects of the HIV envelope glycoproteins with which they interact. Channel activation occurred as a saturable function of the concentrations of the chemokines SDF-1α, MIP-1α, MIP-1β, and RANTES in oocytes that coexpressed the appropriate chemokine receptor (Figure 3). These currents were completely blocked by pertussis toxin (Figure 4), consistent with previous evidence for chemokine receptor coupling to $G_i$ proteins (38). The properties of the conductance activated by these ligands, including its potassium selectivity, voltage-dependence, and sensitivity to block by $Ba^{++}$ ions, are in accord with the properties of Kir 3.1 (11, 39). Because opening of Kir 3.1 channels is mediated by direct interactions with Gβγ subunits that are released upon activation of heterotrimeric G proteins (8, 9, 55), this assay reflects primary G-protein activation and does not depend on complex downstream signaling events such as $Ca^{+2}$ release from intracellular organelles or protein kinase cascades. Accordingly, the response of the system is highly reproducible, making this method exceptionally useful for quantitative studies and for

examining modulations of signaling caused by the gp120 glycoproteins of immunodeficiency viruses.

**Kinetics of chemokine receptor signaling in oocytes**

Desensitization of current responses developed with time constants of 5-8 minutes in the continuous presence of chemokine. Moreover, when both chemokine receptors were coexpressed, heterologous desensitization was observed, as has been observed previously for coexpressed μ-opioid and serotonin-1A receptors in oocytes (38). Desensitizations reached steady-state levels that appeared receptor-specific (e.g., see Figure 5). For example, exposure to a saturating concentration of SDF-1α resulted in 75% desensitization of CXCR4 signaling that developed with a time constant of approximately 6 minutes and recovered with a time constant of approximately 13 minutes. Similarly exposure to a saturating concentration of MIP-1α caused approximately 50% steady-state desensitization of CCR5 that developed with a time constant of 5 minutes and with a recovery time constant of 19 minutes. The heterologous desensitization of mGluR 2, CCR5, and CXCR4 by agonists specific for any one of these receptors implies that the oocyte down-modulatory mechanism is induced by activation of all of these receptors.

This mechanism may promiscuously inhibit all G protein-coupled receptors or it may inhibit a post-receptor step that is common to their

signaling pathways. Desensitization of chemokine receptors occurs differently in mammalian cells. In that case, exposure to a chemokine causes a rapid down-modulation and cessation of the signal within approximately 1-2 minutes (47, 48, 50). Moreover, this rapid inhibition of signaling is specific for the particular receptor since other coexpressed receptors remain fully active (61). Activation of chemokine receptors in mammalian cells results in rapid receptor phosphorylation, binding of arrestin, and endocytosis and that these events inactivate only the specific receptor (4, 25, 28, 29, 32, 47, 50, 56).

**Effects of gp120 on signaling by CXCR4 and CCR5**

In contrast to the currents induced by chemokines in oocytes that coexpress CD4 and Kir 3.1 with CXCR4 or CCR5, brief superfusion of relatively high concentrations of highly purified gp120 preparations derived from T-cell-tropic or M-tropic strains of HIV-1 did not induce detectable signals in these same oocytes (see Figure 6). Moreover, significant signaling was also not seen when the applications of gp120 were increased up to 20 minutes (results not shown). Although these gp120s did not activate CXCR4 or CCR5 signaling in the conditions of our assays, it was conceivable that gp120-induced receptor activation might occur more slowly than the time scale of these experiments. This slow activation might occur as a consequence of the relatively slow binding of gp120 onto CD4 as demonstrated in figure 1C,or to a slow recruitment of coreceptors into ternary complexes, or both. Clearly, however,

our gp120 preparations were competent for binding to CD4 (see Figure 1) and for CD4-dependent interactions with specific coreceptors (see below). Moreover, the JR-FL gp120 preparation that was used in our investigation was generously donated by James Arthos and Anthony Fauci, who recently reported that Env gp120 induced rapid CCR5-mediated $Ca^{+2}$ mobilization in activated CD4-positive B10 lymphocytic cells and in activated CD4-positive T-lymphocytes (61).

To address the absence of gp120-induced coreceptor activations in the oocyte superfusion assay, we preadsorbed gp120s for 1-2 hrs prior to assaying responses to chemokines. The baseline $K^+$-currents were unaffected by the gp120 adsorptions, suggesting an absence of chemokine receptor activation. Interestingly, however, we found that extensive preadsorption of gp120 from the T-cell-tropic HIV-1 isolate IIIB strongly inhibited the subsequent response of CXCR4 to SDF-1$\alpha$ (see Figures 7A). Similarly, extensive preadsorption of gp120 from the M-tropic HIV-1 strain JR-FL specifically inhibited CCR5-mediated signaling (see Figure 7B). These gp120-induced inhibitions were CD4-dependent and they were completely specific for the coreceptors that mediate infections by these isolates of HIV-1. Thus, the IIIB gp120 inhibited CXCR4 response to SDF-1$\alpha$ but did not have an effect on CCR5 response to MIP-1$\alpha$ even when both coreceptors were expressed in the same oocytes (Figure 7A). Conversely, JR-FL gp120 inhibited CCR5 responses to MIP-1$\alpha$

but not CXCR4 responses to SDF-1α (Figure 7B). The specificity of these inhibitions clearly distinguishes them from activation-induced desensitization of signaling which inhibits bystander G protein-coupled receptors (see Figure 5), suggesting instead a direct inhibitory action. Moreover, the gp120 IIIB-mediated inhibition of CXCR4 signaling was partially overcome by using high concentrations of SDF-1α (see Figure 8A), implying that this inhibition involves a competitive mechanism. For example, 80% inhibition of signaling was observed at 0.1 nM SDF-1α, whereas the inhibition declined to approximately 20% when the SDF-1α concentration was 5 nM (see Figure 8A). Thus, gp120 IIIB shifts the SDF-1α response curve to the right and causes it to have a sigmoid shape. Similar results were obtained when gp120 Ba-L were incubated with oocytes (Figure 8B). Together, these observations suggest that gp120-CD4 complexes may bind directly to the appropriate chemokine receptors to inhibit their activations by chemokines. This interpretation is concordant with previous evidence that gp120-CD4 complexes bind to chemokine receptors (40) and competitively inhibit their interactions with [$^{125}$I] chemokines (30, 31, 46, 60, 62). However, these previous papers did not quantitatively measure the residual affinities of ternary gp120-CD4-coreceptor complexes for chemokines. Therefore, it remained theoretically possible that these ternary complexes might be supersensitive rather than inhibited in their abilities to signal in response to chemokines. Our evidence

suggests that the net effect of gp120 adsorption is to reduce rather than to amplify signaling in response to chemokines.

After completion of this research, Weissman *et al.* (60) reported that gp120s from some M-tropic isolates of HIV-1 can induce CCR5-mediated $Ca^{+2}$-mobilization in activated B10 lymphocytic cells and in activated CD4-positive T-lymphocytes. Notably, however, gp120s from some viruses that use CCR5 as a coreceptor failed to activate signaling. Moreover, gp120s from T-cell tropic HIV-1 isolates did not activate CXCR4. In addition, only a fraction (c.a., 15-30%) of the cells in their assay samples responded to gp120 and the gp120 response in these cells was only partially inhibited by Leu-3a antibody that blocks gp120 interactions with CD4. Several of the gp120 responses that were shown also appeared to be substantially weaker than the responses of the same cells to MIP-1β. In addition, the recent report by Davis *et al.* (12) indicated that gp120 preparations from T-cell-tropic and M-tropic isolates of HIV-1 increased the phosphorylation of Pyk2 tyrosine kinase but did not induce detectable $Ca^{+2}$ mobilization in activated CD4-positive T-lymphocytes. In contrast, SDF-1α and MIP-1β induced substantial $Ca^{+2}$ mobilization in the same cells (12). These effects of gp120 are difficult to reconcile because activation of phospholipase C (PLC-β) with resultant $Ca^{+2}$ release is a proximal effect of activated Gβγ subunits (8, 33), whereas Pyk2 phosphorylation is a downstream response that stringently depends upon an

increase in cytosolic $Ca^{+2}$ concentration (13, 41). Consequently, it can be inferred that gp120s induced $Ca^{+2}$ mobilization to a small but undetectable extent in the cell population. Based on these considerations, it seems likely that the gp120s used by Davis *et al.* (12) were only weak CXCR4 and CCR5 agonists or that they functioned as strong agonists in only a small proportion of the chemokine-responsive cells. The latter interpretation is compatible with the observation of Weissman *et al.* (61) that gp120 induced significant $Ca^{+2}$ mobilization only in a fraction of CD4-positive T-lymphocytes. Although additional studies will be required, we believe that the differences in our results derive from our distinct experimental systems and techniques. For example, the activations seen by Weissman *et al.* (61) and Davis *et al.* (12) might only occur in proliferating cells at one stage of the cell cycle, or they might require *Lck* tyrosine kinase or other factors that are absent in oocytes.

The major conclusion of our investigation is that gp120s from T-cell-tropic and M-tropic isolates of HIV-1 can bind to CD4 on oocyte surfaces and then specifically inhibit CXCR4 and CCR5 signaling by the corresponding chemokines. Although our results are compatible with recent evidence that some gp120s can induce a degree of chemokine receptor activation (thus acting as partial agonists) in some cells (12, 61), the inhibitory effects on chemokine signaling that we have detected appear to involve direct interactions of gp120-CD4 complexes with coreceptors in the same membranes and are therefore

likely to occur in all cells, including the natural targets for HIV-1 infections. In close agreement with our results and interpretations, previous evidence has indicated that specific gp120-CD4 complexes competitively inhibit chemokine binding to CXCR4 and CCR5 (30, 31, 46, 60, 62). Thus, gp120s can have both agonist and antagonist effects on CXCR4 and CCR5 signaling, and both effects will need to be considered in future investigations. These effects could occur simultaneously or they could possibly each predominate in specific cells. We emphasize that the recent studies of Weissman *et al.* (61) and Davis *et al.* (12) did not address potential inhibitory effects of gp120s.

Based on these results, we suggest that gp120s shed from virions and from infected cells may bind to uninfected CD4-positive cells and strongly inhibit their responses to specific chemokines. This inhibition could possibly have a major influence on the immune and inflammatory responses of infected individuals. Further investigations will be needed to understand the importance of this inhibition and of the recently reported gp120-mediated CXCR4 and CCR5 activations (12, 61) on immune suppression and AIDS pathogenesis.

## Experimental Procedures

### Reagents and cDNA clones

Chemokines were purchased from PeproTech Inc. (Rocky Hill, NJ). Pertussis toxin was obtained from Calbiochem (San Diego, Ca). Purified monomeric gp120 (IIIB) was from Bristol-Myers Squibb Pharmaceutical Research Institute (Seattle, WA), kindly donated by Dr. Shiu-lok Hu. Macrophage-tropic gp120 Ba-L was a generous gift of Dr. Ray Sweet at SmithKline Beecham Pharmaceuticals (King of Prussia, PA) and JR-FL gp120 was generously provided by Dr. James Arthos and Anthony Fauci (National Institute of Allergy and Infectiouse Diseases, Bethesda, MD). A sample of SDF-1 was kindly provided by Dr. Ian Clark-Lewis (University of British Columbia, Vancouver, Canada). The vectors and cDNA clones were the generous gifts of the following investigators; Kir 3.1 was provided by Dr. Henry Lester, mGluR2 was provided by Dr. Shigetada Nakanishi, pBF expression vector was from Dr. John Adelman, CXCR4 cDNA was provided by Dr. Frank R. Jirik, CCR5 cDNA was provided by Dr. John P. Moore. CD4 polyclonal antibody was obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, National Institute of Allergy and Infectiouse Disease, and was contributed by Dr. Michael Phelan. Bolton-Hunter Reagent [$^{125}$I] kit was purchased from ICN Radiochemicals (Costa Mesa, Ca).

**$[^{125}I]$gp120 binding to *Xenopus* oocytes and HeLa cells**

$[^{125}I]$Bolton-Hunter reagent was used to iodinate 25 μg of T-cell tropic gp120 (IIIB) as well as 25 μg of SDF-1α. Iodinated gp120 and SDF-1α were purified using Sephadex G-10 (Pharmacia Biotech Inc., Piscataway, NJ). For the binding studies, oocytes were incubated in a frog Ringer solution (96 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM Hepes, pH 7.5) containing $[^{125}I]$gp120 for 1-2 hrs at room temperature with gentle shaking. Each oocyte was then separately washed for 5 min in the Ringer solution and counted in a gamma counter. $[^{125}I]$gp120 binding to mammalian cells was done as follows: HeLa and HeLa-CD4 expressing cells (clone H1-J) (34) were plated at $1 \times 10^5$ cells/1 $cm^2$ well of a 48 well culture plate the day before the assay. The cells were incubated with $[^{125}I]$gp120 and various concentrations of unlabelled gp120 IIIB for times indicated at 37 $^oC$. Cells were then washed several times with Dulbecco's modified Eagles medium (DMEM) with 10% fetal bovine serum (FBS) and once with phosphate buffered saline (PBS) before they were solubilized with 0.1 N NaOH and counted in a gamma counter. An aliquot of each sample was assayed for protein using the Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, Ca.).

**Oocyte expression**

Stage V-VI oocytes were collected from anesthetized *Xenopus laevis* and defolliculated using collagenase (Boehringer Mannheim, Indianapolis, IN). Oocytes were incubated at 16 $^{o}C$ in a frog Ringer solution supplemented with 2.5 mM sodium pyruvate (Sigma, St. Louis, MO), 0.5 mM theophylline (Sigma, St. Louis, MO), and 50 μg/ml gentamycin (Life Technologies, Grand Island, NY). cDNAs were subcloned into oocyte expression vectors (pOG-1 or pBF) at a site between 5' and 3' untranslated *Xenopus* β-globin sequences (36). Linearized plasmids were transcribed *in vitro* with T7 or SP6 polymerase and oocytes were microinjected with 5-50 ng of capped cRNA on the day of harvest.

**Oocyte membrane preparations**

Oocytes (6-8) were homogenized in lysis buffer [7.5 mM sodium phosphate pH 7.4, 10 mM EDTA, 0.1 mM PMSF (Sigma Chemicals, St. Louis, MO)] followed by certrifugation at 2,000 rpm for 5 minutes in a Beckman microcentrifuge. The supernatants were then centrifuged at 15,000 rpm for 30 min at 4 $^{o}C$. The pellets containing the membrane proteins were dissolved, warmed at 37 $^{o}C$ for 5 min and analyzed by electrophoresis in a 10% polyacrylamide gel in the presence of 0.1% sodium dodecyl sulfate followed by Western immunoblotting (42). Sheep anti human CD4 antibody was used

at a 1:1000 dilution followed by biotinylated protein-G (BioRad, Ca). For detecting CCR5 expression, CCR5 antibody (Kuhmann *et al.*, 1997) was used at 1:300 dilution followed by biotinylated protein-A (BioRad, Ca). The immunoblots were developed with the Phototope-HRP Western Blot Detection Kit (New England Biolabs, Beverly MA). CD4 in extracts from 8 x $10^4$ HeLa and HeLa-CD4 (H1-J) cervical carcinoma cells were also analyzed on the immunoblots.

**Electrophysiology**

Electrophysiological recording was done 2-5 days after cRNA injection. Two-electrode voltage clamp was performed with a GeneClamp 500 amplifier interfaced to a Digidata 1200 A/D. The interface was controlled with an IBM-compatible computer running pCLAMP 6.0 (Axon Instruments Inc., Foster City, Ca.). Microelectrodes were filled with 3 M KCl and had tip resistances of 0.1-1.5 MΩ. The oocytes were placed in a small chamber continually perfused with high $K^+$ Ringer's solution (100 mM KCl, 2 mM NaCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM HEPES, pH 7.5). Agonists and blockers were applied by bath perfusion. The holding potential was set at -30 mV and current-voltage records were obtained during 250 msec voltage jumps to potentials between +40 and -100 mV. Desensitization kinetics were determined by least squares fit to single exponential functions. The kinetics of recovery from

desensitization were determined by measuring the responses in different oocytes at specific time intervals. The responses were then normalized to the peak response and fitted to an exponential function. Where indicated, oocytes were incubated with 1 μg/mL of pertussis toxin in Ringers for 48 hrs before recording.

### Acknowledgments

The last two authors (M.P.K. and D.K.) contributed equally to this work. This research was supported by NIH grant CA67358 and by NS33270. N.M. is partially supported by an NIH predoctoral fellowship in Molecular Hematology and Oncology (T32HL07781). We are grateful to Drs. Shiu-lok Hu (Bristol-Myers Squibb Pharmaceutical Research Institute, Seattle, WA), James Arthos and Anthony Fauci (National Institute of Allergy and Infectious Diseases, Bethesda, MD) and Raymond Sweet (Smith-Kline and Beecham, King of Prussia, PA) for generous gifts of gp120s. Special thanks are due to Jacques Wadiche for discussion, Ali Nouri for expert technical assistance, and our colleagues in both laboratories for advice and encouragement.

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# Chapter 5

## Conclusions

We investigated two mechanisms of HIV-1 infectivity. Our goals were to elucidate the function of HIV-1 Vif, and to characterize signal transduction by dual-function CXCR4 and CCR5 chemokine receptors/HIV-1 coreceptors. Both goals concerned factors that control viral infectivity for specific target cells.

### I. Mechanisms of Vif action

In order to characterize *vif* function in HIV-1, it was important to produce a substantial amount of *vif*-deleted virus from nonpermissive cells. We devised a coculturing method to produce a large amount of *vif*-deleted virus from nonpermissive cells. This method involved infecting adherent cultures of permissive HeLa-CD4 cells with replication competent wild-type or *vif*-deleted virus. These cells were then cocultured with nonpermissive H9 leukemic T-cells, and the suspensions of H9 cells were later recovered from the culture media. The viral proteins of wild-type and *vif*-deleted virions released from H9 cells were compared by Western blot analyses. We showed that the *vif*-deleted virions had normal levels of Gag proteins and only a slight reduction in the envelope protein gp120. The difference in our results compared to earlier work may derive from our experimental methods (10, 105, 108). For example, we were able to isolate *vif*-deleted virions from H9 cells within 24-48 h of infection before any cytopathology was seen among the cultures. We also showed that the low infectivity of *vif*-deleted HIV-1 was

not enhanced by increasing the CD4 content of the target cells. In addition, pseudotyping with a MLV envelope did not rescue the Vif defect (see below). In agreement with a previous report (121), these results suggest that the lack of infectivity in *vif*-deleted virions is not caused by a deficiency in envelope function.

The viral specificity of Vif function was also examined. Members of the well studied oncogenic murine leukemia viruses lack a *vif* gene, and their full infectivity depends only on *gag*, *pol*, and *env* genes. However, Simon and coworkers (111) reported that Vif enhanced the infectivity of MLV particles released from nonpermissive cells. To further test this viral specificity, we used replication competent xenotropic BV2 virus to infect nonpermissive H9 cells. We then superinfected these H9/BV2 cells with wild-type or *vif*-deleted HIV-*gpt*. These H9 cells produced MLV particles as well as HIV-1 provirus that was pseudotyped with the xenotropic envelope. The H9 cells that contained both *vif*-deleted HIV-1 and BV2, simultaneously released noninfectious pseudotyped HIV-1 and fully infectious MLV. In addition, Vif was fully functional since the H9/BV2 cells infected with wild-type HIV-*gpt* released infectious pseudotyped HIV-1. The infectivity of the pseudotyped virus was tested on HeLa cells by the quantitative focal infectivity assay. The results of two independent experiments showed that Vif failed to enhance the infectivity of BV2 virus. The difference between our results and the earlier report (111) may be due to the differences in our experimental approaches. For example, we measured the effects of Vif directly at the level of infection by using a focal infectivity assay, whereas Simon *et al.* measured the viral spread

10-15 days following infection. Based on our data, Vif's effects are specific to HIV-1.

The cellular specificity of Vif was also investigated. Vif is required for the production of infectious virions in nonpermissive cells, but is not necessary in permissive cells. It had been hypothesized that either permissive cells contain a cellular factor that functionally complements Vif, or that nonpermissive cells have a viral inhibitor that is counteracted by Vif (48, 105, 109, 119). Although these possibilities had been proposed by several investigators, they had not been addressed experimentally. To examine this, we produced wild-type and *vif*-deleted pseudotyped HIV-*gpt* virions in permissive COS-7 cells and used them to generate H9 cells that produced these proviruses. The resulting H9 cells were fused with permissive HeLa cells expressing gp120-gp41 complexes and the virions released from these heterokaryons were assayed. If permissive cells contain a cellular factor that functions like Vif, the *vif*-deleted virus from the heterokaryons would be infectious. However, if nonpermissive H9 cells contain a viral inhibitor, the virus released from the heterokaryons would be noninfectious. Nine independent experiments were performed and in all cases the wild-type virions released from the heterokaryons were much more infectious than the *vif*-deleted virions. These results are critical to the understanding of HIV-1 pathogenesis and suggest that nonpermissive cells, the natural targets of HIV-1, contain an endogenous factor that inhibits HIV-1 replication and is overcome by Vif. Future work should focus on identifying this inhibitor and further characterizing the function of Vif.

Considered together, I believe that my work has contributed substantially to an improved understanding of the HIV-1 Vif protein. Pervious workers in this field had largely focused on analyzing the step(s) of infectivity that were defective when *vif*-deleted virus was used to infect target cells. Perhaps not surprisingly, the results depended on the specific target of cells examined and they have remained somewhat uncertain and controversial. In my opinion this problem derives from the fact that mutations in specific genes typically cause complex pleiotropic down-stream effects. Moreover, the cellular specificity of Vif function as described above clearly demonstrates that Vif acts in producer cells and that its down-stream consequences become manifest in the target cells. The major contribution of my research was to identify the presence of a yet unknown cellular factor which causes the production of noninfectious HIV-1 particles in the absence of Vif. This observation provides a novel function for Vif which offers substantial promise for improving our understanding of HIV-1 and of AIDS. In addition, my results strongly suggest that this putative cellular inhibitor acts specifically on HIV-1 and perhaps on other lentiviruses rather than other enveloped viruses.

## II. Signal transduction by chemokine receptors CXCR4 and CCR5

The signal transduction properties of chemokine receptors CXCR4 and CCR5 were analyzed by directly measuring their responses at the cell membrane rather than by analyzing downstream signaling events such as $Ca^{+2}$ mobilization. Human CD4, a G protein-activated potassium channel (Kir 3.1), and chemokine receptors CXCR4 or CCR5 were coexpressed in *Xenopus laevis* oocytes, and activation of Kir 3.1 was measured by electrophysiological

recordings. Application of a specific chemokine resulted in an induction of current by Kir 3.1. Consistent with previous reports, these currents were inhibited by pertussis toxin, suggesting that the chemokine receptors were coupled to Gi proteins (70). Control studies showed that activation of Kir 3.1 was blocked by $Ba^{+2}$, was potassium selective, and was voltage dependent, which is consistent with the properties of this channel (26, 71). Inward currents induced by SDF-1$\alpha$ and MIP-1$\alpha$ in oocytes coexpressing CXCR4 and Kir 3.1, or CCR5 and Kir3.1, respectively, were dose dependent and saturable. Prolonged application of chemokines resulted in desensitization of current responses. This desensitization was heterologous in oocytes coexpressing CXCR4, CCR5, and Kir3.1, which is consistent with previous evidence with $\mu$-opioid and serotonin-1A receptors in oocytes (67). This observation suggests an inhibition of a downstream pathway that is common to all these receptors. The data obtained by this electrophysiological approach was highly reproducible and quantitative, which made it an excellent method to measure the effects caused by gp120 of HIV-1.

Next, we investigated the modulation caused by the gp120 envelope glycoprotein of HIV-1. We showed activation of Kir 3.1 in response to chemokines in oocytes that coexpressed Kir 3.1, CD4, and CXCR4 or CCR5. In contrast to chemokine application, the application of highly purified R5 or X4 isolates of gp120 did not induce any current. Prolonged applications of gp120 to the oocytes also failed to induce currents. We further investigated the absence of gp120-induced coreceptor activation by preincubating oocytes with saturating levels of X4 or R5 isolates of gp120 for 1-2 h. The activation of chemokine receptors by their specific ligands was then assayed.

The baseline potassium induced currents remained unchanged, which suggested that preadsorption of gp120 did not cause an activation of chemokine receptors. Importantly, we observed a tropism specific inhibition of chemokine receptors by their natural ligands in oocytes coexpressing Kir 3.1, CD4, CXCR4, and CCR5. These inhibitions were completely CD4 dependent and were specific to each coreceptor. This specificity was different from the activation-induced desensitization that inhibits the bystander G protein-coupled receptors, which further confirmed the lack of gp120 activation of chemokine receptors and suggests a direct inhibitory effect. This inhibitory effect was also shown to be overcome at high concentrations of SDF-1$\alpha$ for CXCR4 inhibitions, and MIP-1$\alpha$ for CCR5 inhibitions, implicating a competitive mechanism. These observations suggest that gp120 binds CD4 and the appropriate coreceptor, which competitively inhibits the activation of the coreceptor by its specific chemokine ligand. This finding is consistent with previous data that the gp120-CD4 complex binds chemokine receptors (75) and displaces $^{125}$I-labeled chemokines (56, 58, 86, 118, 126). However, the previous studies were deficient in that the inhibitory effects were not quantitatively measured. Furthermore, it was not shown whether this displacement was a direct effect or resulted from the down-modulation of chemokine receptors. The work described here is direct.

Our findings differed from two reports that suggested gp120s for X4 and R5 isolates of HIV-1 caused increased phosphorylation of the Pyk2 tyrosine kinase (27), and that some R5 isolates of HIV-1 induced $Ca^{+2}$ flux in activated T-lymphocytes (123). However, both reports contained discrepancies. For example, Davis *et al.* reported that $Ca^{+2}$ was mobilized in

activated T-lymphocytes in response to application of chemokines, whereas gp120s from R5 and X4 HIV-1 isolates failed to induce $Ca^{+2}$ flux but increased phosphorylation of the Pyk2 tyrosine kinase (27). However, it is known that activation of Pyk2 tyrosine kinase is a downstream response that is dependent on $Ca^{+2}$ flux (29, 77). In addition, Weismann *et al.* reported that gp120s from R5 isolates of HIV-1 induced $Ca^{+2}$ mobilization in activated human T-lymphocytes. However, similar gp120 preparations from some R5 and all X4 isolates of HIV-1 failed to activate $Ca^{+2}$ fluxes (123). Furthermore, only a small fraction of cells responded to the R5 gp120s. A possible complication in these studies could be that gp120 binding to CD4 in T-lymphocytes causes activation of p56-*Lck* tyrosine kinase thereby activating the Raf-MAP kinase pathways in T-lymphocytes (5, 13, 46, 97). In our assay system, since Kir 3.1 is directly activated by $G\beta\gamma$ subunits, we are measuring chemokine receptor activation specifically at the cell membrane. This assay does not depend on complex downstream processes such as $Ca^{+2}$ fluxes or phosphorylation of protein tyrosine kinases. Moreover, the $Ca^{+2}$ mobilization assay used to measure the chemokine receptor activation generally has high background and low signal-to-noise ratios (see refs 38, and 117). We do not rule out the possibility that gp120 can serve as a weak agonist of chemokine receptors. However, we report that gp120 is a specific and a competitive inhibitor of chemokine receptor, a finding that was not investigated by the previous reports. We have developed a system that is exceptionally useful for quantitative and direct monitoring of G protein activation of CXCR4 and CCR5.

This method was used to measure the signal transduction properties of some CCR5 mutants (Appendix I). Previous work has demonstrated the importance of the multiple extracellular regions of CCR5 for HIV-1 infection (37, 72, 102, 104, 127). Residues in the amino terminus of CCR5 are thought to be critical for gp120 binding and HIV-1 infection (37, 72). Two reports have demonstrated the importance of ECL2 in mediating HIV-1 infection (107, 127). Even though AGM CCR5 has a high homology (98% identity) to human CCR5, it binds gp120s from R5 isolates poorly and it serves as a weak coreceptor for R5 isolates of HIV-1 (72). AGM CCR5 differs from human CCR5 by only nine amino acids; four of them reside extracellularly. Based on sequence comparisons with other primate species (72) the signaling properties of a specific substitution that occurred in an extracellular domain of the receptor was examined. This position is occupied by a glycine in human CCR5, but by an arginine in AGM CCR5. Human CCR5(G163R) had weak coreceptor activity and attenuated binding of gp120s derived from R5 isolates of HIV-1 (107). Conversely, AGM(R163G) CCR5 bound gp120 and served as a coreceptor for R5 isolates of HIV-1 significantly better than the wild-type AGM CCR5. However, these substitutions failed to alter the CCR5 mediated signal transduction response to MIP-1$\alpha$ (Appendix I, Figure 1 A and B). These results suggest that the arginine at positition 163 results in significantly decreased ability of CCR5 to bind gp120 and to serve as a coreceptor for HIV-1 infection. However, the chemokine receptor activity of CCR5 is unaffected by this mutation.

The signaling ability of two more mutants of human CCR5, CCR5(Y14N) and CCR5(D125N) was also tested. Chemokine receptors

contain several tyrosines in their N-termini that are usually flanked by acidic residues (14, 101). A recent report has demonstrated that the tyrosines in the N-terminal region of CCR5 are sulfated and that this sulfation contributes to gp120 binding and HIV-1 infections (40). The Y14N mutation resides in the N-terminus of the receptor and substantially decreases its coreceptor function (to approximately 5% of wild type) (107). Cell surface expression of the CCR5(Y14N) mutant was similar to the wild-type CCR5 (Appendix I, Figure 2). However, its maximal signal transduction response to MIP-1$\alpha$ was significantly decreased, although the $EC_{50}$ of activation was not significantly affected (Appendix 1, Figure 3). This surprising observation implies that this region is critical for both signaling as well as coreceptor abilities of CCR5.

The signal transduction ability of another CCR5 mutant was also tested. The CCR5 aspartic acid to asparagine (D125N) mutation resides in the second intracellular loop (ICL2); more specifically it is predicted to lie at a juncture of TM3 and ICL2 of the receptor, and is believed to be involved in G protein-coupling (39, 66, 88, 106, 123a). Surprisingly, we showed that this CCR5 mutant is capable of signaling; however, its maximal signal transduction in response to MIP-1$\alpha$ was decreased, and its $EC_{50}$ of activation was significantly lower than the wild-type CCR5 (Appendix 1, Figure 3).

In addition, we measured the agonist and antagonist properties of a specific CXCR4 inhibitor, AMD3100 (33). AMD3100 belongs to a family of bicyclam molecules that have been implicated in inhibition of HIV-1 infection (28). In our electrophysiological assay system, AMD3100 was not an agonist of CXCR4 (data not shown); however it was a potent inhibitor of activation of this chemokine receptor by SDF-1$\alpha$ (Appendix 1, Figure 4) with an $IC_{50}$ of

approximately 40 *p*M. These results suggest that our electrophysiological method is excellent for quantitatively measuring the affinites of chemokine receptor mutants and for testing the properties of inhibitors of chemokine receptor signaling.

Chemokine receptor signaling was measured in the human astroglioma cell line, U87MG (Appendix II). Because these cells lack the p56-*Lck* tyrosine kinase, they are useful for analyzing gp120 effects on coreceptor signaling. U87MG cells were used to construct stable U87MG/CD4, U87MG/CD4/CXCR4, and U87MG/CD4/CCR5 cell clones (69). Phosphorylation of MAP-kinase was used as our assay since it is known that chemokine receptors as well as tyrosine kinases can activate MAP-kinases (29, 82, 100). We analyzed this activation by blotting with an antibody specific for MAP-kinase that had been activated by dual phosphorylation at Thr-202 and Tyr-204 by MAPK-kinase. Incubation of cells with MIP-1$\alpha$ or SDF-1$\alpha$ resulted in an increase in phosphorylation of MAP-kinase in U87MG/CD4/CCR5 and U87MG/CD4/CXCR4 cells, respectively (Appendix II, Figure 1). Phosphorylation of MAP-kinase by incubation of SDF-1$\alpha$ with U87MG/CD4/CXCR4 cells was saturated by 4 min and was blocked by pertussis toxin (Appendix II, Figure 2), suggesting the involvement of Gi family of proteins. Incubation of cells with monomeric R5 Ba-L, or X4 IIIB gp120 also resulted in an increase in phosphorylation of MAP-kinase (Appendix II, Figure 1). Additionally, gp120 IIIB activation was found not to be inhibited by pertussis toxin (Appendix II, Figure 3). Moreover, gp120 IIIB induced activation of MAP-kinase in U87MG/CD4 but not control U87MG cells, suggesting that this activation is mediated by CD4 (Appendix II, Figure

3). This activation was surprising since U87MG cells lack p56-*Lck* tyrosine kinase which associates with CD4 (13, 97, 98). Furthermore, U87MG/CD4 cells are completely resistant to HIV-1 infections (69). These results suggest that the MAP-kinase activation is not dependent on a gp120-CD4 complex with a chemokine receptor. Moreover, it implies the existance of a CD4 signaling pathway that does not involve p56-*Lck*. These alternative signaling pathway(s) could be important in understanding HIV-1 pathogenesis. Additional studies are required to characterize the signaling pathways in U87MG/CD4 cells.

## III. Future Directions

Considered together, my results illustrate the complex interactions of viral and cellular proteins that control the critical initial stages of HIV-1 infections. These critical steps of infection proceed integration of proviral DNA into a host cell chromosomes, and they include virus' attachment to cells, fusion of viral and cellular membranes, reverse transcription of the viral RNA, and movement of the preintegration complex into the cell nucleus. My results highlight the importance of both virus-specific and cell-specific factors during the early steps of HIV-1 infection.

Concerning Vif, I believe that the most important goal would now be to identify the putative cellular inhibitor that occurs specifically in nonpermissive cells and is overcome or counteracted by Vif. This factor will presumably be difficult to identify by a simple genetic screen, in part because the putative cellular inhibitor blocks release of infectious HIV-1 but does not prevent infection of the cells by *vif*-deleted virus that is derived from permissive cells.

Consequently, nonpermissive cells and permissive cells can both be infected and killed by *vif*-deleted virus made in permissive cells. Moreover, *vif*-deleted virus made in nonpermissive cells is noninfectious for both permissive and nonpermissive cells. This complexity has made Vif very difficult to study and has inhibited its investigation.

Our result that Vif counteracts a cellular inhibitor specifically present in nonpermissive cells implies that Vif directly or indirectly interacts with the inhibitor. An example of an indirect interaction would be one in which the inhibitor causes a post-translational modification of another protein (perhaps a viral protein) and Vif reverses this modification. A direct interaction of Vif with the putative inhibitor could potentially be detected by a yeast two-hybrid screen (43) or by identifying cellular proteins that bind to Vif in a GST-pulldown assay (47) or in a cDNA expression library study (60). The inhibitor candidates should occur specifically in nonpermissive cells. We have initiated a yeast two-hybrid screen, and have identified a potential candidate that specifically binds to Vif (R. Millette and N. Madani, unpublished data). Another approach that I believe might be useful would be to carefully compare the proteins of wild-type and *vif*-deleted virions released from nonpermissive cells for differences in their post-translational modifications. This approach could be difficult because the virions are difficult to purify and because the post-translational modifications might be subtle.

During the last few years it has become evident that coreceptors play a critical and complex role in controlling HIV-1 infections and pathogenesis. The degree to which gp120 signaling via CD4 and/or coreceptors contributes to infections or disease has been very difficult to evaluate. One lead provided

by my research concerns a CD4-mediated and *Lck*-independent signaling induced by gp120 in astroglial U87MG-CD4 cells (see Appendix II). Presumably, there may be another tyrosine kinase that can substitute for *Lck* in mediating this signal transduction. Such kinases can usually be inhibited by genistein, tyrphosin and herbimycin A (4, 29, 51, 77, 82). In contrast, if a chemokine receptor were involved, pertussis toxin would be expected to inhibit the MAP-kinase response. If the response that we observe is inhibited by a tyrosine kinase inhibitor, it would be important to determine whether PI-3 kinase is involved using LY294002 (13, 64) and to identify the tyrosine kinase by a standard autophosphorylation method. I believe that this investigation would be important because such alternative tyrosine kinase(s) might occur in lymphocytes or macrophages and they might be responsible for some of the pathogenic effects of gp120 *in vivo*.

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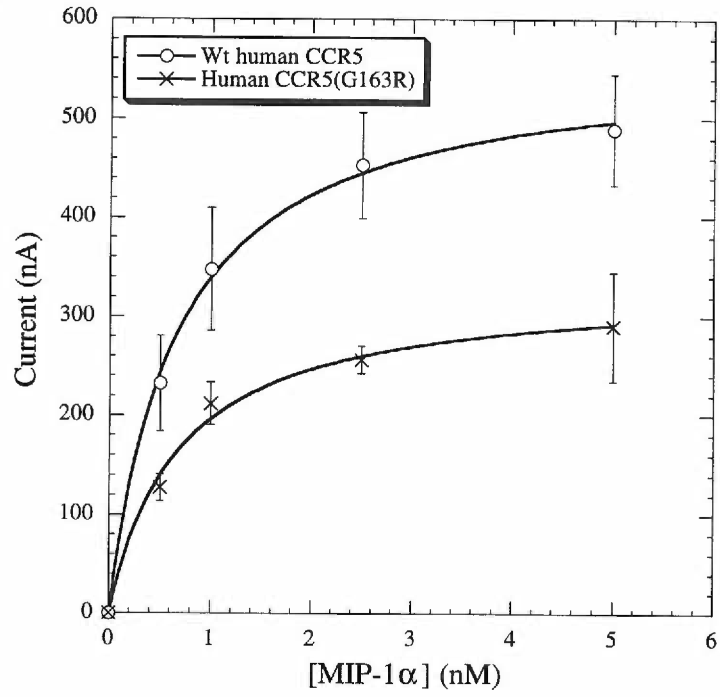
# Appendix I

## Signal transductions by wild-type and mutant CCR5 chemokine receptors

**Figure 1: Effects of substitution at amino acid 163 on activation of CCR5 by MIP-1$\alpha$.** *Xenopus* laevis oocytes were injected with cRNA for CCR5 and Kir 3.1. 3-4 days later, inward currents were measured by two electrode voltage clamp analyses. (A). Activation of human CCR5 by MIP-1$\alpha$ was compared to the corresponding activation of human (G163R) mutant CCR5. (B). Activation of AGM CCR5 by MIP-1$\alpha$ was compared to AGM (R163G) mutant CCR5. Inward potassium currents were measured at -80 mV during voltage pulses in two (B) or three (A) oocytes. Error bars in (A) represent the S.E. The $EC_{50}$ values of activation were as follows: human 0.74 $\pm$ 0.28 nM; human (G163R), 1.22 $\pm$ 0.13 nM AGM, 0.3 $\pm$ 0.06 nM; and AGM (R163G), 0.3 $\pm$ 0.01 nM.

A .




B .

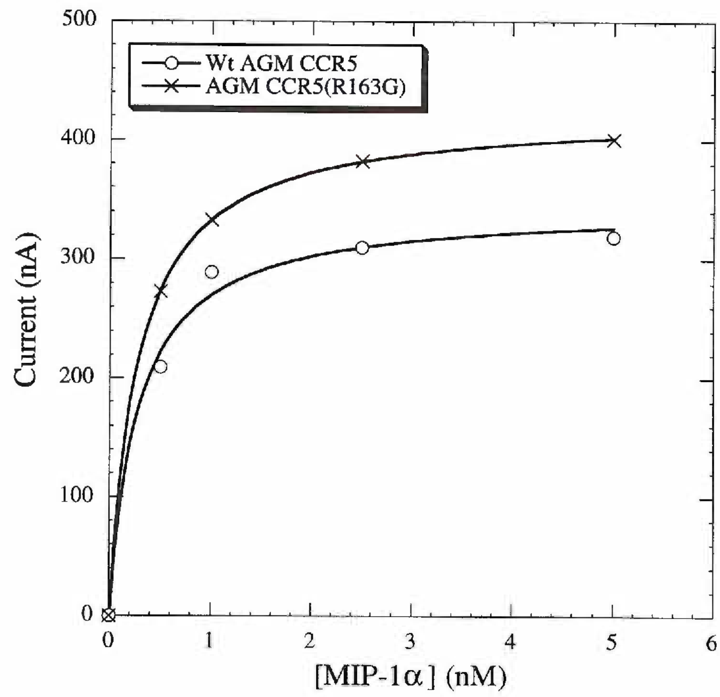
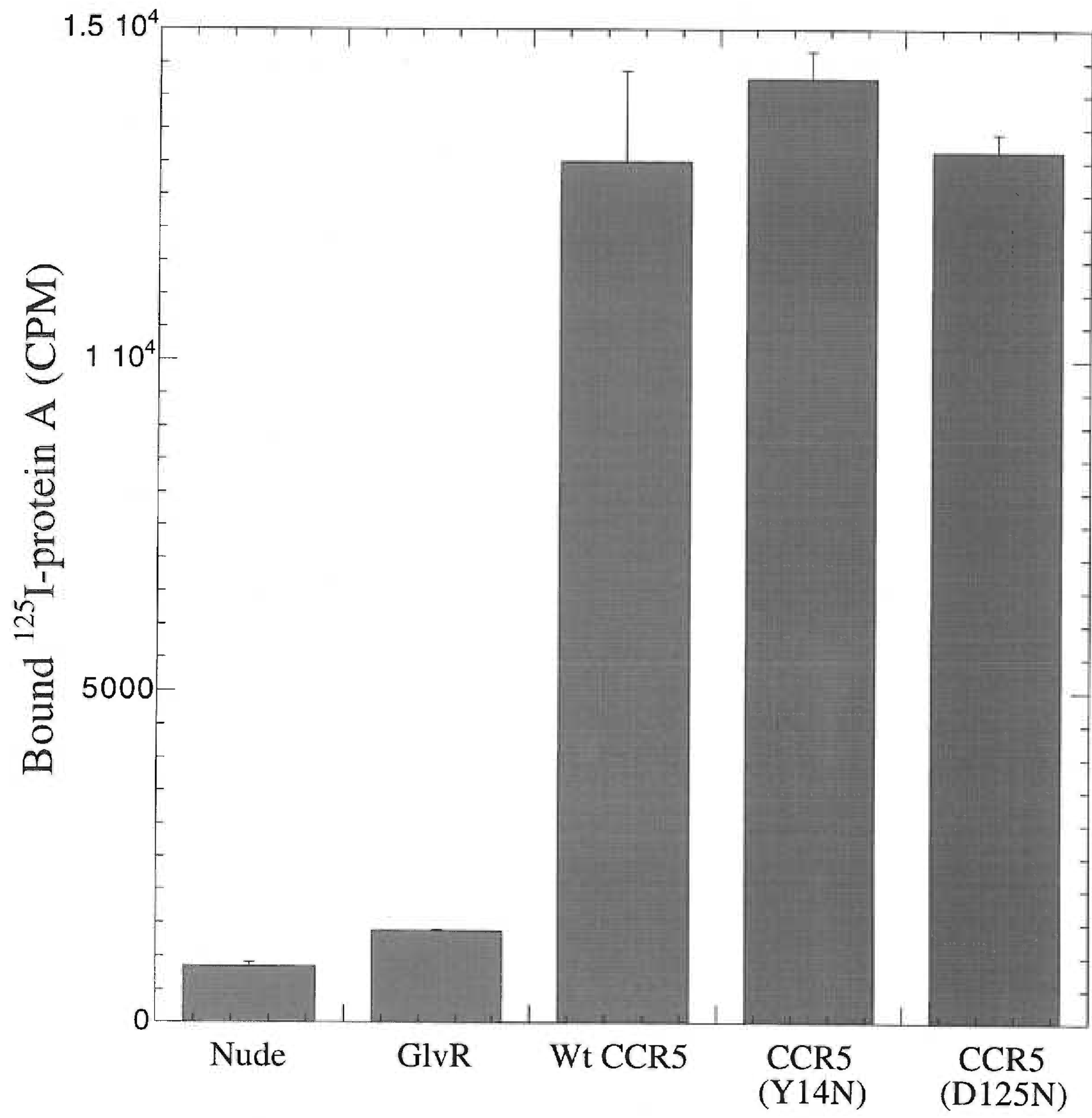


**Figure 2: Cell surface expression of CCR5 is unaffected by the substitutions at amino acids 14 and 125.** cRNA injections were as before. $^{125}$I-protein A binding was measured 4 days later. Water injected, or oocytes expressing GlvR (Gibbon-ape leukemia virus receptor injected to determine nonspecific binding), wild-type or mutant CCR5, were incubated with a mouse monoclonal antibody 2D7 specific for CCR5 (PharMingen, San Diego, CA) at a dilution of 1:100 for 60 min, washed for 5 min, incubated with goat anti-mouse IgG serum (Organon Teknika Corp., Durham, NC) at a 1:500 dilution for 45 min, washed, and then incubated with $^{125}$I-protein A (NEN Life Science Products) for 45 minutes. Following this incubation the oocytes were washed as before, lysed in 10% SDS, and counted in a gamma counter. All the incubations were done with excess amounts of reagents in oocyte Ringer's solution (100 mM KCl, 2 mM NaCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM HEPES, pH 7.5) containing 10% fetal bovine serum (FBS). Results are the average of six oocytes from a single representative experiment. Error bars represent the S.E..



1.5 10^4
1 10^4
5000
0
Bound 125I-protein A (CPM)
Nude
GlvR
Wt CCR5
CCR5
(Y14N)
CCR5
(D125N)

**Figure 3: Effects of substitutions at amino acid 14 and 125 on activation of CCR5 by MIP-1$\alpha$.** *Xenopus* oocytes were injected with cRNAs as in figure 1. Inward currents were measured 4 days following injections. Activation of human CCR5 by MIP-1$\alpha$ was compared with the activation of human (Y14N) and human (D125N) mutants. As in figure 1, inward currents were measured at -80 mV during voltage pulses in three oocytes. The $EC_{50}$ values of activation were as follows: wild-type human 1.3 $\pm$ 0.4 nM; human(Y14N) 3.7 $\pm$ 1.1 nM; human(D125N) 7.0 $\pm$ 3.5 nM.

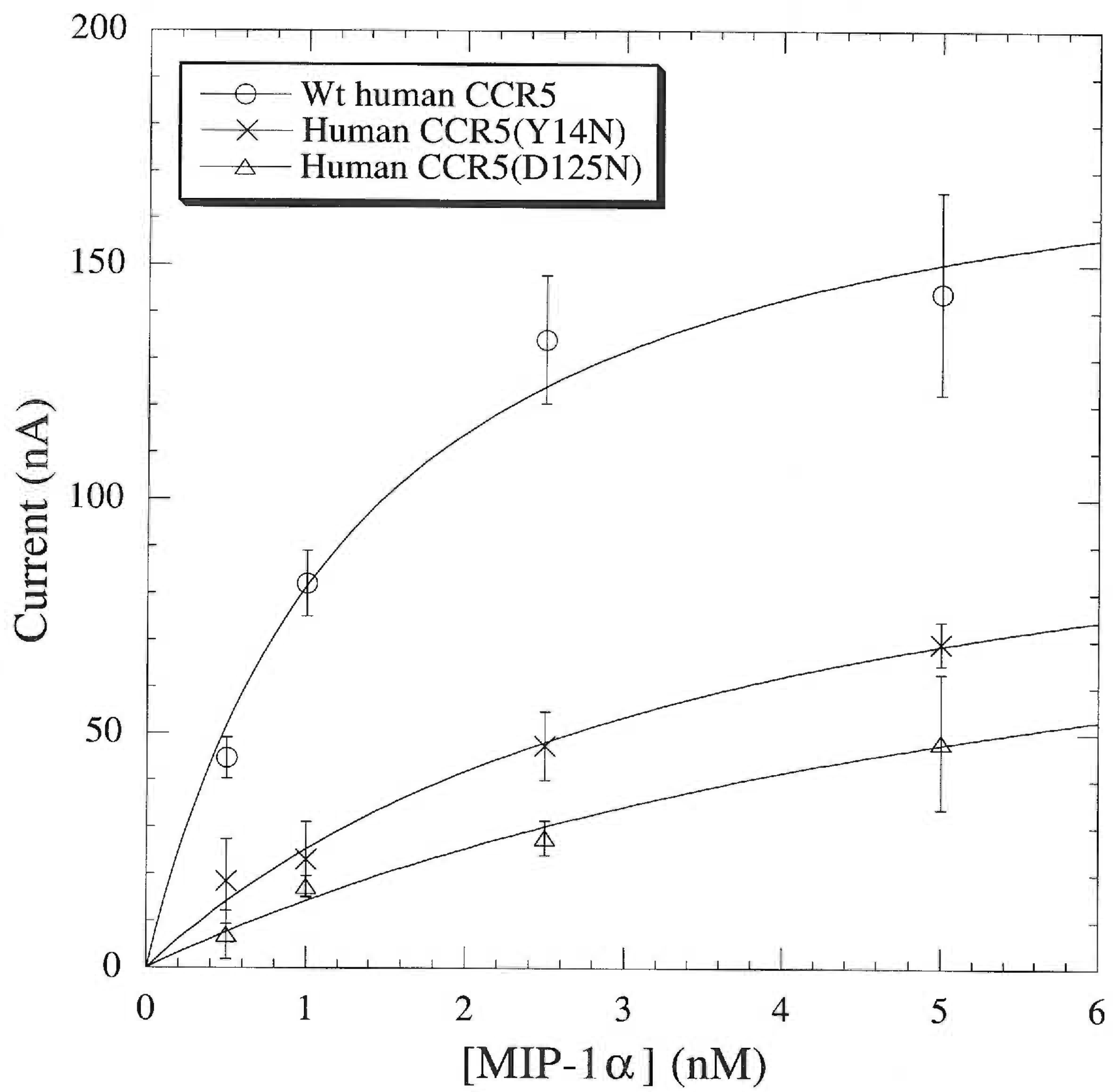
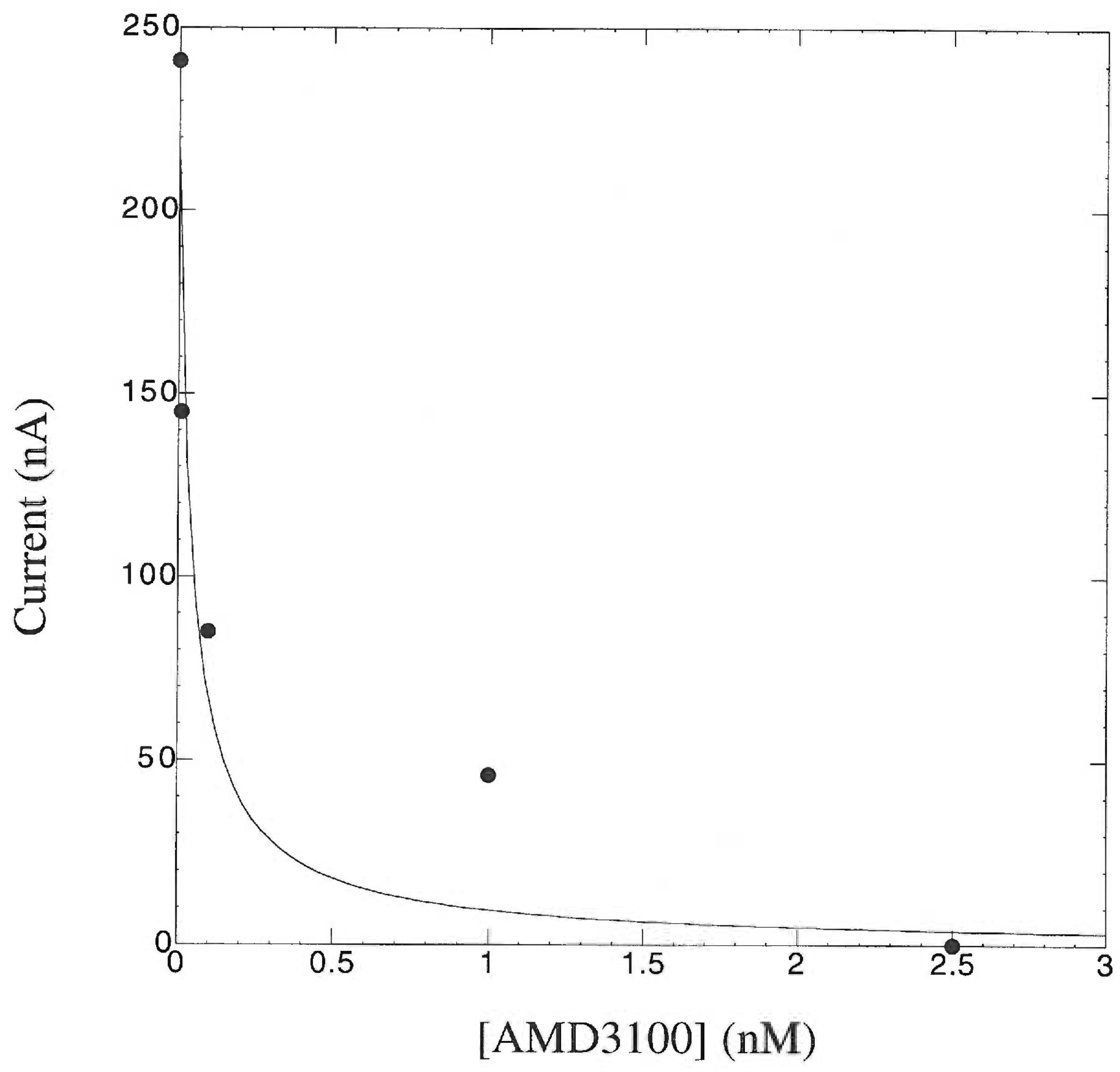

Wt human CCR5
Human CCR5(Y14N)
Human CCR5(D125N)
Current (nA)
[MIP-1α] (nM)
200
150
100
50
0
0
1
2
3
4
5
6

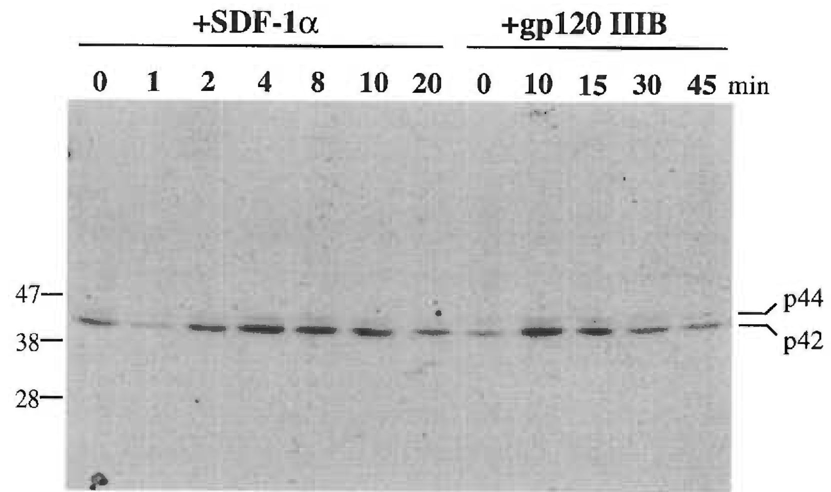
**Figure 4: Inhibition of CXCR4 activation by AMD3100.** *Xenopus* oocytes were injected with CXCR4 cRNAs as in figure 1. Inward currents were measured 4 days following injections. Activation of human CXCR4 by 2.5 nM SDF-1$\alpha$ was measured in the presence of increasing concentrations of AMD3100 (generous gift of Dr. E. DeClercq). As in figure 1, inward currents were measured at -80 mV during voltage pulses. The $IC_{50}$ values of inhibition were determined to be approximately 40 $\pm$ 30 *p*M. Each point represents an average of two oocytes.


250
200
150
100
50
0
Current (nA)
0
0.5
1
1.5
2
2.5
3
[AMD3100] (nM)

# Appendix II

## gp120 stimulates CD4 mediated signaling in U87MG/CD4 cells

**Figure 1: Transient phosphorylation of MAP-kinase by incubation of chemokines and purified gp120 with stable clones of U87MG cells.** (A) U87MG/CD4/CXCR4 were seeded at 5 x $10^4$ cells in 60-$cm^2$ wells of a 6-well culture dish in Dulbecco's Modificed Eagle's medium (DMEM) containing 10% Fetal Bovine Serum (FBS). 8 h later their medium was changed and they were incubated in DMEM containing 5% FBS for 12 h. The cells were then incubated for specific length of time with either 120 nM SDF-1$\alpha$ or 5 $\mu$g/mL of purified gp120 IIIB. After each incubation, total cell extracts were obtained by first washing the cells in phosphate-buffered saline (PBS) (Life Technologies), followed by cell lysis in sodium dodecyl sulfate-polyacrylamide gel electrophoresis sample buffer (62.5 mM Tris-HCl [pH 6.8], 10% glycerol, 2% sodium dodecyl sulfate, 0.1% bromophenol blue, 10% 2-mercaptoethanol). The samples were then boiled, and equal amounts were loaded onto 10% polyacrylamide gels in the presence of 0.1% sodium dodecyl sulfate and subjected to electrophoresis. The proteins were then electrotransferred to nitrocellulose membranes and used for immunoblotting. Phosphorylation of MAP-kinase was detected by blotting with an antibody specific to dually phosphorylated MAP-kinase at Thr202 and Tyr204 (New England Biolabs). Antibody binding was then detected with a phototope-HRP Western blot detection kit (New England Biolabs). (B) U87MG/CD4/CCR5 were seeded as in A. The cells were then incubated for specific lengths of time with either 120 nM of MIP-1$\beta$ or 5 $\mu$g/mL of purified gp120 Ba-L. Activation of MAP-kinase and its detection was done as in A. Mrs are indicated on the left, in thousands. As a control for protein loading each blot was stripped and reblotted with anti-MAP-kinase antibody (data not shown).


A.
+SDF-1α
+gp120 IIIB
0 1 2 4 8 10 20 0 10 15 30 45 min
47—
38—
28—
p44
p42

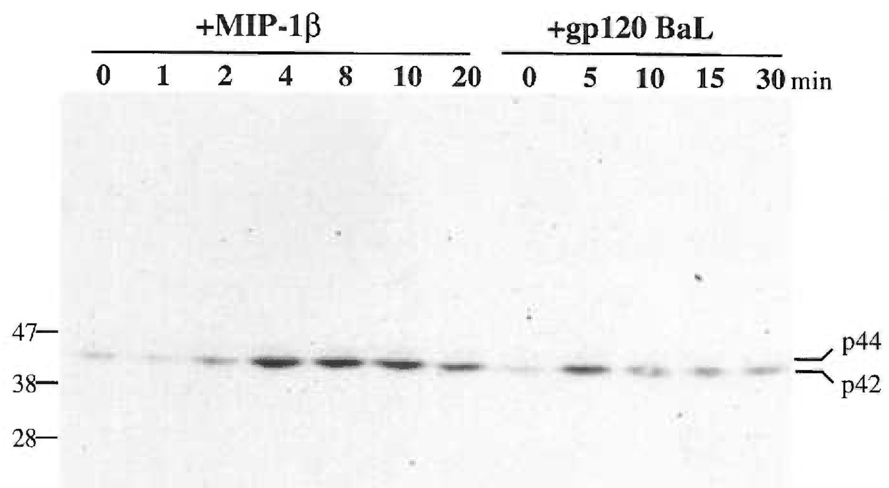
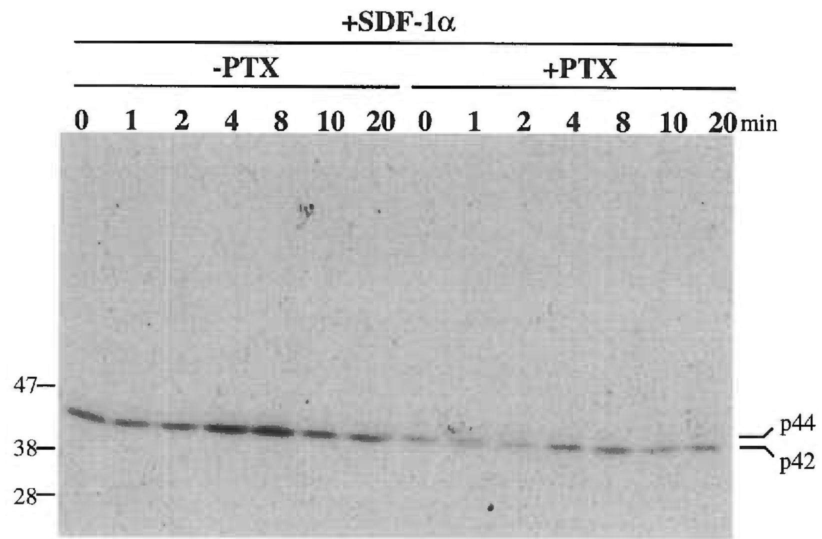

B.
+MIP-1β
+gp120 BaL
0 1 2 4 8 10 20 0 5 10 15 30 min
47—
38—
28—
p44
p42

**Figure 2: Inhibition of SDF-1$\alpha$ but not gp120 IIIB activation of MAP-kinase by pertussis toxin.** (A) and (B). U87MG/CD4/CXCR4 cells were seeded as in figure 1. 6-8 h later the medium was changed and DMEM containing 0.5% FBS was added to all wells. Pertussis toxin (PTX) treatment of cells was done by incubating cells with 200 ng/mL of PTX for 12 h. Control and PTX treated cells were then incubated for specific lengths of time with either 120 nM of SDF-1α (A) or 5 $\mu$g/mL of purified gp120 IIIB (B). Cell lysis, electrophoresis, electrotransfer and immunoblotting was done as in figure 1.

A.




B.

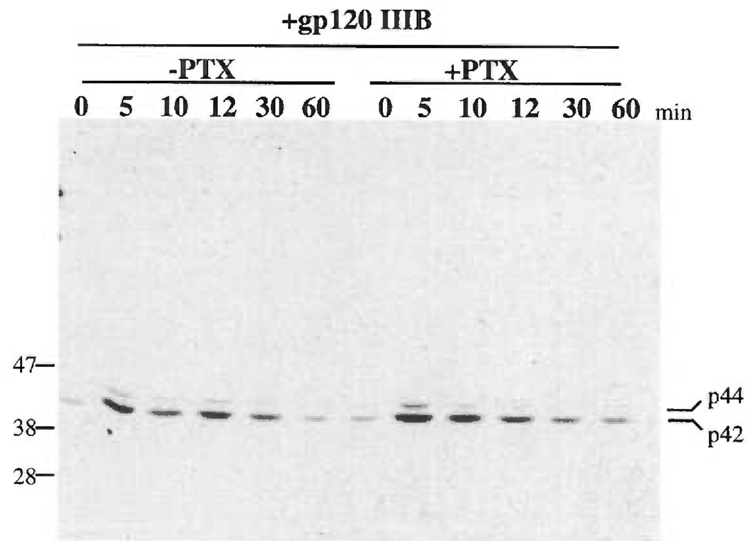
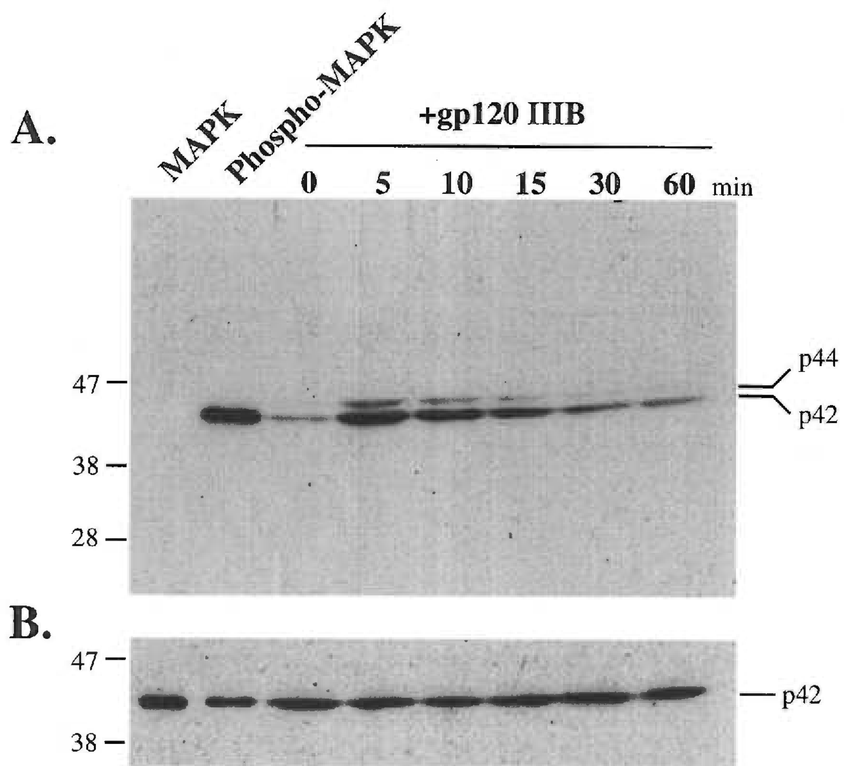


**Figure 3: Activation of MAP-kinase by incubation of gp120 IIIB with U87MG/CD4 cells.** (A). U87MG/CD4 cells were seeded and incubated with serum free medium as before. 12 h later, the cells were incubated with 5 $\mu$g/mL of gp120 IIIB for specific lengths of time. The first two lanes are control lanes with 5 ng of unphosphorylated and phosphorylated MAP-kinase, respectively. The cell extracts were obtained, electrotransferred and immunoblotted as before. (B). As a control for protein loading the blot was stripped and reblotted with MAP-kinase antisera.


A.
MAPK
Phospho-MAPK
+gp120 IIIB
0
5
10
15
30
60
min
47
38
28
p44
p42
B.
47
38
p42