HIV-1 Envelope-Induced Signaling Mediates Actin Cytoskeleton Rearrangements Necessary for Fusion and Entry

Brooke Harmon

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ABSTRACT OF THE DISSERTATION

HIV-1 Envelope-Induced Signaling Mediates Actin Cytoskeleton Rearrangements Necessary for Fusion and Entry

By

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Doctor of Philosophy in Biology and Biomedical Sciences (Molecular Microbiology and Microbial Pathogenesis)

Washington University in St. Louis, 2009

Professor Lee Ratner, Chairperson

Human immunodeficiency virus type-1 (HIV-1) initiates infection by direct fusion of the virus membrane with the plasma membrane of the target cell. This fusion event is a multi-step process mediated by the envelope (Env) surface subunit gp120 and the transmembrane subunit gp41 which anchors gp120 into the viral membrane. First the surface subunit gp120 binds to the primary receptor CD4. This interaction promotes actin cytoskeletal rearrangements in the target membrane that bring the chemokine coreceptor, CCR5 or CXCR4, into close proximity for binding and induces conformational changes in gp120 that allow it to couple to the coreceptor. Formation of the gp120-CD4-coreceptor complex triggers conformational changes in the transmembrane gp41 subunit that allows gp41 to insert into the target cell membrane, allowing lipid mixing or hemifusion, and pore formation. Previous studies from our lab have shown that reorganization of the actin cytoskeletal network is required for multiple steps in HIV-1 fusion. Additional data from our lab show that HIV-1 Env induced activation of Rac is
critical for HIV-1-mediated membrane fusion. HIV-1 Env activates multiple signaling pathways that could potentially lead to Rac activation. In an effort to determine which signaling molecules were required both upstream and downstream of Rac activation, we utilized small interfering RNAs (siRNAs) and various small-molecule inhibitors to disrupt signaling pathways activated by HIV-1 Env through CD4 and coreceptors. Published data from our lab demonstrated that the Goq pathway, activated by CCR5, is required for Env-mediated Rac activation and fusion. Further studies have shown that the downstream effectors of Rac that promote fusion are components of the Wave2 signaling complex and at least some of these signaling components are required at a post-hemifusion step. Other studies from our lab and other labs, with mutant CD4 constructs, have shown that signaling through CD4 is also playing a role in membrane fusion, most likely at the step of forming the gp120-CD4-coreceptor complex. These results suggest a model in which gp120 signaling though CD4 promotes gp120 binding to and signaling through the coreceptor, which is necessary for full fusion and release of the viral nucleocapsid into the cell’s cytosol.
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Chapter 1

Introduction
HIV AND AIDS

Human immunodeficiency virus (HIV), a complex retrovirus and the etiologic agent of acquired immunodeficiency syndrome (AIDS), has infected more than sixty million people worldwide since its discovery in the early 1980s. AIDS was first identified in the United States (US) in 1981. Clinicians in New York and California noted an unusual clustering of rare diseases such as Kaposi sarcoma and other opportunistic infections in young homosexual men. It soon became apparent that there was a common immunological deficit in cell-mediated immunity, resulting from a loss of circulating CD4$^+$ T cells in these patients. In 1983, a research team in France published data indicating an association between a retrovirus and AIDS. At the time there were two known retroviruses, human T-lymphotrophic virus (HTLV)-1 and HTLV-II that were recognized as viruses known to preferentially infect CD4$^+$ T cells. The transmission pattern among individuals with HTLV was similar to that seen among AIDS patients. In 1984 the French group and researchers at the National Institutes of Health (NIH) led by Robert C. Gallo published evidence that the virus known as HIV was the cause of AIDS (1).

The discovery of HIV as the agent that causes AIDS led to intense research in the field of molecular virology that is still ongoing (1). This research has led to the development of potent antiretroviral drugs, 30 of which are currently approved by the Food and Drug Administration (FDA) to treat people infected with HIV. These drugs are prescribed in combination in a treatment known as highly active antiretroviral therapy (HAART). This treatment, although effective at suppressing the virus does not cure the infection so it must be maintained for life, and prolonged use often results in significant
toxicity. Any interruption in treatment results in the rapid rebound of replicating virus which can lead to drug resistance. An increase in heart disease, diabetes, liver disease, and many forms of cancer has also been observed in aging HIV-infected patients who are receiving treatment (2). Due to the high cost of HAART therapy and the lack of an adequate delivery method, it is not available in developing countries where it is most needed. According to the 2008 UNAIDS/WHO report, there are 33 million people estimated to be currently living with HIV/AIDS worldwide, with 22 million residing in sub-Saharan Africa. Out of the 9.7 million people in immediate need of life-saving AIDS drugs, only 2.99 million (31%) are receiving the drugs. This information clearly shows that current treatment strategies are not sufficient and that understanding the molecular biology of HIV and its relationship with the host is imperative to further progress in drug discovery, vaccine development and prevention methods.

**HIV GENES AND THEIR PROTEIN PRODUCTS**

Lentiviruses are a separate genus of the family *Retroviridae*. They include equine infectious anemia virus, visna/maedi virus, caprine arthritis-encephalitis virus, bovine immunodeficiency virus, feline immunodeficiency virus, simian immunodeficiency virus (SIV), and HIV. There are two types of HIV, HIV-1 and HIV-2. HIV-1 is found globally, whereas HIV-2 is found predominantly in West Africa. HIV-2 has a longer clinical latency period than HIV-1 and a lower morbidity rate. HIV-1 and HIV-2 have 40-60% genomic similarity and are thought to have evolved from SIV. Lentiviruses possess a relatively large genome, with more genes than other retroviruses. Lentiviral genomes encode the three structural proteins common to all retroviruses, Gag, Pol, and Env, as
well as many additional auxiliary proteins. The \textit{gag} gene encodes the 55kD Gag polyprotein precursor, and due to a ribosomal frameshift event, the 160kD Gag-Pol polyprotein that is cleaved to produce viral protease (PR), integrase (IN), and reverse transcriptase (RT). RT transcribes viral DNA from the viral RNA genome, IN catalyzes integration of viral DNA into the host chromosome, and during maturation of virus particles, PR cleaves the Gag polyprotein into matrix (MA), capsid (CA), and nucleocapsid (NC). The product of the \textit{env} gene is a monomeric glycoprotein precursor (gp160) that is processed into the gp120 surface subunit and the gp41 transmembrane subunit (2-4).

The auxiliary proteins, transcriptional activator (Tat) and regulator of viral gene expression (Rev), perform regulatory functions necessary for viral replication \textit{in vitro} and \textit{in vivo}. The negative effector (Nef), viral infectivity factor (Vif), viral protein R (Vpr), and viral protein U (Vpu), are not essential for viral replication in vitro, and are known as the accessory proteins. Tat and Rev are translated early in infection from multiply spliced RNA, so that they may function to stimulate transcription and facilitate mRNA export, respectively. The accessory protein Nef is also translated early from multiply spliced RNA, so that it may induce signaling pathways that enhance viral replication. Nef promotes the production and release of more infectious virions by activating T cells, activating expression of FasL which induces apoptosis in cytotoxic T cells that express Fas, and by downregulating expression of CD4 and major histocompatibility complex (MHC) class I molecules on the cell surface. Nef also inhibits apoptosis in the infected cell by inhibiting p53 and by blocking Fas and TNFR death signaling pathways (2, 3).
The rest of the accessory proteins, Vif, Vpr, Vpx, and Vpu are translated late in infection from singly spliced mRNAs. Vif is required early in infection to stabilize the reverse transcription complex (2, 3). Vpr is associated with the nucleocapsid of the virion and has multiple functions in the cell. Vpr promotes G2 cell cycle arrest, plays a role in reverse transcription, promotes entry of viral DNA into the nucleus, regulates apoptosis and transactivates the HIV-LTR (5). The HIV-2 and SIV genomes encode the accessory protein Vpx in addition to Vpr. Similar to Vpr, Vpx is associated with the nucleocapsid and is essential for replication of SIV and HIV-2 in non-dividing macrophages. However the role of Vpx in the HIV-1 life cycle is less clear. The current hypothesis for the role of Vpx is that it interacts with a ubiquitin ligase complex to inactivate a restrictive host factor in macrophages (6). The final accessory protein Vpu is unique to HIV-1 and SIV<sub>CPZ</sub> and is involved in viral release and degradation of CD4 (2, 3).

**HIV-1 LIFE CYCLE**

HIV-1 entry is mediated by the envelope (Env) glycoproteins gp120 and gp41. Entry is a multistep process that begins with binding of gp120 on the virion surface to the receptor CD4 on the surface of the target cell. This gp120-CD4 interaction induces conformational changes in the gp120/gp41 complex that expose coreceptor binding regions of gp120 and regions of gp41 necessary for insertion into the membrane. Next, one of two chemokine coreceptors, CCR5 or CXCR4, is localized to the binding site and gp120 binds the coreceptor, leading to dissociation of gp120 and gp41. Subsequently the fusion peptide gp41 is inserted into the target membrane leading to establishment of the
fusion pore and pore expansion (4, 7). The process by which HIV-1 virions enter the host cell will be discussed in great detail below.

Once fusion occurs and the NC is released inside the cell, viral uncoating occurs, revealing the reverse transcription complex. This complex includes the diploid viral positive sense RNA genome, tRNA\textsuperscript{lys} primer, RT, IN, MA, NC, Vpr and various host proteins. Phosphorylated MA then facilitates docking of this complex on actin microfilaments which is necessary for DNA synthesis. Completion of reverse transcription results in double stranded DNA that assembles with IN, MA, Vpr, RT, and host proteins to form the preintegration complex (PIC), which is then transported on microtubules to the nucleus. The PIC has a 28nm radius making it approximately the same size as a ribosome and making it twice the size of the center aqueous channel in the nuclear pore, indicating that nuclear import is not a simple process. Many of the proteins in the PIC have nuclear targeting signals, so these proteins could be functioning in a cooperative manner to facilitate import or they could be playing unique roles based on the target cell. Once the PIC is inside the nucleus, integration of viral DNA into the host chromosome is mediated by IN, and this establishes a functional provirus. Alternatively, the ends of the viral DNA may be joined to form 2-LTR circles, the virus may undergo homologous recombination resulting in a single LTR circle, or the viral DNA may auto-integrate into itself. None of these processes result in production of infectious virus; however these circular forms may be transcriptionally active leading to production of Tat and Nef (2, 3).

Once the viral DNA is integrated, it is transcribed into proviral mRNAs, and the mRNA is transported to the cytoplasm. Transcription of viral DNA into mRNA is a
multi-step process involving both host and viral proteins. First, the host-cell transcription machinery transcribes short nonpolyadenylated transcripts that form an RNA stem loop called the transactivation response (TAR) element. In association with the cellular protein cyclin T1, the viral protein Tat binds TAR and recruits cyclin-dependent kinase 9 (Cdk9) and other activating host proteins to the HIV LTR, where they form the positive transcription-elongation factor b (P-TEFb) complex. The P-TEFb complex positively regulates the host RNA polymerase II, allowing efficient elongation of the viral genome. During the transcription process, more than a dozen specific transcripts are generated. Some of these transcripts are unspliced or singly sliced and they stay in the nucleus, whereas the multiply spliced transcripts that encode Nef, Tat, and Rev are transported into the cytoplasm early in infection (2, 3).

Once transcribed, the small shuttling protein Rev binds to the Rev response element (RRE) in the env gene and in association with host proteins exports the unspliced and singly spliced transcripts from the nucleus to the cytoplasm (2, 3). The singly spliced transcripts encode the structural and accessory proteins Env, Vif, Vpr, and Vpu, whereas the unspliced transcripts encode the viral RNA genome, Gag-Pol mRNA, and pre-mRNA. Once in the cytoplasm Gag and Gag-Pol polyprotein precursors are translated by cytoplasmic ribosomes, Env mRNA is translated by ribosomes bound to the endoplasmic reticulum (ER), and full-length RNA molecules become encapsidated as progeny viral genomes. Gag and Gag-pol precursors are transported to the plasma membrane by multivesicular bodies, whereas the Env proteins are transported through the Golgi apparatus where they are glycosylated and cleaved to form the mature gp120 + gp41 complex, and delivered to the plasma membrane. At the plasma membrane myristoylated
Gag associates tightly with the inner leaflet of the membrane, oligomerizes with additional Gag molecules, and recruits Env gp120 and gp41 proteins as trimers to the site of assembly. Also recruited to the site of assembly are two copies of the viral RNA genome, Vpr, and Nef which are then encapsidated. Formation of the immature HIV capsid requires approximately 1,500 Gag polyproteins. The host protein human ATP-binding protein HP68 acts a molecular chaperone for Gag, mediating conformational changes in Gag necessary for the assembly of HIV capsid. Once assembled at the plasma membrane the virions bud from the surface of the cell. This process involves the host-cell budding machinery and is directed by Gag. At this point the immature virion is noninfectious; maturation requires that PR cleave Gag and Gag-Pol, at which point Gag molecules rearrange to form mature infectious virus which involves condensation of the virion cores (2, 3).

**HIV-1 ENTRY**

HIV-1 enters cells by fusion at the plasma membrane and endocytosis, and it is thought that only fusion at the plasma membrane leads to productive infection (2). However, a recent study has shown that complete HIV-1 fusion can occur in endosomes resulting in efficient infection (8). Fusion at both the plasma membrane and in endosomes is mediated by the Env glycoproteins expressed on virions and CD4 and coreceptors expressed on the target cell surface. On the virion gp120 is organized into trimeric spikes that associate noncovalently with trimeric gp41 which anchors gp120 into the viral membrane (4). HIV-1 fusion is a complicated process that is dependent on actin cytoskeleton rearrangements in the target cell and multiple conformational changes in Env. The initial attachment of the mature virion to the target cell is due to the interaction
of positively charged regions on Env with negatively charged heparan sulfate proteoglycans. This attachment brings gp120 into close proximity to the primary receptor CD4 (4, 9). Gp120 then binds to CD4 and this engagement induces conformational changes in the gp120/gp41 complex and actin cytoskeleton rearrangements in the target cell. Multiple regions of gp120 undergo conformational changes upon binding to CD4. One set of conformational changes results in exposure of the coreceptor binding domain on gp120. This domain includes conserved epitopes near or within the bridging sheet and the V3 loop. CD4 binding has been implicated in promoting exposure of the bridging sheet by moving the V2 loop. Additional conformational changes occur in the C1-C4/5 regions of gp120 that are occluded due to gp41 interactions. CD4 binding also promotes conformational changes in gp41 that induce formation and exposure of the gp41 pre-hairpin intermediate (4). The actin cytoskeleton rearrangements induced in the target cell by CD4 binding promotes recruitment of chemokine receptors CXCR4 or CCR5, cholesterol, ganglioside GM1, adhesion molecules, and GPI-anchored proteins to contact sites (10, 11). Once the coreceptor binding site on gp120 is exposed and the coreceptor is in close proximity gp120 can bind to coreceptor. Binding of gp120 to the coreceptor induces signaling through the coreceptor that results in actin cytoskeleton rearrangements necessary for fusion. Binding to the coreceptor also induces additional conformational changes in gp41 and subsequent insertion of the hydrophobic N-terminus of gp41 into the target membrane (4, 12).

To follow gp41 progression through intermediate conformations, the formation/exposure of novel gp41 epitopes has been assessed by antibody reactivity or by binding measurement of gp41-derived peptides that are complementary to the heptad
repeat region (HR) 1 and HR2 domains of gp41 (7, 13-21). Intermediate conformations where HR domains are exposed after binding to CD4 and coreceptor and prior to six-helix bundle (6HB) formation are referred to as pre-bundles (7). During fusion gp41 folds into three intermediate prebundle conformations that mediate different steps of membrane fusion before folding into a stable 6HB as depicted in Figure 1. The first gp41 early prebundle forms after gp120 binding to CD4. Further conformational changes in gp120 and gp41 occur after binding to coreceptors which result in dissociation of gp120 and gp41. At this stage HR1 and HR2 of gp41 are exposed. Additional folding of gp41 results in formation of a second bridging prebundle that inserts itself into the target cell’s plasma membrane and further folding of gp41 into the fusogenic intermediate results in hemifusion (lipid mixing). The energy from the final folding of gp41 into the 6HB conformation is thought to facilitate pore formation and enlargement while the 6HB, which forms after pore formation, is though to prevent pore closure. In the 6HB conformation the HR2 domains are packed in antiparallel orientation against the trimeric HR1 coiled coil and this interaction forms a thermodynamically stable 6HB structure (7, 22, 23). The actin cytoskeleton rearrangements induced by Env signaling via coreceptor in cooperation with folding of multiple gp41 intermediates into 6HBs mediates the pore formation and enlargement necessary to allow delivery of the nucleocapsid to the cytoplasm (7).

The initial steps of Env binding to and forming ternary complexes with CD4 and coreceptor can occur at blocked temperatures (below 18°C when membrane fusion does not occur) without promoting hemifusion or fusion. HR1 and HR2 domains of gp41 are also exposed after receptor binding at blocked temperatures. The formation of the Env-
CD4-coreceptor complexes and the intermediate gp41 conformations at this lower temperature is referred to as temperature arrested stage (TAS). The next stage of lipid mixing which occurs at optimal temperature can be blocked by lyso-lipids that inhibit fusion by disfavoring monolayer bending. This arrested stage is termed lipid-arrested stage (LAS) and is used to identify the gp41 intermediate structure that forms directly before membrane merger (7). At this stage the gp41-derived peptides inhibit fusion demonstrating that the 6HB has not yet formed. The gp41 conformations formed at optimal temperature but prior to membrane merger (LAS) directly engage the target membrane and gp41 folding into 6HB is completed after but not before pore formation (7). Based on these observations it is thought that gp41 pre-bundles can form small nascent fusion pores and 6HBs play a role in stabilizing and possibly expanding the pore.
Fig. 1. Model of intermediate steps of HIV-1 Env-induced fusion progressing through early, bridging, and fusogenic prebundles toward 6-helix bundles that form after opening of the fusion pore adapted from Melikyan 2008 (7). As mentioned in the text actin cytoskeletal rearrangements are necessary in the target cell first to localize the coreceptor to the site of gp120-CD4 binding and then again at a post-binding step most likely to facilitate pore formation and enlargement.
THE ACTIN CYTOSKELETON AND HIV-1 ENTRY

In the case of HIV-induced membrane fusion as well as other types of membrane fusion, actin remodeling is probably required for at least 3 steps in the membrane fusion process (12, 24). The actin filament capping drug that interferes with actin assembly, Cytochalasin D (CD), inhibits Env-CD4-coreceptor complex formation (25) but has little to no effect on fusion post-binding (12, 25). Jasplakinolide (JP), an actin filament stabilizing drug and Lactrunculin A (LA), a monomer sequestering drug both inhibit HIV-1 induced membrane fusion at a post-binding step (12).

Rho GTPases are involved in multiple cell signaling pathways leading to cytoskeleton rearrangements, cell adhesion, cell polarity, endocytosis, vesicle trafficking, progression through the cell cycle, differentiation, oncogenesis, and gene transcription (26, 27). Rho GTPases are regulated by three groups of proteins, guanine nucleotide exchange factors (GEFs), which increase exchange of GDP for GTP, GTPase activating proteins (GAPs), which stimulate intrinsic GTPase activity, and guanine nucleotide dissociation inhibitors (GDIs), which inhibit the dissociation of GDP from GTPases (27-30). Membrane fusion is mediated by Rho GTPase activation of multiple downstream effector complexes involved in cytoskeletal rearrangement (24). There are three main subfamilies of Rho GTPases, each controlling a signal transduction pathway connecting membrane receptors to assembly and disassembly of the actin cytoskeleton. Members of the Rho subfamily stimulate myosin based contractility resulting in stress fibers and focal adhesions. The Rac subfamily stimulates lamellipodia and membrane ruffles and the Cdc42 subfamily stimulates the formation of filopodia (27, 29, 31, 32).
Upon incubation with HIV-1 R5 Env expressing cells, target cells expressing CD4 and CCR5 underwent changes to the membrane structure resulting in membrane ruffles, lamellipodia and filapodia extensions, indicating activation of Rac and Cdc42 in target cells (12). However, only the downstream effects of Rac are required for Env-induced membrane fusion, as expression of dominant negative (DN) Rac completely abolished fusion but DN Cdc42 had no effect. On the other hand, expression of DN Rho, in target cells caused a 40% reduction in syncytium formation and cell fusion as measured by dye transfer, but no stress fibers were observed in cells incubated with HIV R5 Env expressing cells (12). In addition, both Rac and Rho have been shown to be activated in target cells incubated with HIV R5 Env expressing cells or with HIV-1, respectively (12, 33).

The study demonstrating Env-mediated RhoA activation also showed a 50% reduction in single cycle infection and cell-cell fusion with cells expressing DN RhoA (33). A more recent study described a role for Env-mediated filamin-A association with CD4 and coreceptor in Env-CD4-coreceptor complex formation. Interaction of filamin-A with CD4 and coreceptor induces phosphorylation and inactivation of cofilin through a RhoA and ROCK dependent mechanism (11). The activation of Rac and Rho by HIV-Env and the inhibition of fusion by blocking this activation is evidence that HIV-1 induced signaling to Rho GTPases is required for fusion. However, the studies with CD, LA and JP demonstrated that actin cytoskeleton rearrangement is necessary at multiple steps in the membrane fusion process (12, 25). Therefore it is unclear whether Rac and Rho are required for the same or different steps in the fusion process.
Rho activation mediates integrin clustering and promotes actin polymerization, however the level of actin polymerization in response to Rho is relatively small (27). Outside of integrin clustering Rho activation has been shown to have a stabilizing effect on the actin cytoskeleton. Its activation leads to suppression of cofilin, an actin depolymerizing protein, and enhancement of myosin activity which results in a rigid cortex (27, 34, 35). On the other hand, Rac is a potent activator of polymerization and has been shown to activate multiple actin remodeling proteins leading to nucleation of actin polymerization, and uncapping of actin filaments (35). Stimulation of Rac has also been shown to actively antagonize Rho resulting in disassembly of stress fibers and focal adhesions (27, 36).

Rho has also been shown to mediate inactivation of Rac via the filamin-A-binding Rac GAP, FilGAP. FilGAP antagonizes Rac in response to phosphorylation by the RhoA-activated ROCK, and the binding of FilGAP to filaminA is thought to position FilGAP where upstream factors can activate and inactivate it (37). This study and the 2007 Jimenez-Baranda study describing a role for filamin A, RhoA and ROCK in Env-CD4-coreceptor complex formation, suggest that RhoA activation is required at this initial step. Interestingly another study has shown that the C terminus of gp41 interacts with and inhibits p115 Rho GEF, suggesting that Rho is turned off and unnecessary for fusion events after gp41 insertion (38). Therefore it seems likely that Rho is involved in the initial step of Env-induced fusion (Env-CD4-coreceptor complex formation) before it is inactivated by the C-terminus of gp41, and active Rac can then mediate later steps in membrane fusion.
HIV-1 Env induces multiple signaling pathways through CD4 and the coreceptors that could potentially lead to Rac activation and actin cytoskeleton reorganization (27, 32, 39-43). Preliminary studies from our lab indicate that Env-induced Rac activation is mediated by the coreceptor as determined by inhibition of Rac activation with the CCR5 small molecule inhibitor TAK779, and the inability of X4 Env to induce Rac activation in CCR5 expressing cells (12). Additional studies with cells lacking expression of CD4 indicate that CD4 also plays a role in Env induced Rac activation (preliminary studies in chapter 5). Also, as described in chapter 2, a small molecule inhibitor (Rac GEF Inhibitor) that blocks recognition of Rac by Rac GEFs dependent on a specific Rac tryptophan (Trp56) eliminated Env-dependent fusion (44, 45). The Rac GEFs shown to be sensitive to this inhibitor are the Rac specific GEF Tiam-1 and the Rac and RhoG specific GEF Trio (44). Rac can also be activated and stimulated to promote actin rearrangements by an unconventional Rac GEF DOCK180/Elmo/CrkII/p130Cas (DOCK180 complex) (28, 39, 46-48). The DOCK180 complex requires cooperation with multiple proteins to mediate Rac activation including RhoG and Trio, suggesting that this GEF, in addition to Tiam-1 and Trio, would also be inhibited by the Rac GEF inhibitor and is possibly required for Env-induced Rac activation and fusion (39).

**CHEMOKINE RECEPTORS CXCR4 AND CCR5**

Chemokines were first identified as chemoattractant cytokines that regulated leukocyte trafficking to sites of inflammation. However chemokines are involved in more than just chemotaxis. Chemokines have been shown to be involved in angiogenesis, hematopoiesis, embryonic development, and metatasis (40). Some chemokines and chemokine receptors are highly specific for each other, but in general chemokine
receptors can bind to more than one chemokine and chemokines can interact with more
than one receptor (49). CXCR4 and its ligand, stromal cell derived factor (SDF-
1/CXCL12) are highly specific for one another, whereas CCR5 is associated with four
inflammatory chemokines MIP-1\(\alpha\) (CCL3), MIP-1\(\beta\) (CCL4), RANTES (CCL5), and
MCP-2 (CCL8), and each of these chemokines can also associate with other receptors
including CCR1, CCR4, and CCR2B (49, 50).

Activation of CXCR4 by SDF-1 induces several signaling pathways that lead to
chemotaxis, migration, and secretion of angiopoietic factors. CXCR4 is expressed by
many cell types and SDF-1, a member of the alpha family of chemokines, is a potent
lymphocyte chemoattractant but is also constitutively secreted by fibroblasts in bone
marrow, lymph nodes, lung, liver, and muscle. During embryogenesis signaling through
CXCR4 is involved in both hematopoiesis and organogenesis (51). CXCR4 is also
involved in the homing and retention of hematopoietic/lymphopoietic stem cells, pre T
and B lymphocytes in the bone marrow, and the trafficking of these cells to sites of tissue
inflammation and damage. A recent study provides evidence that SDF-1 may also signal
through another chemokine receptor, CXCR7/RDC1, but its role in cell trafficking and
migration remains undefined (51).

CCR5 is expressed on subpopulations of lymphocytes and monocytes/macrophages
in blood, primary and secondary lymphoid organs and in non-inflamed tissues. CCR5 is
also expressed in the central nervous system on astrocytes, neurons, and microglia and in
other tissue on epithelium, endothelium, vascular smooth muscle, and fibroblasts (52).
Unlike CXCR4 the role of CCR5 in vivo is not very well understood. Studies in knockout
mice have shown only minor defects in cell mediated immune responses and individuals
that lack CCR5 surface expression on their cells due to a 32-base pair deletion in the CCR5 gene are seemingly healthy. However, recent results show a role for CCR5 in control of infection by West Nile virus (WNV). WNV is a mosquito-borne flavivirus that primarily infects birds but can also infect humans. It was first isolated in humans in Uganda in 1937 and has caused periodic outbreaks of encephalitis in Africa and Asia since then. The first reported case in North America was in a flamingo in 1999 and by 2005 it had caused 19,369 cases of human symptomatic illness and 782 deaths in the US alone. Symptoms associated with the virus include mild-flu like illness, and more rarely meningitis, encephalitis, acute flaccid paralysis, and post-neurologic conditions (53, 54).

In CCR5 knockout mice, infection was uniformly fatal, in constrast to wildtype mice where the majority of infected mice survived. In humans with the CCR5Δ32 homozygous mutation, two association studies were performed with a total of four cohorts. These studies showed that the majority of CCR5Δ32 individuals infected with WNV became symptomatic. In one of the cohorts homozygous CCR5Δ32 was also associated with death. This study also suggested that CCR5 controls WNV infection by mediating leukocyte recruitment to the infected central nervous system (53, 55). These studies with WNV were the first to associate an antimicrobial function with CCR5 in humans, however in mouse models of infection CCR5 has been implicated in defense against Trypanosoma cruzi, Toxoplasma gondii, Cryptococcus neoformans, Chlamydia trachomatis and herpes simplex virus 1 and 2 (54).

**CHEMOKINE RECEPTORS AND HIV**

Although there are many different chemokine receptors that could potentially act as coreceptors for HIV, CXCR4 and CCR5 are the receptors preferentially used in the
human host. CCR5 is used by macrophage-tropic viral isolates (R5 viruses) and CXCR4 is used by T-cell tropic isolates (X4 viruses). Transmission of HIV is mediated by R5 viruses which are present in early stages of the disease, and preferentially infect CCR5-expressing macrophages and dendritic cells. R5 viruses are particularly abundant in the gut-associated lymphoid tissue, one of the primary sites of HIV and SIV replication. X4 viruses are present at later stages of disease progression when virus preferentially infects CD4+ T cells (2, 56, 57).

The switch from CCR5 to CXCR4 usage by HIV-1 strains late in the infection course is likely due to selective pressure of endogenous CCR5 chemokines. There is not always a complete switch meaning that viruses can use both CCR5 and CXCR4, and patients may harbor R5 viruses, X4 viruses, and dual-tropic (R5X4) viruses that can use both CCR5 and CXCR4. In a subset of patients, during the terminal stages of disease, R5 and dual-tropic viruses seem to be lost and are replaced by pure X4 viruses. X4 variants emerge in only about 40-60% of HIV-1 positive individuals. X4 variants are associated with expanded cell tropism, increased viral replication rate, faster disease progression, and the onset of AIDS. It is still not clear whether the X4 phenotype is a cause or consequence of HIV disease progression.

**HIV-1 ENTRY INHIBITORS**

Currently there are two entry inhibitors, approved by the FDA for treatment of HIV-1, maraviroc (Selzentry, Pfizer) an antagonist of CCR5, and enfuvirtide (Fuzeon, Roche) also known as T-20, which competitively binds to HR1 on gp41 preventing association of HR1 with HR2. The efficacy of enfuvirtide is limited by the delivery route (subcutaneous injection) and cost. In addition, enfuvirtide is only prescribed in
combination with HAART because it has also been shown to drive viral evolution and resistance (9, 58, 59). There are many others entry inhibitors including attachment inhibitors, co-receptor binding inhibitors, and fusion inhibitors that are currently in clinical trials (9, 58, 59). The risk with inhibitors that block chemokine receptors and CD4 is that they could also block natural ligand binding and signaling, inhibiting essential immune cell functions.

Most coreceptor inhibitors either approved by the FDA or in clinical trials target CCR5. The CCR5 inhibitors that are in clinical trials or beyond, block binding of CCR5 to its chemokine ligands and block CCR5-mediated signaling events. CCR5 is thought to be a better drug target than CXCR4 because individuals harboring the naturally occurring mutation $\text{CCR5}^\Delta32$ have no known health defects due to this deletion. However as mentioned above, CCR5 has been associated with defense against infection with WNV and has been implicated in defense against *Trypanosoma cruzi, Toxoplasma gondii, Cryptococcus neoformans, Chlamydia trachomatis* and herpes simplex virus 1 and 2 (54). These results demonstrate that complete blockade of CCR5 could put patients at an increased risk for numerous infections some of which are potentially fatal. In addition there is the possibility that treatment with CCR5 antagonists would promote emergence of X4 viruses, thereby accelerating disease progression.

Development of antagonists of CXCR4 is also complicated due to its role in multiple processes including cerebral development, embryonic survival and B-cell lymphopoiesis (51, 60, 61). In addition, investigations with the X4 inhibitor AMD3100 revealed that CXCR4 blockade releases myeloid cells and CD34+ stem cells from the bone marrow into the blood, and the backup compound AMD-11070 causes abnormal
liver histology in animals during long term dosing studies (9, 58, 59). These results show that there are many possible disadvantages to blocking the normal functioning of coreceptors. In addition, it is well known that the envelope glycoprotein is among one of the most variable HIV proteins with diverse phenotypes making evolution of entry inhibitor-resistant viruses likely. Therefore, a better understanding of chemokine receptor and CD4 signaling, function, and mechanism of action, in response to natural ligands and in response to infectious diseases is necessary to develop novel approaches to limiting HIV entry without impairing the normal immune functions of CD4 and chemokine coreceptors.

**CD4 SIGNALING**

The primary cellular receptor for HIV-1, CD4, is a component of the molecular complex that facilitates the interaction of the T cell receptor (TCR) with MHC class II molecules and is involved in TCR signaling (43, 62). The CD4 molecule is a 58 kDa transmembrane (TM) glycoprotein that consists of a 370 amino acid containing extracellular region, a 25 amino acid containing TM region, and a cytoplasmic tail of 38 amino acids. The extracellular region is folded into four distinct domains, D1-D4. CD4 is post-translationally modified by formation of disulfide bonds that stabilize D1, D2, and D4, and by N-linked glycosylation of D3 and D4. There is extensive species conservation of the cytoplasmic tail of CD4, indicating that it is functionally important. In addition to T lymphocytes, CD4 is expressed on monocytes/macrophages, B cells, and certain specialized cells of the central nervous system (63). In CD4$^+$ T cells, CD4 plays multiple roles in response to antigens. The binding site on MHC II for CD4 is distinct from the binding site for the TCR so CD4 can interact with the MHC class II antigen presenting
complex (APC) at the same time as the TCR increasing responsiveness to any particular antigen. Upon binding, CD4 undergoes conformational changes that lead to activation of the constitutively associated Src tyrosine kinase Lck. Activated Lck phosphorylates immunoreceptor tyrosine activation motifs (ITAMs) located on accessory chains of the T-cell receptor complex. Once ITAMs are phosphorylated, they can bind the Syk family kinase ZAP-70 which is then phosphorylated and activated by Lck. Once activated ZAP-70 phosphorylates the linker of activation in T cells (LAT) and SLP-76.

Once these proteins are activated there are three downstream pathways that can be induced. LAT and SLP-76 bind to and activate Tec kinases, which phosphorylate and activate phospholipase C-γ (PLC-γ). PLC-γ then cleaves inositol phospholipids to generate diacylglycerol (DAG) and inositol trisphosphate (IP₃). LAT binds to the adaptor protein GADS, a homolog to Grb2, which then recruits the Ras guanine nucleotide GEF SOS-1 that then activates Ras. Another GEF, Vav, also binds to SLP-76 and LAT and this association leads to Rac activation. Ras is also activated downstream of PLC-γ by the GEF Ras-GRP which is activated by DAG and phorbol esters. Other downstream effectors of PLC-γ include protein kinase C (PKC) and Calcineurin. Once activated, PKC, Calcineurin, Ras, and Rac mediate activation of transcription factors that influence cell proliferation and differentiation and activation of proteins involved in actin cytoskeleton reorganization (43). Lck is not expressed in non-lymphocytes, but CD4 induces signaling through unknown proteins that lead to Ca⁺ flux, PLC and phospatidylinositol-3-kinase (PI3K) activation (64).
CHEMOKINE RECEPTOR SIGNALING

The chemokine coreceptors belong to a family of seven-transmembrane (TM)-spanning-receptors termed G-protein coupled receptors (GPCRs). GPCRs share a common structural theme that consists of an extracellular amino (N) terminal segment, a seven TM \(\alpha\)-helical segment forming the transmembrane core, three extracellular loops (ECL) three intracellular loops (ICL) and a carboxy (C) terminal cytoplasmic segment. Chemokine receptors contain an acidic N terminus, the amino acid sequence DRYLAIVHA in ICL2, a short basic ICL3, and a cysteine in each of the 4 extracellular domains (49). GPCRs associate with heterotrimeric (het) G proteins. The GDP bound inactive form of \(G\alpha\) interacts with \(G\beta\gamma\) and the GPCR which upon ligand binding acts as a GEF and causes dissociation of GDP from \(G\alpha\). This results in immediate association of \(G\alpha\) with GTP, which leads to dissociation from \(G\beta\gamma\) and activation of downstream effectors of both \(G\alpha\) and \(G\beta\gamma\). Signaling is ended by hydrolysis of GTP to GDP.

There are four classes of \(G\alpha\) proteins including \(G\alpha_i\), which is sensitive to ADP ribosylation by pertussis toxin (PTX), \(G\alpha_s\), \(G\alpha_q\), and \(G\alpha_{12/13}\). Dissociation of \(G\alpha_i\) and \(G\beta\gamma\) leads to signaling by both subunits. \(G\alpha_i\) signaling results in inhibition of adenylyl cyclases (ACs) and subsequent inhibition of cAMP production, activation of potassium (\(K^+\)) ion channels, inhibition of Ca\(^{2+}\) channels, and activation of phosphodiesterases. Signaling through the \(G\beta\gamma\) subunit associated with \(G\alpha_i\) also results in activation of \(K^+\) ion channels, and inhibition of Ca\(^{2+}\) channels. Unlike the \(G\alpha_i\) subunit, \(G\beta\gamma\) activation can also stimulate PI3K activation, PLC-\(\beta\) activation, activation of some ACs, and can recruit Rho and Rac to the plasma membrane. Signaling through \(G\beta\gamma\) is usually observed only at high agonist concentrations. \(G\alpha_s\)-mediated signaling results in activation of ACs and
stimulation of cAMP production, stimulation of c-Src, and activation of the GTPases activity of tubulin. Gaq-mediated activation of PLC-β, results in production of IP3 and DAG, which then stimulate intracellular Ca\(^{2+}\) release and PKC activation. The downstream effectors of the Ga\(_{12/13}\) subunits mediate activation of multiple Rho GEFs, stimulate E-cadherin and activate a GAP of Ras (65-69). A few of the signaling effectors of each class are illustrated in figure 2 (49).

While many GPCRs are known to associate with only one class of het G proteins, some are capable of interacting with several classes of het G proteins. Different agonists for one receptor and different concentrations of agonist can result in different active states of the GPCR which are recognized by different G proteins (65). GPCRs are also capable of signaling independently of het G proteins through interactions with β-arrestin, a scaffolding protein involved in internalization of GPCRs, or by direct binding with the PDZ domain of GEFs, and various other binding partners (67, 70).
Fig. 2. Signaling effectors activated by ligation of G-protein-coupled receptors
adapted from Marinissen and Gutkind 2001 (71).
Our lab and others have shown that HIV-1 Env mediated signaling is necessary for membrane fusion and entry (12, 25, 33, 72-74). Although multiple signaling components are known to be activated by HIV-1 Env through the chemokine coreceptors and through CD4, many of these components are not required for Env-induced fusion. Multiple studies with soluble CD4 and CD4 tailless have shown that signaling through CD4 is not essential for Env-induced fusion (75, 76). However these studies were done in cell lines where CD4 and coreceptor were over-expressed. More recent studies done in primary cells and cell lines expressing physiologically relevant levels of CD4 and coreceptor have shown that signaling through CD4 is required to promote formation of the Env-CD4-coreceptor complex (10, 11, 25, 53, 54, 77).

PTX experiments as well as mutational studies have shown that Env-induced fusion is independent of signaling pathways mediated by G\(_{\alpha_i}\), and either the conserved DRY motif in ICL2 or the C terminus of the coreceptors (78-85). Therefore, the receptor signaling events that mediate fusion are induced by Env, independent of G\(_{\alpha_i}\). CXCR4 and CCR5 are capable of inducing multiple PTX-insensitive pathways either through G proteins coupled to ICL3 or ICL1, or \(\beta\)-arrestin. Some PTX-insensitive signaling events that are induced by Env engagement of chemokine coreceptors or CD4 are phosphorylation and activation of proline-rich tyrosine kinase 2 (pyk2) and p38 MAPK in both T cells and primary macrophages (41, 78, 86-93). PTX insensitive p38 MAPK has also been shown to be activated by Env in B cells (94). Env-induced pyk2 phosphorylation is dependent on coreceptor signaling and on extracellular Ca\(^{2+}\) flux (88). CCR5 and focal adhesion kinase (FAK) phosphorylation in T cells is also PTX
insensitive and CD4 signaling dependent (89). Furthermore, chemokine mediated PTX-sensitive pathways are blocked by Env, but PTX-insensitive signaling to p38MAPK is not (90, 94-97) suggesting that PTX-insensitive signaling pathways are important for HIV-1 infection.

The signaling mediators required for the Env-mediated activation of these pathways are unknown, and it is also unknown whether these pathways lead to Rac activation and fusion. Various studies have shown that distinct intracellular signals are elicited by Env stimulation in different cells, by Env engagement of different coreceptors, and by Env versus the receptors’ natural ligands (49, 92, 98, 99). For example, signaling through R5 Env but not X4 Env induces elevations of Ca$^{2+}$ in lymphocytes (100). Neither CXCL12 nor the natural ligands of CCR5 activate nonselective cation ion channels that are opened by X4 and R5 Env (98). In addition Env activated Cl$^-$ currents more frequently and Ca$^{2+}$ activated K$^+$ currents less frequently than the corresponding chemokines (98). Furthermore, co-engagement of CD4 along with coreceptors may induce unique signals not elicited by either receptor alone and/or the presence of CD4 may serve to enhance signaling through coreceptors or vice versa. In previous literature it has been shown that CD4 constitutively interacts with CCR5 and this interaction leads to modulation of signaling via CCR5 (101, 102). CXCR4 has also been shown to interact with CD4 in the presence and absence of Env, however the physiological role of this interaction has not been fully determined (60, 103, 104). In addition, signaling through CD4 via Env or via IL16 has also been shown to inhibit natural ligand mediated chemotaxis via CXCR4 and CCR5 without altering expression of either receptor (95, 102, 105).
It is also possible that the necessary signaling for fusion can occur through either CD4 or coreceptor as both are capable of eliciting similar signaling pathways (49). In fact unpublished data from the Broder lab demonstrated diminished Env-mediated syncytia in cells expressing both tailless CD4 and tailless CXCR4 (49). In conclusion, it is possible that although Env mediated signaling appears to be different for CXCR4 vs. CCR5, there are similar components activated by both coreceptors that are required for Rac activation and fusion. As demonstrated in chapter 3, X4 and R5 Env-mediated fusion with primary cells expressing CXCR4 and CCR5 was dependent on the same pathway. It is also likely that the interaction of CD4 with CXCR4 or CCR5 is necessary not only to facilitate binding of Env and exposure of the fusion peptide domain but also to facilitate signaling distinct from that induced by chemokines. Therefore, Env-induced Rac activation and subsequent fusion could be dependent on signaling components activated by ligation to CD4 and CXCR4 or CCR5 that are not activated by chemokines or required for host coreceptor functions.

CONCLUSION

In conclusion, the current treatment for HIV, HAART, although effective at suppressing the virus, does not cure the infection, it must be maintained for life, and prolonged use often results in significant toxicity. Interruption in HAART treatment results in the rapid rebound of replicating virus which can lead to drug resistance (2). In addition, due to its high cost, HAART therapy is not available to 31% of the people in immediate need of these life-saving AIDS drugs. This information clearly shows that current treatment strategies are not enough and understanding the molecular biology of
HIV and its relationship with the host is required to make further progress in drug discovery.

New drug development targets the steps in entry. There are many entry inhibitors including attachment inhibitors, co-receptor binding inhibitors, and fusion inhibitors that are currently approved for treatment or in clinical trials (9, 58, 59). The risk with inhibitors that block chemokine receptors and CD4 is that they could also block natural ligand binding and signaling, inhibiting essential immune cell functions. In addition, it is well known that the envelope glycoprotein is among one of the most variable HIV proteins with diverse phenotypes making evolution of entry inhibitor-resistant viruses likely. Therefore, a better understanding of signaling through CD4 and chemokine receptors in response to natural ligands and in response to HIV-1 Env is necessary to develop novel approaches to limiting HIV entry without impairing the normal immune functions of CD4 and chemokine coreceptors.

In chapter 3 we provide evidence that siRNA and inhibitors targeted to Gaq and its downstream effectors prevent HIV-1 infection of TZM-BL cells as well as Env-dependent membrane fusion in U87.CD4.CCR5 cells and PBLs and virus-dependent membrane fusion of U87.CD4.CCR5 cells. In chapter 4, we show that the Rac mediated actin cytoskeletal rearrangements necessary for fusion are dependent on Abl and the Wave2 signaling complex. These results confirm and extend the implication of Rac-mediated actin cytoskeletal rearrangements in HIV-1 infection, and point to new potential target molecules of HIV-1 inhibitory drugs. This strategy of using inhibitors that disable host signaling proteins essential for pathogen survival may have a general efficacy in developing drugs to combat pathogens that acquire drug resistance.
Chapter 2

Antiviral Activity of a Rac GEF inhibitor Characterized with a Sensitive HIV/SIV Fusion Assay
Preface to Chapter 2

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Antiviral Activity of a Rac GEF inhibitor Characterized with a Sensitive HIV/SIV Fusion Assay

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Running Title: Virus-dependent fusion & Rac inhibition

Key Words: HIV-1, envelope, Rac, fusion
Abstract

A virus-dependent fusion assay was utilized to examine the activity of a panel of HIV-1, -2, and SIV isolates, of distinct coreceptor phenotypes. This assay allowed identification of entry inhibitors, and characterization of an antagonist of a Rac guanine nucleotide exchange factor, as an inhibitor of HIV-mediated fusion.
Introduction

HIV infection is initiated by binding of envelope surface protein (SU or gp120) to CD4 and coreceptor, usually either chemokine receptor, CCR5 or CXCR4 (106). This results in conformational changes in transmembrane envelope protein (TM or gp41), resulting in insertion of the fusion peptide into the cell membrane, and subsequent fusion of viral and cell membranes. HIV envelope acts as a chemokine mimic, stimulating responses such as chemotaxis, gene transcription, and phosphorylation (107).

One target of this signaling pathway is the actin filament network (108). Reorganization of the actin cytoskeleton is a critical feature of HIV-induced fusion (12). This is mediated by activation of Rho family GTPases, especially Rac (27). Rac regulates diverse cellular processes, including intercellular adhesion, cytoskeletal membrane ruffling and lamellipodia formation, proliferation, and gene transcription. The active, GTP-bound form of Rac is negatively regulated by Rac GTPases (GAPs) and positively regulated by Rac guanine nucleotide exchange factors (GEFs). Tiam1 is a GEF specific for Rac, while others are more promiscuous in activating multiple Rho GTPases.

In order to further elucidate the role of Rac activation in HIV fusion, we made use of a novel virus-dependent fusion assay (12, 109-111). This is based on the ability of virus particles to bridge at least two cells and allow transfer of cytoplasmic contents. In this assay, we use U87 glioma cells expressing CD4 and CCR5 or CXCR4, as well as vaccinia virus expressing T7 polymerase. The second population of U87 glioma cells, with CD4 and CCR5 or CXCR4, is infected with a vaccinia virus with a β-galactosidase gene under the regulation of the T7 promoter. A three hour incubation of these two cell
populations in the presence of fusion-competent virus particles allows fusion, quantified by β-galactosidase activity. Sensitivity of the assay was found to be enhanced by serum starvation for 24-48 hrs prior to fusion. We show here that this assay is rapid, flexible, and applicable to a wide range of lentivirus isolates. Moreover, this assay is useful for examining the activity of inhibitors of receptor or co-receptor binding, fusion peptide activity, as well as subsequent fusion activities, including Rac activation.

Results

Comparison of virus-dependent fusion and infection assays and the env-dependent fusion assay

The virus-dependent fusion assay was directly compared to the env-dependent fusion assay (Fig 1). For the env-dependent fusion assay, a macrophage-tropic virus, derived from the YU2 envelope (WT), was compared to one with a mutation in gag, resulting in substitution of L12E within the MA protein, resulting in a defect in envelope incorporation in virus particles (112, 113). Both proviral clones expressed similar amount of cell-surface envelope, as demonstrated by the fusion assay (Fig 1, left-hand bars). However, in the virus-dependent fusion assay the WT virus is capable of inducing fusion, whereas the L12E virus, defective in envelope incorporation, fails to induce fusion activity in this assay (Fig 1, right-hand bars).

The virus-dependent fusion and infection assays were also compared with isogenic viruses that differed only in the sequence of their V3 envelope domain (Fig 2) (114). Virus p2027 includes the V3 loop from R5 strain SF162. In contrast, virus IDI has a V3 loop derived from X4 strain HXB2, with the exception of substitutions at positions 27, 29, and 30 of the V3 loop that are found in SF162. Virus EIDI is identical to virus IDI
with the exception of an additional substitution at position 25. Twenty or 50 ng of virus was tested in the virus-dependent fusion assay, as described above. In contrast, 10 or 50 ng of virus was tested for infection of Magi.CD4.CCR5 cells (115). The viruses exhibited dose-dependent levels of infection and fusion in these assays, and the results were quite similar.

The virus-dependent fusion and infection assays were also tested with a panel of 40 primary HIV isolates with differences in coreceptor tropism, as well as viruses derived from 14 HIV-1 molecular clones, 2 HIV-2 molecular clones, and 2 SIV molecular clones (Table 1, Fig 3). For this purpose, fusion assays were performed with U87.CD4.CCR5 and U87.CD4.CXCR4 cells, whereas infection assays were performed with Magi.CD4 or Magi.CD4.CCR5 cells (which also express CXCR4). In each case, the virus-dependent fusion and infection assays gave comparable results, demonstrating the utility of this assay to screen a wide variety of laboratory and primary isolates.
## Table 1

### Panel of Isolates Tested with Virus-Dependent Fusion Assay and Magi Assay

<table>
<thead>
<tr>
<th>Virus or Panel</th>
<th>Coreceptor Usage</th>
<th>Novel Properties</th>
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<tbody>
<tr>
<td>Primary Isolates</td>
<td></td>
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</tr>
<tr>
<td>BaL, SF162</td>
<td>R5</td>
<td></td>
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<tr>
<td>LAI, 8 primary isolates</td>
<td>X4</td>
<td></td>
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<tr>
<td>89.6, MN</td>
<td>R5 X4</td>
<td></td>
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<tr>
<td>Sequential Isolates</td>
<td>R5→X4</td>
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<tr>
<td>Molecular Clones</td>
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<td></td>
</tr>
<tr>
<td>ADA, BaL, SF162, YU2 (V3 mutant</td>
<td>Variable R5</td>
<td></td>
</tr>
<tr>
<td>HXB2 chimeras)</td>
<td></td>
<td>Env chimeras and mutants that express V3 loop or minimal sequence determinants</td>
</tr>
<tr>
<td></td>
<td></td>
<td>for CCR5 usage in HXB2 backbone</td>
</tr>
<tr>
<td>HXB2</td>
<td>X4</td>
<td></td>
</tr>
<tr>
<td>HIV-2 (ES, ROD10)</td>
<td>R5 X4</td>
<td></td>
</tr>
<tr>
<td>SIV (mne, pbj)</td>
<td>R5 X4</td>
<td></td>
</tr>
<tr>
<td>Luciferase Reporters</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ADA, SF162, YU2 chimeras</td>
<td>R5</td>
<td>Multiple mutants with minimal sequence determinants for R5 use</td>
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<td></td>
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### Use of virus-dependent fusion assay for studies of HIV entry inhibitors

The virus-dependent fusion and infection assays were compared in studies of entry inhibitors that specifically block SU-mediated CD4 binding (Sim2) or CCR5 binding (2D7), or TM-mediated fusion (T20, Fig 4). U87.CD4.CCR5 cells were pre-incubated with antibody or drug, at the indicated concentrations for 12 hrs. For fusion assays, the cells were also infected with either vaccinia viruses expressing β-galactosidase or T7 polymerase, as described above. The cells were then exposed to 100 ng of HIV-luc virus (YU2-derived), for either 3 hrs for the fusion assay or 24 hrs for the infection assay (116).
Fusion activity was determined by β-galactosidase activity, as described above. Infectious virus was quantified by luciferase activity. In each case, a similar concentration-dependence was seen for inhibition, although the infection assay was slightly less sensitive than the fusion assay at high concentrations of the anti-CCR5 antibody (2D7, Fig 4a). Similar assays were carried out with two different preparations of T-20, a TM-fusion inhibitor, and inhibitory concentrations identified in the fusion assay were similar to those reported previously using infection assays (Fig 4b) (117).

Identification of a Rac GEF inhibitor that blocks HIV entry

The virus-dependent fusion assay was utilized to identify novel HIV entry inhibitors. Based on previous observations of a role of Rac activation during HIV fusion, we examined an inhibitor of the Rac 1 GEFs, Trio and Tiam, NSC23766 (44). This drug blocked HIV fusion, as measured by the Env-dependent fusion assay (Fig 5a,b), infection (Fig 5c), or virus-dependent fusion (Fig 5d). The concentration range of HIV inhibitory activity was similar to that required to block Rac activation (not shown).

In order to examine the specificity of the effects of the Rac GEF inhibitor, a constitutively active Rac mutant, RacV12, was used to overcome the inhibitor effects by activating the signaling pathway downstream of the Rac GEFs. RacV12 overcame the effects on Rac activation as measured by the amount of Rac-GTP in cell lysates resulting from HIV Env-mediated fusion (Fig 6a). No effects of RacV12 were seen on Rho activation resulting from HIV Env mediated fusion (not shown). Moreover, RacV12 overcame the effects on Env-mediated fusion of the Rac GEF inhibitor (Fig 6b).

Conclusions
The virus-dependent fusion assay provides a rapid, sensitive, flexible method to examine the biological activity of laboratory or natural isolates of HIV-1, -2, or SIV, and may have applications to many other viruses that mediate pH-independent fusion. It compares favorably with other assays of virus, such as one that depends upon transfer of a virus core containing a Vpr-β-lactamase fusion protein into a target cell, which requires a longer incubation time and FACS analyses (118, 119). It is likely that the virus-dependent fusion assay can also examine pseudotyped HIV particles, to identify inhibitors of other viral glycoproteins, as shown with the β-lactamase assay (120). This assay has advantages over various env-dependent fusion assays, since it utilizes relevant levels of virus-associated glycoprotein and does not require multiple receptor contacts for initiating the activity (121).

The rapidity of the virus-dependent assay is ideal for screening panels of potential HIV entry inhibitors, and is readily adaptable for high throughput screens. The assay is effective at identifying inhibitory small molecules and antibodies. It may have applications for studies of neutralizing antibodies directed against HIV-1. Lastly, differences in results of virus-dependent fusion and infection assays may provide new insights into biological differences between different virus isolates.

Rac activation is critical in other aspects of HIV biology besides entry. HIV-1 Nef binds the DOCK-ELMO complex and p21-activated kinase 2 complex to initiate Rac activation, inhibit lymphocyte chemotaxis, and induce merlin phosphorylation (122, 123). Rac activation is also important for other viruses. Rho and cdc42 mediate adenovirus endocytosis (124). Early steps in herpes simplex virus type 1 infection are dependent
upon regulated Rac and Cdc42 signalling (125). In contrast, Rho activation facilities Kaposi’s sarcoma herpesvirus entry (126).

The activity of the inhibitor of Rac GEF, Trio, further substantiates the role of Rac activation in the fusion process. Additional studies of downstream mediators of Rac activity in HIV fusion have shown no effects of chemical inhibitors of NADPH oxidase, MEK, PI-3 kinase, or myosin light chain kinase, or dominant-negative inhibitors of Pak-2 or PI-3 kinase (our unpublished findings). It is quite possible that Rac activation through the WAVE, Arp 2/3 complex is important for HIV fusion. Further studies of the entire signaling pathway involved in HIV-fusion should be informative in developing new strategies to block infection.

Materials and methods

Inhibitors

The CCR5 and CD4 monoclonal antibodies 2D7 and Sim.2, and T-20 drugs were obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases (117, 127, 128).

Viruses

Recombinant vaccinia viruses expressing β-galactosidase (vCB21R), T7 polymerase (vPT7-3), and CD4 (vCB-3) were obtained from the AIDS Research and Reference Reagent Program, vaccinia viruses encoding an uncleaved HIV envelope (UNC; vCB-16), ADA envelope (vCB-39), and HXB2 envelope (vSC60) as well as wild type vaccinia virus (vWT) were gifts from Dr E Berger, vaccinia viruses encoding the YU2 envelope (vSP-5) and constitutively active Rac GTPase (vRacV12) were gifts from Drs C Broder and S Wei, respectively. HIV virus stocks were prepared by lipofection of plasmid DNA
encoding full-length proviral molecular clones, with the Env gene of the R5 YU2, ADA, or SF162 strains or the X4 HXB2 strain in the HIV NL4-3 backbone (12, 116), or with a mutation in the Gag gene at residue 12 (L12E; our unpublished data). HIV Luc viruses encode the firefly luciferase gene in place of Nef. Transfected 293T cell supernatants were harvested after 48 hrs, filtered, and tittered for p24 antigen content by ELISA. Twenty-seven low-passage isolates obtained at serial visits from participants in the Baltimore site of the Multicenter AIDS Cohort Study (MACS) are also included (129).

**Fusion and infection assays**

The HIV-1 envelope-mediated fusion assay was modified from that developed by Dr Berger (130). The U87 cell lines were serum-starved for 36 hrs, then infected overnight at 37°C with vCB21R at MOI=10, and in some cases recombinant vaccinia virus encoding the constitutively active GTPase RacV12 mutant (vRacV12). Fusion partner BSC40 cells were infected overnight with vPT7-3 and vCB-39 encoding the ADA envelope. Alternatively, in some experiments 10^6 BSC40 cells were transfected with 5-10 ug of proviral plasmids and infected after 1 hr with vPT7-3. Cells were then lightly trypsinized, washed, and incubated with or without the Rac GEF inhibitor for 1 hr at 37°C. Cells (10^5) of each type are then mixed 1:1 in triplicate wells, incubated for 3 hrs at 37°C, and fusion stopped by the addition of NP-40 to a final concentration of 1% and freeze thawing at -20°C. β-galactosidase activity was determined using chlorophenol red-β-d-galactopyranoside (CPRG, Calbiochem) and the absorbance of each sample determined at 579 nm (116).

The virus-dependent fusion assay was performed with free virus particles obtained 48 hrs after transfection of 293T cells (116). Virus was incubated in the presence of 20
ug/ml DEAE-dextran with 10^5 U87.CD4.CCR5 cells infected for 18 hrs with vPT7-3 and 10^5 U87.CD4.CCR5 cells infected for 18 hrs with vCB21R. Assays were performed in triplicate wells. Fusion activity was quantified as described above.

Infection assays were performed with Magi.CD4 or Magi.CD4.CCR5 cells and number of blue foci determined 2 days post-infection (115). Alternatively, for some assays, viruses with luciferase expressed in place of Nef were utilized, and luciferase activity measured 1-2 days after infection.

**Rac and Rho Activation Assays**

These assays were performed with 2x10^6 serum starved U87.CD4.CCR5 cell mixed at a ratio of 1:1 with BSC40 cells infected with vaccinia virus expressing Env or vWT. In some cases, one population of U87.CD4.CCR5 cells was infected with vRacV12. Rac GEF inhibitor was added at the indicated concentrations to the target cells 1 hr prior to mixing and again at the time of mixing. Reactions were incubated at 37°C for 30 min, washed two times with ice cold PBS, and cells lysed. Lysates were snap frozen and later equal amounts of protein per well were analyzed using a G-LISA Rac activation or Rho-A activation assay kit according to the manufacturer’s instructions (Cytoskeleton, Denver, CO).

**Acknowledgements**

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Figures

Fig. 1. Comparison of Env-dependent and virus-dependent fusion assays, using an Env packaging-defective mutant proviral clone (L12E). Virus particles from HIV-1 MA mutant L12E have diminished levels of envelope incorporation and demonstrate little virus-dependent fusion activity. In contrast, transfection of these proviral clones into BSC40 cells result in similar levels of Env-induced fusion when cells are mixed with U87-CD4 cells.
Fig. 2. Virus-dependent fusion assay results are comparable to levels of infection of HeLa.CD4.CCR5 cells containing an LTR-lacZ reporter using viruses with Env V3 mutations. These viruses include amino acid substitutions in the V3 envelope domain that affect their efficiency of use of CCR5 (114).
Fig. 3. Virus dependent fusion assay and infection results with HIV-2 and SIVmne.

Different amounts of virus produced from infectious molecular clones by transfection of 293T cells, were used in fusion assays with U87.CD4.CCR5 cells or infection assays with Magi.CD4.CCR5 cells.
Fig. 4. Inhibition of virus-dependent fusion with antibodies or peptide inhibitors. a) Antibody neutralization studies were performed with anti-CCR5 antibody 2D7 or anti-CD4 antibody Sim2. Virus-dependent fusion assays (top) are compared to infection assays performed for 24 hrs with U87.CD4.CCR5 cells (bottom). b) Dose-dependent inhibition of virus-dependent fusion with T-20 was examined. Two different sources of T-20 were obtained from the NIH AIDS Research and Reference Repository, derived originally from DAIDS (with free N and C-terminal amino acids) and Trimeris (N-acetylated derivative). RLU, relative light units.
b

![Graph showing the effect of T-20 concentration on A579 activity. The graph plots T-20 Concentration (µg/mL) on the x-axis and A579 on the y-axis. Two lines are shown: one for DAIDS (closed circles) and one for Trimeris (open circles). The y-axis ranges from 0 to 1.4, and the x-axis ranges from 0.0001 to 10.]
Fig. 5. Dose dependent inhibition of R5-Env dependent fusion or infection of U87.CD4.CCR5 cells using a Rac GEF inhibitor. a) Rac GEF inhibitor was added at the time of cell mixing or b) target U87 and/or HIV-1 ADA Env-expressing BSC40 cells were pretreated for 1 hr, washed, and mixed prior to measuring fusion activity by β-galactosidase activity. c) Cells were infected with 10 ng p24 of HIVluc derived from HIV-1 ADA, with drug added at the time of virus addition. Luciferase activity was determined 16 hr post-infection, expressed as relative light units (RLU). d) Rac GEF inhibitor was added at the time of virus in a virus-dependent fusion assay. Experiments were repeated 3 times and representative data shown.
Fig. 6. Constitutively active RacV12 overcomes the effects of the Rac GEF inhibitor.

a) Effects of RacV12 on the ability of the Rac GEF inhibitor to block the ability of HIV ADA mediated fusion to activate Rac activation, as measured by the level of Rac-GTP in the cells. b) Effects of RacV12 on the ability of the Rac GEF inhibitor to block HIV-1 ADA Env-dependent fusion.
Chapter 3

HIV Envelope Induction of the Gαq Signaling Cascade

Is Required for Virus Entry
Preface to Chapter 3

Chapter 3 was published in the September 15, 2008 Journal of Virology. In Volume 82, Issue 18, Pages 9191-9205 by Brooke Harmon and Lee Ratner. All experiments and data were performed and collected by Brooke Harmon. Suzanne Pontow and Nancy A. Campbell gave important technical guidance for completion of the study and Intelly Lee gave technical assistance with one experiment shown in figure 11A.
HIV Envelope Induction of the Gαq Signaling Cascade Is Required for Virus Entry

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Key Words: HIV, Gαq, Rac, Fusion, Ras

Running Title: Gαq Signal Cascade for HIV Entry
Abstract

Binding of human immunodeficiency virus type 1 (HIV-1) envelope glycoprotein (Env) with the primary receptor CD4 and one of two coreceptors, CXCR4 or CCR5, activates a signaling cascade resulting in Rac GTPase activation and stimulation of actin cytoskeletal reorganizations critical for HIV-1 mediated membrane fusion. The mechanism by which HIV-1 Env induces Rac activation and subsequent actin cytoskeleton rearrangement is unknown. In this study, we show that Env-mediated Rac activation is dependent on the activation of G\textsubscript{a}q and its downstream targets. Fusion and Rac activation are mediated by G\textsubscript{a}q and phospholipase C (PLC), as shown by attenuation of fusion and Rac activation in cells either expressing siRNA targeting G\textsubscript{a}q or treated with the PLC inhibitor U73122. Rac activation and fusion were also blocked by multiple protein kinase C (PKC) inhibitors, by inhibitors of intracellular Ca\textsuperscript{2+} release, by Pyk2 targeted siRNA, and by the Ras inhibitor S-trans,trans-farnesylthiosalicylic acid (FTS). Fusion was blocked without altering cell viability or cell surface localization of CD4 and CCR5. Similar results were obtained when cell fusion was induced by Env expressed on viral and cellular membranes and when cell lines or primary cells were the target. Treatment with inhibitors and siRNA specific for G\textsubscript{ai} or G\textsubscript{as} signaling mediators had no effect on Env-mediated Rac activation or cell fusion, indicating that the G\textsubscript{a}q pathway alone is responsible. These results could provide a new focus for therapeutic intervention, with drugs targeting host signaling mediators, rather than viral molecules, a strategy which is less likely to result in resistance.
Introduction

Entry of HIV-1 is mediated by sequential binding of the trimeric HIV envelope glycoprotein (Env) to CD4 and one of two primary chemokine coreceptors, CXCR4 or CCR5. This binding triggers a series of conformational changes in Env that expose the fusion peptide, which then induces the merger of viral or infected cell membranes with target membranes (2, 4, 49). The HIV-1 Env interaction with CD4 and coreceptor also stimulates various intracellular signaling events, similar to those initiated by their natural ligands, such as phosphorylation of Pyk2, Ca²⁺ mobilization, activation of RhoGTPases, and actin cytoskeleton rearrangements (12, 33, 49, 86, 87, 89, 99). Actin cytoskeletal remodeling and RhoGTPases play a central role in regulating fusion of biological membranes (24, 27). In the case of HIV-1-induced membrane fusion, activation of the RhoGTPase Rac and subsequent actin cytoskeletal reorganizations are required for efficient virus entry and infection (12, 25). The exact mechanism of Env-induced Rac activation that mediates actin cytoskeletal rearrangements and induces membrane fusion has not been investigated.

Binding of both CD4 and the coreceptors elicit signaling pathways that result in Rac activation. However, previous results suggest that Env-induced Rac activation is mediated via the coreceptor rather than CD4 (12). The coreceptor CCR5 is the principal receptor for HIV-1 transmission, whereas CXCR4 binding viral isolates are primarily found in late stages of disease (131). Coreceptors CCR5 and CXCR4 belong to a family of seven-transmembrane-spanning receptors termed G-protein coupled receptors (GPCRs). Ligand induced conformational changes in GPCRs leads to activation of heterotrimeric (het) G proteins (40, 49, 67, 69). GPCRs associate with four classes of het
G proteins, \( \text{G}\alpha_i \), which is sensitive to ADP ribosylation by pertussis toxin (PTX), \( \text{G}\alpha_s \), \( \text{G}\alpha_q \), and \( \text{G}\alpha_{12/13} \) (49, 67). Most documented signaling through chemokine receptors goes through the PTX sensitive \( \text{G}\alpha_i \), however fusion has been shown to be PTX insensitive (78, 82-85). Additional studies have shown that mutation of the DRY domain of the second intracellular loop of CCR5, the domain thought to interact with \( \text{G}\alpha_i \), also had no effect on HIV-1 induced membrane fusion and entry (78, 79, 82-85). These results suggest that Env-induced fusion is independent of signaling pathways mediated by \( \text{G}\alpha_i \). However, GPCRs can induce PTX-insensitive pathways by activating other het G proteins; by signaling independently of het G proteins through interactions with \( \beta \)-arrestin, a scaffolding protein involved in internalization of GPCRs; or by direct binding with the PDZ domain of guanine nucleotide exchange factors (GEFs), and various other binding partners (67, 70).

The signaling method utilized depends on multiple factors, including cell type, receptor, the activation state of the cell, and availability of signaling partners. In addition to \( \text{G}\alpha_i \), CCR5 has been shown to couple to \( \text{G}\alpha_s \) and \( \text{G}\alpha_q \) and the interaction with \( \text{G}\alpha_q \) has been mapped to the third intracellular loop of CC chemokine receptors (49, 80, 132). Signaling through \( \text{G}\alpha_s \) leads to activation of adenylyl cyclase, calcium (\( \text{Ca}^{2+} \)) channels and cyclic AMP (cAMP)-dependent protein kinase A (PKA), whereas signaling though \( \text{G}\alpha_q \) results in activation of phospholipase C\( \beta \) (PLC\( \beta \)), \( \text{Ca}^{2+} \)channels, and protein kinase C (PKC). Signaling components that participate in both \( \text{G}\alpha_s \) and \( \text{G}\alpha_q \) mediated pathways and het G protein-independent pathways are activated by HIV-1 Env interaction with CCR5 (87, 88, 133, 134). Since the likely domains involved in G protein dependent and independent signaling pathways are also required for normal surface expression,
generation of CCR5 molecules unable to couple with signaling intermediates was not possible. In addition, similar signaling pathways are activated via CD4 and CCR5 suggesting that required signaling may occur through one receptor if the other is impaired (49, 64, 89). In this study, we utilized small interfering RNA (siRNA) and various small molecule inhibitors to determine which signaling processes are required for Rac activation and subsequent membrane fusion (Fig. 1). The data presented demonstrate that HIV-1 Env mediates activation of the Gαq pathway via CCR5 and this activation is critical for HIV-1 induced cell-cell fusion.

Materials and Methods

Cell Lines. U87.CD4.CCR5 is an astroglioma cell line engineered to express CD4 and a CCR5 construct that is tagged at its C terminus with green fluorescent protein (GFP). The maintenance of BSC40 cells (African green monkey kidney cells), U87.CD4.CCR5 cell lines and peripheral blood lymphocytes (PBLs) has been described (116). The TZM-BL (also known as JC53-BL) reporter cell line was a gift from John C. Kappes. This is a HeLa cell clone that expresses CD4, CCR5 and CXCR4 and was engineered to express Escherichia coli β-galactosidase (β-Gal) and firefly luciferase under the transcriptional control of the HIV-1 long terminal repeat (LTR) as described (135). TZM-BL cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 4 mM L-glutamine, 1 mM sodium pyruvate, 100 units of penicillin per ml, 100 µg of streptomycin per ml (complete DMEM; Mediatech) supplemented with 15% fetal bovine serum.
(FCIII). Unless noted, tissue culture supplies were obtained from Mediatech, Manassas, VA.

**Reagents.** The control siRNA constructs (non-targeting 20-25 nt siRNA designed as a negative control), the siRNA constructs targeted to $G_{\alpha_q}$, $G_{\alpha_i}$, $G_{\alpha_s}$, Pyk2 and Rac and the rabbit anti-$G_{\alpha_q}$, goat anti-$G_{\alpha_i}$, goat anti-$G_{\alpha_s}$, goat anti-Pyk2, goat anti-actin, and HRP conjugated donkey anti-goat antibodies were obtained from Santa Cruz Biotechnology, Inc (Santa Cruz, CA.). The siRNA constructs were transfected using GeneEraser siRNA Transfection Reagent according to the manufacturer's instructions (Stratagene, La Jolla, CA). The rabbit anti-Pyk2 antibody and thapsigargin were obtained from Sigma (St. Louis, MO). The monoclonal anti-human CCR5 antibody (MAB182) was obtained from R&D systems (Minneapolis, MN). The rabbit polyclonal anti-Pyk2 (pYpY579/580) phosphospecific antibody and Cytochalasin D (CD) were obtained from Invitrogen (Carlsbad, CA). 100X GDP, 100X GTP$_\gamma$S, and anti-Rac and anti-Ras antibodies were obtained from Millipore (Billerica, MA). U73122 was obtained from Tocris Bioscience (Ellisville, MO), U73343 and the cyclopiazonic acid were obtained from BioMol International (Plymouth Meeting, PA), Xestospongin C was obtained from Cayman Chemical (Ann Arbor, MI) dantrolene was obtained from Axxora (San Diego, CA), and all other inhibitors were obtained from EMD Chemicals (San Diego, CA).

**Viruses.** Wild-type (WT) vaccinia (WR strain) and recombinant vaccinia viruses expressing $\beta$-galactosidase (vCB21R), and T7 polymerase (vPT7-3) were obtained from the AIDS Research and Reference Reagent Program. Vaccinia viruses encoding an
uncleaved HIV envelope (UNC; vCB-16), ADA envelope (vCB-39), and HXB2 envelope (vSC60), were gifts from Edward Berger, and the vaccinia virus encoding the HIV envelope from the YU2 (vSP-5) strain and constitutively active Rac GTPase (vRacV12) were gifts from Drs C Broder and S Wei, respectively (45). The HIV stocks used in the following assays are prepared by the lipofection of plasmid DNA encoding full-length proviral molecular clones, which contain the Env gene of the R5 YU2 strain in the HIV$_{NL4-3}$ backbone. To produce pseudotyped HIV-1, either amphotropic MLV (A-MLV) envelope or vesicular stomatitis virus G (VSV-G) expressing plasmids were cotransfected with the proviral DNA of HIV-1. Transfected 293T cell supernatants are harvested 48 hours postlipofection, filtered, and assayed for p24 antigen content by enzyme-linked immunosorbent assay (116).

**Envelope-dependent fusion assay.** The HIV-1 envelope-mediated fusion assay used was a modification of the assay developed by Dr. Berger's laboratory (130). The target cells (PBMCs or U87.CD4.R5 cells) were serum starved for 36 hours (h) then infected overnight with vCB21R or with recombinant vaccinia virus encoding the constitutively active GTPase RacV12 mutant (vRacV12). Fusion partner BSC40 cells were co-infected with vPT7-3 and vaccinia virus expressing HIV-1 Env. Cells were infected overnight (multiplicity of infection [MOI], 10 for U87.CD4.R5 cells and BSC40 cells and MOI of 200 for PMBCs) at 37°C in complete media. The next day infected cells were lightly trypsinized, and washed with PBS prior to mixing. Unless otherwise indicated inhibitors were added at the indicated concentrations to U87.CD4.CCR5 cells 1 h prior to mixing and again at the time of mixing. To allow fusion, $10^5$ cells were mixed 1:1 with a fusion partner in triplicate wells and incubated 3 h at 37°C. To account for any effect of
inhibitors on vaccinia virus infection and/or on T7 polymerase function, vCB21R and vPT7-3 co-infected cells were similarly treated with inhibitors. Concentration curves were performed with all of the inhibitors to determine the concentration that resulted in the maximum decrease in fusion without altering vaccinia virus infection or T7 polymerase activity. Cells were lysed by addition of NP-40 to a final concentration of 1% and freeze thawing at -20°C. β-galactosidase activity of reaction lysates is determined using chlorophenol red–β-d-galactopyranoside (CPRG, Calbiochem) and the absorbance of each sample is determined at a wavelength of 570nm (12).

**Western Blot Analysis.** Cell lysates were prepared using mammalian cell lysis buffer (50mM Tris-Cl, PH 8, 5mM EDTA, 100mM NaCl, 0.5% Triton X100 plus protease and phosphatase inhibitors. Fifty micrograms of lysate was separated by 12% SDS-PAGE for siRNA transfected samples and by 10% SDS-PAGE for Pyk2 samples and transferred to PVDF membranes, blocked with 0.01% Tween 20 and 5% dry milk in TBS, and probed overnight with rabbit polyclonal anti-Pyk2 (pYPY579/580) antibody (1:1,000) or for 2h with anti-Gαq (1:500), anti- Gαi (1:500),anti-Gαs (1:500), anti-Pyk2 (1:500) or anti-Rac (1:2,000) antibodies. After washing with 150 mM NaCl, 10 mM Tris-HCl, pH 7.5 (TBS), horseradish peroxidase-labeled secondary antibody was added (1:1,000 donkey anti-goat IgG; Santa Cruz, 1:2,000 dilution, goat anti-rabbit IgG; Pierce, or 1:10,000 dilution anti-mouse IgG; Amersham). Proteins were visualized in an Alpha Innotech (San Leandro, CA) imager by using supersignal west femto sensitivity substrate (Pierce). The blots were then stripped for 30 minutes at 50 degrees in stripping buffer (2% SDS, 6% Tris-Cl, PH 7.5, 0.7% β-mercaptoethanol in H2O), washed 3X in TBS, blocked and probed with anti-
actin (1:500) or anti-Pyk2 (1:1,000) overnight, washed, and appropriate secondary antibodies were added prior to visualization on Alpha Innotech imager.

**Rac and Ras Activation Assay.** Rac and Ras activation assays were performed with $2 \times 10^6$ serum-starved U87.CD4.CCR5 cells or $1 \times 10^7$ serum-starved U87.CD4.CCR5 cells per sample respectively. These cells were mixed at a ratio of 1:1 with BSC40 cells which were infected with vCB-39 (HIV$_{ADA}$ Env), vSC60 (HIV$_{HXB2}$ Env), or WT vaccinia virus (no Env) as described above. Inhibitors were added at the indicated concentrations to the target cells 1 h prior to mixing and again at the time of mixing unless otherwise noted. Reactions were incubated at 37 °C for 30 min, washed two times with ice cold PBS, and cells were lysed. Lysates were snap frozen and later equal amounts of protein per sample (determined by Bio-Rad Protein Assay, Bio-Rad Laboratories, Hercules, CA) were analyzed using a G-LISA Rac activation assay kit (Cytoskeleton, Denver, CO) or Ras activation assay kit according to the manufacturer's instructions (Cell Biolabs, San Diego, CA), with equal amounts of protein for column loading (Bio-Rad).

**Virus-dependent fusion assay.** The virus-induced cell fusion assay was performed as previously described (45). Briefly, U87.CD4.CCR5 cells were infected with vCB21R or vPT7-3 cultured overnight, harvested by trypsin treatment, washed with PBS, and $10^5$ cells were mixed (1:1) with DEAE-dextran (20ug/ml) and 100ng p24 HIV$_{YU2}$. The assay was performed in triplicate using a 96-well microtiter plate. Unless otherwise indicated inhibitors were added at the indicated concentrations to both populations of U87.CD4.CCR5 cells 1 h prior to virus addition and again at the time of virus addition.
Fusion reactions were incubated for 3 h at 37°C and reactions were assayed for β-galactosidase activity as described (12).

**JC53-BL assay.** JC53BL cells were serum starved for 12h then plated overnight in complete media in 96 well plate at 1X10^4 cells per well. Cells were treated for 1h with indicated concentrations of inhibitors prior to addition of media alone or 150ng p24 of HIV_YU2 or VSVG or A-MLV-pseudotyped HIV in the presence of 20ug/ml DEAE-dextran for 3h at 37°C. After 3h cells were washed 3 times with PBS and inhibitors were added in fresh media. Following a 24h incubation cells were lysed and luciferase units determined. Infected wells and uninfected wells with inhibitor were compared to wells with no inhibitor.

**cAMP assay.** cAMP assay was performed with 5 x 10^6 serum-starved U87.CD4.CCR5 cells per condition. Half of the U87.CD4.CCR5 cells were pre-treated with 200ng/ml pertussis toxin (PTX) for 18h prior to treatment with 1uM forskolin (EMD Chemicals) and 500ng/ml CCL4 (PreproTech Inc., Rocky Hill, NJ), for 30 minutes and cAMP assay was performed with equal amounts of protein per sample (Bio-Rad) according to manufacturer’s instructions (Cayman Chemical, Ann Arbor, MI).

**PKA assay.** PepTag assay for nonradioactive detection of cAMP-dependent protein kinase (Promega) was used with whole cell lysates prepared from U87.CD4.CCR5 cells treated with indicated inhibitors for 1h. The assay was performed in the presence of inhibitors with equal amounts of protein per sample (Bio-Rad) according to the
manufacturer's protocol. PKA activity was quantitated by scanning densitometry of phosphorylated peptide substrate resolved in 0.8% Tris · HCl agarose (pH 8.0).

**Flow Cytometry and Confocal microscopy.** U87.CD4.CCR5 cells were treated with indicated concentrations of inhibitors for 3h. For detection of CD4 and CCR5, Phycoerythrin (PE)-conjugated anti-CD4 (Q4120, Sigma), and monoclonal anti-human CCR5 antibodies (MAB182, R&D systems, Minneapolis, MN) were used at 1:20 fold dilution and 10ng/ml respectively. Cells were detached by incubation in 5 mM EDTA (in PBS) for 5min at 37 °C. The incubation time for the primary and secondary (goat anti-mouse PE conjugated IgG F(\(ab\))2, R&D systems) antibodies was 30 minutes on ice for CCR5 and room temperature (RT) for CD4. Cells were then fixed with 1% paraformaldehyde and analyzed using FACS Calibur flow cytometer (BD Biosciences) with CellQuest software (BD Biosciences).

For confocal microscopy analysis, U87.CD4.CCR5 cells were plated on coverslips at 2X10^5 cells per well in 6 well plate. The next day cells were treated with indicated concentrations of inhibitors for 3h and with CD for 15 minutes. Cells were fixed with 4% paraformaldehyde for 30 minutes at RT, blocked with 5% BSA in PBS and stained with anti-CD4-PE for 1h at RT (CCR5 has C-terminal GFP tag). Cells were washed with PBS and incubated with 1:500 dilution of TO-PRO3 (Invitrogen) in permeabilization buffer (0.2% saponin, 5% BSA in PBS) for 15 minutes. Cells were washed three times in PBS, mounted on slides using Gold Anti-fade mounting media (Invitrogen), and 10 random fields recorded for each condition. Cells were analyzed by confocal fluorescence microscopy using a 510 Meta LSM confocal microscope (Zeiss, Thornwood, NY).
**Statistical Analysis.** Raw data for fusion and infectivity assays were compared using two-tailed *t*-test. P values are considered significant when they are <0.05, and very significant when they are <0.01.

**Results**

**HIV-1 Env induces Rac activation and membrane fusion via a PTX-insensitive and PKA-independent pathway.** In order to determine whether HIV-1 Env-dependent Rac activation is mediated by G\(_{\alpha_{i/o}}\) family members, U87.CD4.CCR5 cells were pretreated with 200ng/ml PTX, 18 hours prior to incubation with BSC40 cells expressing Env from R5 HIV\(_{\text{ADA}}\) strain or cells lacking Env. Exposing cells to HIV\(_{\text{ADA}}\) Env in the presence or absence of PTX resulted in a 2.25 fold increase in Rac activation as determined by quantitation of levels of GTP-bound Rac1 purified by Rac-G-LISA (Fig. 2A). Activity of PTX was confirmed by demonstrating that MIP-1\(\beta\)-mediated inhibition of forskolin-induced cAMP formation was sensitive to PTX (not shown). In addition, Env-dependent cell-cell fusion was also unaffected by treatment with PTX (78, 82-85). These results indicate that HIV-1 stimulates Rac activity and Env-dependent cell-cell fusion by a pathway that does not involve G\(_{\alpha_{i/o}}\) proteins.

Next, we investigated the importance of PTX-resistant G proteins G\(_{\alpha_s}\) and G\(_{\alpha_q}\). The major downstream effector of G\(_{\alpha_s}\) is adenylyl cyclase, which results in increased cellular levels of cAMP and leads to activation of cAMP-dependent PKA. PKA is a likely candidate for the pathway described in this study, because its activity is stimulated by HIV-1 Env and PKA has been shown to be upstream of Rac activation and subsequent cell migration (133). To test the role of PKA, U87.CD4.CCR5 cells were pre-treated with two different inhibitors of PKA, inhibitory peptide PKA-I (14-22) and chemical inhibitor
H89 and the effects were studied in a quantitative Env-dependent cell-cell fusion assay. Although these PKA inhibitors effectively inhibited PKA activity, there was no effect on Env-mediated cell fusion (Fig 2B). These data confirm a previous report that synthesis of an early product of reverse transcription is not affected by PKA inhibition (136). In this study none of the inhibitors had an effect on vaccinia virus infection or on T7 polymerase activity at the concentrations described (not shown). In addition, H89 had no effect on Env-dependent Rac activation (Fig 2C). In this experiment, controls included a mismatched X4 Env that did not induce Rac activation in U87.CD4.R5 cells, and a CCR5-inhibitor, TAK-779, that completely inhibited Rac activation (12). These data suggest that although the cAMP-dependent PKA pathway is activated by HIV-1 Env, it is not required for Env-dependent Rac activation and cell-cell fusion.

**HIV-1 Env mediates Rac activation and membrane fusion via G proteins from the** **Ga<sub>q</sub>/11 family.** In addition to Ga<sub>s</sub>, the Ga<sub>q</sub>/11 family of PTX-resistant G proteins are known to associate with CCR5, (40, 49). To investigate the importance of the Ga<sub>q</sub>/11 family of G proteins, Ga<sub>q</sub> expression was down-regulated by RNAi in U87.CD4.CCR5 cells. Ga<sub>i</sub> and Ga<sub>s</sub> were also down-regulated to confirm that these proteins do not play a role in Env-mediated fusion and to show that knockdown of irrelevant pathways has no effect on Env-mediated fusion. siRNA expressing cells were then mixed with BSC40 cells expressing different Env subtypes. Maximal Rac activation was measured after 30 min, whereas maximal Env-dependent fusion was measured after 3 h (12). Transfection of cells with Ga<sub>q</sub>-directed siRNAs decreased levels of Env-dependent cell-cell fusion by an average of 73±3.6% for both HIV-1 R5 and Dual-tropic Env subtypes (Fig. 3A left
panel). There was no significant fusion observed with cells expressing the X4 Env and U87.CD4.R5 target cells with or without siRNA expression as was expected. Rac activation was similarly decreased in cells transfected with Ga-q-directed siRNAs (Fig 3C), whereas siRNA directed against Ga-i and Ga-s had no effect on Env-induced cell-cell fusion or Rac activation (Fig 3A and 3C). The decrease in levels of fusion and Rac activation correlated well with the decreased steady state level of Ga-q and each siRNA specifically downregulated its target protein with no effects on expression of the other Ga proteins as detected by immunoblot (Fig. 3B). Vaccinia virus infection and T7 polymerase activity was also measured in siRNA transfected cells and Env-dependent cell-cell fusion in these cells was normalized accordingly. In order to determine if Ga-q acts upstream of Rac in Env-mediated cell-cell fusion, a constitutively active Rac mutant, RacV12, was used (Fig. 3A right panel). RacV12 overcame the effects on Env-mediated fusion resulting from siRNA targeted to Ga-q (Fig. 3A). These results show that the effects of the siRNA are overcome by expression of a downstream signaling component. Thus the Ga_q/11 proteins are involved in HIV-1 Env-dependent cell-cell fusion upstream of Rac activation.

**HIV-1 Env-induced Rac activation and cell-cell fusion depends on PLC activation**

Activation of the Ga_q leads to activation of inositol phospholipid-specific phospholipase C beta (PLCβ) which catalyzes the hydrolysis of phosphatidylinositol-4,5-bisphosphate (PtdIns(4,5)P2) to produce the secondary messenger molecules diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP3) (137). To test for the involvement of PLC in HIV-1 induced fusion and Rac activation, U87.CD4.CCR5 cells
were pretreated for 1h with 3µM of the PLC inhibitor U73122, or the inactive analog U73343 prior to mixing with HIV-1 Env-expressing cells, and measurement of cell-cell fusion and Rac activation (Fig. 4). This concentration of U73122 has previously been shown to be specific for PLC (138), and does not increase intracellular Ca\(^{2+}\), whereas higher concentrations of U73122 inhibit PLC and also lead to a release of Ca\(^{2+}\) from intracellular stores (139). The PLC inhibitor U73122 suppressed Env-dependent cell-cell fusion by an average of 73 ± 7.6% (Fig. 4A top panel) and Rac activation by 62 ± 1.5% (Fig. 4B). The same concentration of the inactive analog U73343 had no significant effect on fusion or Rac activation (Fig. 4). Inhibitory effects of U73122 were overcome by expression of constitutively active RacV12 (Fig. 4A bottom panel). The phosphatidylinositol-specific PLC inhibitor ET-18-OCH\(_3\) also suppressed Env-dependent cell-cell fusion and Rac activation by approximately 45% (data not shown). To validate the effects of U73122 and U73343 on Env-mediated fusion in a relevant HIV-1 target cell, peripheral blood lymphocytes (PBLs) which express both CCR5 and CXCR4 were used as the target cell in a quantitative Env-dependent cell-cell fusion assay. PBLs were pretreated with U73122, U73343 and TAK-779 for 1h prior to mixing with BSC40 cells expressing different HIV-1 Env subtypes and measurement of cell-cell fusion. The PLC inhibitor U73122 suppressed Env-dependent cell-cell fusion in PBLs by an average of 74 ± 12.8% and U73343 had no effect (Fig 4C). U73122 inhibited fusion mediated by HIV-1 R5 Envs, Dual-tropic Envs and X4 Envs suggesting that PLC is required for Env-induced fusion mediated by both CCR5 and CXCR4. TAK-779, as expected completely inhibited fusion mediated by HIV-1 R5 Env-expressing cells but inhibited fusion mediated by the
Dual-tropic Env by only 59 ±5.4% and had no significant effect on fusion mediated by X4 Env.

**Intracellular Ca\(^{2+}\) release is required for Env-dependent cell-cell fusion and Rac activation.** The second messengers IP3 produced by hydrolysis of PtdIns(4,5)P2 activates the IP3 receptor (IP3R) on the membrane of the sarcoplasmic/endoplasmic reticulum (SER) which opens a Ca\(^{2+}\) channel resulting in intracellular Ca\(^{2+}\) release. This Ca\(^{2+}\) release leads to activation of the ryodine receptor (RyR)-operated Ca\(^{2+}\) channel leading to a further increase in intracellular Ca\(^{2+}\). It has been shown previously that HIV-1 Env interaction with chemokine receptors results in a PTX-insensitive elevation of intracellular Ca\(^{2+}\) (89). However the role for intracellular Ca\(^{2+}\) release in HIV-1 Env-mediated fusion is unknown. To test for the involvement of intracellular Ca\(^{2+}\) release, multiple inhibitors of intracellular calcium release were utilized. These inhibitors include xestospongin C (XC), which specifically blocks IP3R-induced Ca\(^{2+}\) release and dantrolene, which blocks RyR-induced intracellular Ca\(^{2+}\) release. In addition, thapsigargin (TG) and cyclopiazonic acid (CPA), irreversible and reversible inhibitors, respectively, of the sarcoplasmic/endoplasmic reticulum Ca\(^{2+}\) ATPase (SERCA) pumps, were used to deplete internal Ca\(^{2+}\) stores. Inhibition of the SERCA pumps prevents Ca\(^{2+}\) from being sequestered in the ER and it is gradually lost from the cell via passive leakage into the cytosol and subsequent extrusion through the plasma membrane. TG and CPA are structurally different and are unlikely to elicit similar cellular responses unless specifically acting on SERCAs (140).
Target cells were pretreated for 1hr with 100uM dantrolene, 10uM CPA, 2uM TG, or 5uM XC. The cells were subsequently incubated with HIV-1 Env expressing cells and cell-cell fusion and Rac activation were measured. Dantrolene, CPA, TG and XC treatment reduced Env-mediated cell-cell fusion by an average of 93±2.2%, 79±2.5%, 97±2.6%, and 76±3.5% respectively in U87.CD4.CCR5 cells which is similar to the inhibition observed in PBLs (Fig. 5A, left panel, Fig 5C). In the case of PBLs this inhibition was observed with both CCR5 and CXCR4 tropic Envs. The effects of these intracellular Ca\(^{2+}\) modulators on Env-induced Rac activation in U87.CD4.CCR5 cells also correlate with their effects on Env-induced cell-cell fusion. Although all of these intracellular Ca\(^{2+}\) modifiers inhibit Env-dependent Rac activation and cell-cell fusion to similar degrees, the effects on cell-cell fusion were not completely reversed by expression of RacV12 (Fig 5A, bottom panel). Env-dependent cell-cell fusion in cells treated with dantrolene, CPA, TG, and XC and expressing RacV12 was 50±1.6%, 40±2.7%, 25±2.6%, and 32±6%, that of untreated cells, suggesting a role for intracellular Ca\(^{2+}\) flux both upstream and downstream of Rac activation.

**HIV-1 Env mediates Rac activation and cell-cell fusion via a PKC-dependent pathway.** Production of the second messenger DAG and the IP3 induced Ca\(^{2+}\) release facilitate membrane translocation and activation of the serine/threonine kinase, PKC. To investigate the role of PKC, U87.CD4.CCR5 cells were pre-treated for 1h with small molecule inhibitors of PKC, Bisindolylmaleimide I (Bis I), Calphostin C, and Chelerythrine Chloride (Ch Ch). These cells were then incubated with HIV-1 Env expressing cells, and cell-cell fusion was measured. The PKC inhibitors Calphostin C and
ChCh reduced Env-mediated cell-cell fusion by 87±2% and 89±2% respectively whereas Bis I only reduced fusion by 55±11% (Fig 6A, left panel). Concentration curves were performed with all of the inhibitors used in this study to determine the concentration that resulted in the maximum decrease in fusion without altering vaccinia virus infection or T7 polymerase activity (data not shown). At higher concentrations Bis I had a greater effect on Env-induced cell fusion, however at these concentrations Bis I is no longer specific for PKC (141) (data not shown). Effects of PKC inhibition on fusion were overcome with constitutively active RacV12 (Fig 6A, right panel).

To determine which isoform of PKC is involved in Env-mediated Rac activation and cell-cell fusion, PKC inhibitors were used that are specific for conventional isoforms, PKCα, PKCβ, and PKCγ, which depend on Ca^{2+} and DAG as well as a phospholipid for activation. The specific PKC inhibitors used were Ro-32-0432 which is most specific for PKCα, Go-6976 which specifically inhibits Ca^{2+} dependent PKC, particularly PKCα and PKCβ1, and the inhibitory peptide, PKC-I (20-28), which is a pseudosubstrate that specifically blocks PKCα and PKCβ activation. U87.CD4.CCR5 cells were pre-treated with these inhibitors for 1h prior to mixing with HIV-1 Env-expressing cells, and measurement of cell-cell fusion. Pretreatment with Ro-32-0432, Go-6976, and PKC-I (20-28) decreased Env-mediated cell-cell fusion by 73±4.4%, 70±3.6% and 93±3.5% respectively (Fig 6B, left panel). Expression of the constitutively active Rac mutant, RacV12 overcame the effects of these PKC inhibitors on HIV-1 Env-mediated cell-cell fusion (Fig 6B, right panel).

To determine the effects of PKC inhibitors on Rac activation U87.CD4.CCR5 cells were pre-treated with Calphostin C, ChCh, Go-6976 and PKC-I (20-28) for 1h prior
to mixing with HIV-1 Env-expressing cells. Levels of suppression of Env-dependent Rac activation correlated with levels of inhibition of Env-dependent cell-cell fusion (Fig. 6C). In this experiment, controls included the CCR5-inhibitor, TAK-779 and a specific RacGEF inhibitor, both of which inhibited Env-induced Rac activation (44, 45). In addition to the data with RacV12, this suggests that PKCα and/or PKCβ is required upstream of Rac in Env-mediated cell fusion.

To validate the effects of both general and specific PKC inhibitors on Env-mediated fusion in a relevant HIV-1 target cell, PBLs were pretreated with Ch Ch and Go-6976 for 1h prior to mixing with HIV-1 Env-expressing cells, and measurement of cell-cell fusion. Pretreatment with Ch Ch and Go-6976 decreased Env-mediated cell-cell fusion with HIV-1 R5 Envs, Dual-tropic Env and X4 Env by an average of 98±2.9%, and 75±2% respectively (Fig. 6D). As with the above experiments with the PLC inhibitor and Ca²⁺ inhibitors these results suggest that PKC is required for Env-induced cell-cell fusion in both cell lines and primary cells regardless of Env coreceptor preference.

**HIV-1 Env-induced Pyk2 activation mediated by intracellular Ca²⁺ release is required for Env-dependent cell-cell fusion and Rac activation.** Previous studies have shown that HIV-1 Env-induced intracellular Ca²⁺ release results in activation of the focal-adhesion-related-kinase Pyk2, a downstream target of PKC (88, 89). Pyk2 is a non-receptor tyrosine kinase that can be activated by growth factors, chemokines, and GPCR ligands and provides a link between these ligands and multiple downstream cellular events such as modulation of the cytoskeleton (88, 93, 142, 143). There are no available inhibitors specific for Pyk2 so to test the role of Pyk2 in Env-induced Rac activation and
cell-cell fusion, Pyk2 expression was down-regulated by RNAi in U87.CD4.CCR5 cells. RNAi to Rac was used as a positive control for down-regulation of fusion. siRNA expressing cells were then mixed with BSC40 cells expressing different Env subtypes. Transfection of cells with Pyk2- and Rac-directed siRNAs decreased levels of Env-dependent cell-cell fusion by an average of 77±5.4% and 73±2.3% respectively (Fig. 7A left panel) and Rac activation by 83±2.5 and 81±0.6 respectively (Fig. 7C). The decrease in levels of fusion and Rac activation correlated well with the decreased steady state level of Pyk2 and Rac protein, and each siRNA was specific for its target protein as detected by immunoblot (Fig. 7B). Vaccinia virus infection and T7 polymerase activity was also measured in siRNA transfected cells and Env-dependent cell-cell fusion in these cells was normalized accordingly. RacV12 overcame the effects onEnv-mediated fusion resulting from siRNA targeted to Pyk2 or Rac (Fig. 7A right panel) indicating that Pyk2 acts upstream of Rac in Env-mediated cell-cell fusion and that the effects of the siRNA are overcome by Rac over-expression.

**HIV-1 Env-induced Rac activation and cell-cell fusion depend on Ras activation.**

Pyk2 can lead to Rac activation via multiple signaling partners; however previous data suggests that Env-dependent Rac activation and cell-cell fusion are mediated by a Rac-specific GEF (45). The Rac-specific GEF Tiam1 is directly activated by Ras and is involved in actin cytoskeletal reorganization (42, 144). In addition, Pyk2 activates Ras via Src downstream of G\textsubscript{αq} providing a link between Pyk2 and Rac activation (142). To test the role of Ras, U87.CD4.CCR5 cells were pretreated with the Ras inhibitor S-trans,trans-farnesylthiosalicylic acid (FTS), which blocks membrane association of Ras.
thus preventing Ras activation. FTS inhibition of Env-dependent cell-cell fusion was dose dependent (Fig. 8A, left panel), and concentrations of FTS shown to abolish Env-dependent cell-cell fusion in U87.CD4.CCR5 cells similarly inhibited Env-dependent Rac activation and Env-dependent cell-cell fusion induced by BSC40 cells expressing all HIV-1 Envs with target PBLs (Fig. 8B and Fig. 8C). However, FTS inhibition of cell-cell fusion was not completely reversed by expression of RacV12, suggesting a role for Ras both upstream and downstream of Rac activation (Fig. 8A, right panel).

Ras activation downstream of the chemokine receptor CCR5 has not been shown in previous studies. To determine if Ras is activated via HIV-1 Env interaction with CCR5 and to determine if FTS inhibits this activity, U87.CD4.CCR5 cells were pretreated for 1hr with DMSO alone or with the indicated concentrations of FTS prior to mixing with HIV-1 Env-expressing cells for 30 minutes, and measurement of Ras activation. The activation state of Ras in cell lysates was determined by utilizing Raf-1 binding to separate Ras-GTP from total Ras (Fig. 8D). There was a 3.6 fold increase in activated Ras when U87.CD4.CCR5 cells were mixed with cells expressing Env from R5 HIV_{ADA} strain (lane 6) versus U87.CD4.CCR5 cells mixed with cells expressing no HIV Env (lane 3), or expressing Env from X4 HIV_{HXB2} strain (lane 4). The increase in levels of activated Ras was prevented by TAK-779 (lane 5), suggesting that the Env interaction with coreceptor, rather than CD4 alone, mediates Ras activation. Env-induced Ras activation was inhibited by FTS in a dose dependent manner that correlates with inhibition of Env-dependent cell-cell fusion (Fig. 8A, 8D lanes 7, 8, 9). These data suggest that HIV-1 Env-induced Ras activation is required for Env-dependent Rac activation and cell-cell fusion.
HIV-1 Virus-dependent cell fusion and infection of TZM-BL cells is dependent on the \( \text{Go}_{q} \) signaling pathway. The Env-induced cell fusion assay is a rapid, sensitive, and flexible method used to solely study the HIV-1 Env-mediated fusion step of virus infection. However, this assay involves over-expression of Env on the cell surface of BSC40 cells and does not involve actual virus particles and may not reflect all the factors that mediate fusion. Therefore, we used a virus-dependent fusion assay that is based on the ability of virus particles to bridge two cells and allow transfer of cytoplasmic content (45). This assay has advantages over the Env-induced cell fusion assay since it utilizes relevant levels of virus-associated glycoprotein and is representative of virus-cell fusion versus cell-cell fusion. In this assay we use two populations of U87.CD4.CCR5, one population is infected with vaccinia virus expressing T7 polymerase and the second population is infected with a vaccinia virus expressing the \( \beta \)-galactosidase gene under the regulation of the T7 promoter. Both populations of U87.CD4.CCR5 cells were treated with TAK-779, U73122, U73343, Ch Ch, Go-6976, PKC-I (20-28) dantrolene, CPA, TG, XC, and FTS, for 1h prior to addition of fusion-competent R5 virus HIV\_YU2 for 3h. In this experiment, controls included untreated and inhibitor treated cells that were not incubated with virus (subtracted as background) and the CCR5-inhibitor, TAK-779, that completely inhibited HIV-1 virus-dependent cell fusion. Like Env-dependent fusion, virus-induced fusion was reduced by 73±5% in U73122 treated cells versus cells treated with DMSO alone, and the inactive analog U73343 had no effect (Fig. 9A). The PKC inhibitors, dantrolene, CPA, TG, XC and FTS also inhibited virus-dependent fusion at levels comparable to inhibition seen with the Env-dependent fusion assay (Fig. 9A).
The inhibitory effects of the various inhibitors to the downstream mediators of Gαq on HIV-1 Env-induced cell-fusion and virus-dependent cell fusion suggest an inhibition of virus entry. However, both the Env-dependent and virus-dependent fusion assay only measure the initial step of virus infection, and do not look at the effect of blocking the Gαq pathway, and subsequently fusion, on the entire virus life cycle. It is also unknown whether the inhibitors used in this study specifically block fusion induced by HIV-1 Env or if they block virus-induced membrane fusion and infection in general. To test the specificity of these inhibitors for HIV-1 Env-induced entry and infection we performed the following assay with wildtype HIV\textsubscript{YU2} particles or with VSV-G and A-MLV-pseudotyped HIV-1 virus particles. HIV-1 enters cells by inducing virus-cell membrane fusion at the cell surface. When HIV-1 is pseudotyped with VSV-G or A-MLV Env, the route of viral entry becomes clathrin-mediated or caveola-mediated endocytosis (145, 146). To analyze the effects of these inhibitors on infection with each of these viruses, we performed a single-cycle HIV-1 infection assay using TZM-BL indicator cells (also known as JC53-BL cells), a derivative of HeLa cells, which express surface levels of CD4, CCR5, and CXCR4 comparable to levels produced by peripheral blood mononuclear cells (PBMC) (117). These cells have also been engineered to express β-galactosidase and luciferase under the control of the HIV-1 LTR, which allows quantitative measurement of virus infectivity. TZM-BL cells were pretreated with TAK-779, U73122, U73343, Ch Ch, Go-6976, dantrolene, CPA, TG, XC, and FTS, for 1hr prior to incubation with R5 virus HIV\textsubscript{YU2}, VSV-G-pseudotyped HIV-1 or A-MLV-pseudotyped HIV-1 for 3hrs. Ammonium chloride (NH\textsubscript{4}Cl) (inhibits clathrin mediated endocytosis) and okadaic acid (OA) (inhibits caveola-mediated endocytosis) were
included as controls for inhibition of VSV-G and A-MLV-mediated entry respectively. After 3hrs virus was washed off, the same concentration of inhibitor was added back to the wells, and the cells were incubated at 37° for 24hrs. Figure 9B shows that the reduction in infection with HIV\textsubscript{YU2} in the presence of PKC inhibitors, Ca\textsuperscript{2+} inhibitors, and FTS was comparable to the reduction in Env-dependent and virus-dependent cell fusion (Fig. 9B, upper left panel). The stronger inhibitory effect of U73122 seen in the reporter gene virus infectivity assay, suggests that PLC could also play a role in post-fusion steps in the virus life cycle (Fig. 9B, upper left panel). Interestingly, VSV-G HIV-1 infected inhibitor-treated cells, with the exception of U73122 and NH\textsubscript{4}CL-treated cells, at efficiencies equal to DMSO treated cells (Fig. 9B, right panel). Similarly, A-MLV HIV-1 infected inhibitor-treated cells, with the exception of U73122 and OA-treated cells, as efficiently as it did DMSO-treated cells (Fig. 9B, lower left panel). These data indicated that the PKC inhibitors, Ca\textsuperscript{2+} inhibitors and FTS were unable to block HIV-1 infection when HIV-1 entered cells through VSV-G–mediated or A-MLV-mediated endocytosis. In other words reverse transcription and nuclear import were able to proceed in the presence of these inhibitors. VSV-G HIV-1 and A-MLV HIV-1 infection in U73122-treated cell was inhibited by 57±8.9% and 64±3.6% respectively (Figure 9B). These results support the suggestion PLC plays a role in post-fusion steps in the virus life cycle.

To analyze the effects of the siRNAs targeted to G\textsubscript{q}, G\textsubscript{i}, G\textsubscript{s}, pyk2 and Rac on HIV-1 infection and to show that the effects are not cell-type specific, we performed a single-cycle HIV-1 infection assay using TZM-BL indicator cells transfected with control siRNA and siRNA targeted to G\textsubscript{q}, G\textsubscript{i}, G\textsubscript{s}, Pyk2 and Rac. Figure 9C shows that the reduction in infection with HIV\textsubscript{YU2} in cells expressing siRNA directed against G\textsubscript{q}, pyk2,
and Rac versus cells expressing control siRNA was comparable to the reduction in Env-
dependent cell-cell fusion and Rac activation (Fig. 3 and Fig. 7). Whereas, the siRNA
targeted to G\(_{\alpha_i}\) and G\(_{\alpha_s}\) had no effect (Fig. 9C). The decreased steady state level of G\(_{\alpha_q}\),
G\(_{\alpha_i}\), G\(_{\alpha_s}\), pyk2 and Rac protein in TZM-BL cells expressing targeted siRNA versus
control siRNA, was similar to that observed in U87.CD4.R5 cells (Fig. 3B and Fig. 7B),
and each siRNA was specific for its target protein, as detected by immunoblot (data not
shown).

**Inhibitor treatment does not alter CD4/CCR5 receptor expression or surface localization.** Suppression of Env-dependent Rac activation and cell-cell fusion in the
presence of inhibitors could also be explained by a change in CD4/CCR5 receptor
localization or surface expression. To confirm that there were no changes in CD4/CCR5
receptor localization; U87.CD4.CCR5 cells were incubated with the inhibitors for 3h and
were analyzed by confocal microscopy. No gross differences in cell surface localizations
of CD4 or CCR5 were observed (Fig 10A). Blocking CCR5, with TAK-779 and Rac
activation with the RacGEF Inhibitor also had no effect on CD4/CCR5 localization. In
contrast, CD4 and CCR5 localization could be disrupted by treating cells with the actin
assembly inhibitor Cytochalasin D (CD). U87.CD4.CCR5 cells treated with inhibitors
reconstituted in DMSO were compared to DMSO treated cells, and the cells treated with
inhibitors reconstituted in H\(_2\)O were compared to untreated cells.

To determine the effect of inhibitors on surface expression of the CD4/CCR5
receptors we used flow cytometric analysis (FACS). Incubation of U87.CD4.CCR5 cells
with U73122, U73343, Calphostin C, Ch Ch, PKC-I (20-28), dantrolene, FTS, and
RacGEF Inhibitor for 3h had no effect on cell surface expression of CD4 or CCR5 (Fig. 9B). In this experiment, U87 cells that do not express CD4 or CCR5 were used as a negative control and untreated cells were used as a positive control. TAK-779 a CCR5 inhibitor that interacts with the same domain as the anti-CCR5 antibody blocked CCR5 staining but had no effect on CD4 staining. These results confirm that at the concentrations used in this study these inhibitors do not alter CD4/CCR5 cell surface expression or localization.

**Inhibitor treatment results in specific inhibition of target molecules.** Next we examined whether the decrease in Env-dependent Rac activation and cell-cell fusion in the presence of inhibitor is due to specific inhibition of the target molecule by determining effects on PKA activity and Pyk2 phosphorylation. To measure the effect on PKA activity, U87.CD4.CCR5 cells were pretreated for 1h with U73122, U73343, Ch,Ch, Dantrolene, or FTS then lysed and total lysates were analyzed in the presence of inhibitors using the PepTag assay for the non-radioactive detection of cAMP-dependent protein. PKA activity in U73122, U73343, ChCh, and FTS-treated cells was similar to that in untreated cells (Fig. 11A). PKC inhibitors Bis I, Calphostin C, Go-6976, and Ro-32-0432 were also tested with similar results (data not shown). Treatment of U87.CD4.CCR5 cells with dantrolene decreased PKA activity by 30% suggesting a role for intracellular Ca\(^{2+}\) release in maintaining endogenous PKA activity. This decrease is minimal compared to the 75% reduced PKA activity in cells treated with H89 (Fig. 10A). In addition, dantrolene treated cells show a 90% decrease in Env-induced Rac activation.
and 98% decrease in cell-cell fusion whereas PKA inhibition has no effect (Fig. 6, Fig. 2B).

To determine the effect of inhibitors on HIV-1 Env-induced Pyk2 activation, U87.CD4.CCR5 cells were pretreated with U73122, U73343, PKC inhibitors Calphostin C, Ch Ch, Go-6976 and PKC-I (20-28), dantrolene, or FTS for 1h prior to mixing with HIV-1 Env expressing cells for 5 min and measurement of Pyk2 phosphorylation. As expected, inhibitors targeted to PLC, PKC and intracellular Ca\(^{2+}\) release that are required upstream of Pyk2 decreased the amount of Env-induced Pyk2-phosphorylation whereas FTS had no effect (Fig 11B).

Table 1 summarizes the effects of the PLC inhibitor, general and specific PKC inhibitors, intracellular Ca\(^{2+}\) release inhibitors and FTS on Env-induced cell-cell fusion, Rac activation, virus-dependent cell-cell fusion, infection of TZM-BL cells, PKA activation and Pyk2 phosphorylation. These results confirm that at the concentrations used in this study these inhibitors are specific for their target molecules.
Table 1. Summary of studies with inhibitors

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Target</th>
<th>% EDF$^a$</th>
<th>% EDF$^b$ with RacV12</th>
<th>% Rac Activation</th>
<th>% VDF$^c$</th>
<th>% Infection</th>
<th>% PKA Activation</th>
<th>% Pyk2-P</th>
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<tr>
<td>H89 (10)</td>
<td>PKA</td>
<td>95±2</td>
<td>NT$^d$</td>
<td>100±2</td>
<td>NT</td>
<td>NT</td>
<td>24±0.6</td>
<td>NT</td>
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<tr>
<td>U73122 (3)</td>
<td>PLC</td>
<td>27±1</td>
<td>108±0.2</td>
<td>27±2</td>
<td>28±5</td>
<td>0.8±1</td>
<td>105±5</td>
<td>19</td>
</tr>
<tr>
<td>U73343 (3)</td>
<td>Inactive analog for U73122</td>
<td>95±1</td>
<td>105±0.4</td>
<td>104±4</td>
<td>99±14</td>
<td>96±10</td>
<td>111±4</td>
<td>97</td>
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<tr>
<td>Bis I (10)</td>
<td>PKC</td>
<td>45±11</td>
<td>107±8</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
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<td>NT</td>
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<tr>
<td>Calphostin C (0.5)</td>
<td>PKC</td>
<td>13±2</td>
<td>97±7</td>
<td>14±1</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
<td>16</td>
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<tr>
<td>Ch Ch (50)</td>
<td>PKC</td>
<td>11±2</td>
<td>116±9</td>
<td>17±0.4</td>
<td>7±4</td>
<td>10±2</td>
<td>99±4</td>
<td>14</td>
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<tr>
<td>Go 6976 (1)</td>
<td>Ca2+ dependent PKC</td>
<td>21±4</td>
<td>99±3</td>
<td>27±1</td>
<td>24±6</td>
<td>33±3</td>
<td>NT</td>
<td>26</td>
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<tr>
<td>Ro-32-0432 (10)</td>
<td>Specific for PKC α &amp; β</td>
<td>27±4</td>
<td>108±5</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
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<tr>
<td>PKC-I (20-28) (100)</td>
<td>Specific for PKC α &amp; β</td>
<td>7±3</td>
<td>114±5</td>
<td>6±1</td>
<td>0±3</td>
<td>2±0.2</td>
<td>NT</td>
<td>0</td>
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<tr>
<td>Dantrolene (100)</td>
<td>RyR-Intracellular Ca2+ release</td>
<td>7±2.2</td>
<td>50±1.6</td>
<td>10±5</td>
<td>9±1.8</td>
<td>10±1.2</td>
<td>70±2.5</td>
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<td>CPA (10)</td>
<td>SERCA</td>
<td>21±2.5</td>
<td>40±1.6</td>
<td>19±1.2</td>
<td>14±3</td>
<td>13±1.7</td>
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<tr>
<td>TG (2)</td>
<td>SERCA</td>
<td>3±2.6</td>
<td>25±1.6</td>
<td>21±2.2</td>
<td>9±1.1</td>
<td>0.6±0.5</td>
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<td>XC (5)</td>
<td>IP3R-Intracellular Ca2+ release</td>
<td>24±3.5</td>
<td>32±1.6</td>
<td>25±1.3</td>
<td>20±6.6</td>
<td>3±0.5</td>
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<td>NT</td>
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<td>FTS (50)</td>
<td>Ras</td>
<td>0±0.2</td>
<td>40±4</td>
<td>20±4</td>
<td>0±9</td>
<td>0.3±2</td>
<td>92±24</td>
<td>97</td>
</tr>
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</table>

$^a$ Values are percentages for treated reactions compared to those determined for reactions with untreated controls.

$^b$ EDF, Env-dependent fusion (percent fusion is the average of reactions from cells incubated with HIV$_{ADA}$, HIV$_{YU2}$, and HIV$_{89.6}$ Env-expressing cells).

$^c$ VDF, virus-dependent fusion

$^d$ NT, not tested
Discussion

Earlier studies using PTX and/or mutant CD4 and coreceptors supported the view that signaling is not required for HIV-1 Env-mediated fusion (78, 82-85). However, it is now apparent that signaling via CD4 and the coreceptors is far more intricate than was thought previously. The studies suggesting that signaling is dispensable for HIV-1 entry focused mostly on signaling through $G_{\alpha_i}$ and did not show that $G_{\alpha_i}$-independent pathways were similarly dispensable. A recent study reported that HIV-1 Env-mediated Rac activation and subsequent reorganization of the actin filament network are required to facilitate membrane fusion (12). Another study reported a block of entry and post-entry events with the B oligomer of PTX, which modulates signaling independent of $G_{\alpha_i}$ (147). Additional studies reported that the tyrosine kinase inhibitor genistein blocks HIV-1 infection in primary macrophages and that RhoA and filamin A are required for the actin-dependent clustering of CD4 and coreceptor required for membrane fusion (11, 148, 149). These findings indicate that HIV-1 Env-induced signaling plays a crucial role in Env-mediated membrane fusion.

In the current study, we elucidate that the signal transduction pathway of Env-dependent Rac activation and membrane fusion is $G_{\alpha_q}$, PLC-β, PKC, Pyk2, and Ras dependent (Fig 1). Env-mediated Rac activation and membrane fusion were attenuated in cells expressing siRNA targeted to $G_{\alpha_q}$ and Pyk2 and in cells treated with the PLC inhibitor U73122 but not in cells treated with its inactive analog U73343 or in cells expressing siRNA targeted to $G_{\alpha_i}$ and $G_{\alpha_s}$. Rac activation and fusion were also blocked by multiple PKC inhibitors, by inhibitors of intracellular Ca$^{2+}$ release, and by the Ras inhibitor FTS. The observation that siRNA and inhibitors targeting the $G_{\alpha_q}$ pathway were
inhibitory but inhibitors and siRNA targeting the G\(\alpha_i\) or the G\(\alpha_s\) pathway were not indicates that this pathway is both necessary and sufficient to mediate membrane fusion. Using a constitutively active Rac mutant, RacV12, we have shown that G\(\alpha_q\), PLC-\(\beta\), PKC and Pyk2, are necessary upstream of Rac whereas Ras activation and intracellular Ca\(^{2+}\) release are required for cell-cell fusion both upstream and downstream of Rac activation. Importantly, the inhibitor concentrations employed in our experiments did not alter levels of surface expression or localization of CD4 and CCR5 and also did not show any nonspecific effects on vaccinia virus infection and T7 polymerase or on other signaling pathways. Inhibitors to PKC, intracellular Ca\(^{2+}\) release and Ras suppressed Env-dependent and virus-dependent fusion as well as HIV\(_{\text{YU2}}\) infection of TZM-BL cells to comparable levels. On the other hand, VSV-G HIV-1 or A-MLV HIV-1 infection of TZM-BL cells was not blocked by the PKC, intracellular Ca\(^{2+}\) release, and Ras inhibitors, confirming that the block is specific to HIV-1 Env-mediated membrane fusion. The PLC inhibitor U73122 had a greater effect on HIV\(_{\text{YU2}}\) infection of TZM-BL cells than on Env-dependent and virus-dependent fusion and also decreased VSV-G HIV-1 or A-MLV HIV-1 infection. Taken together this data suggests that while all the inhibitors suppress fusion and therefore infection, U73122 can also suppress post-fusion steps in the virus life-cycle.

It has been shown previously that binding of CCR5 by HIV-1 gp120 activates several signaling pathways including those involving PLC and PKC activation, Ca\(^{2+}\) elevation, Pyk2 phosphorylation, and Rac activation (12, 90, 99). In the present study we show that Env interaction with CCR5 also results in Ras activation. Env-induced Ras activation is mediated specifically by CCR5, and not by CD4, because no increase in
RasGTP was observed in U87.CD4.CCR5 stimulated with X4 Env or TAK-779 treated cells stimulated with R5 Env. Env-dependent Ras activation provides a link between CCR5 stimulation and Rac activation. We have shown previously that Env-mediated Rac activation is dependent on a Rac specific GEF most likely Tiam-1 (45, 144), and Ras has been shown to mediate Rac activation via a direct interaction with Tiam-1 or via phosphatidylinositol 3-OH kinase-mediated activation of Tiam-1. The Env-mediated activation of Ras upstream of Rac indicates that Tiam-1 is the RacGEF responsible for Env-mediated Rac activation. Rac-GEFs and their interacting proteins play an important role in selection of downstream effectors of Rac leading to regulation of different cellular processes (150-152). Therefore, discovery of the Rac-GEF and of downstream targets of Rac required for fusion will provide information on the mechanism of fusion and additional targets for therapeutic intervention.

In summary, we provide evidence that siRNA and inhibitors targeted to Gαq and its downstream effectors prevent HIV-1 infection of TZM-BL cells as well as Env-dependent membrane fusion in U87.CD4.R5 cells and PBLs and virus-dependent membrane fusion of U87.CD4.R5 cells. HIV-1 Env interaction with CCR5 stimulates a signaling cascade involving Gαq, PLC, PKC, intracellular Ca^{2+} release, Pyk2 and Ras that allows activation of Rac and subsequent actin cytoskeleton rearrangements necessary for fusion. These results confirm and extend the implication of Rac in HIV-1 infection, and point to new potential target molecules of HIV-1 inhibitory drugs. Multiple PKC inhibitors are in clinical trials or are approved for clinical use to treat cancer and diabetes including Go-6976 and Bis I used in this study (153). Multiple studies in mice with the Ras inhibitor FTS have given promising results for the treatment of various types of
cancer, neurofibromatosis, and kidney disease (154, 155). In addition, FTS is currently in phase I clinical trials for hematologic malignancies (155, 156). The ability of these inhibitors to specifically down-regulate their target molecules without adverse side effects suggests that these inhibitors might be appropriate drugs for treatment of HIV-1 and other infectious microbes that manipulate this pathway. This strategy of using inhibitors that disable host signaling proteins essential for pathogen survival may have a general efficacy in developing drugs to combat pathogens that acquire drug resistance.

Acknowledgements

We thank S. Pontow, N. Campbell, and I. Lee for assistance with these experiments. This work was supported by PHS grant AI24745 and T32 AI007172.
**Figures**

Fig. 1. **Model of signal transduction pathway elicited by Env interaction with CCR5 required for Rac activation and membrane fusion.** This study establishes the role of various signaling molecules during HIV-1-induced membrane fusion by examining the effects of selective inhibitors and siRNA targeting the Gαq pathway using Rac activation, virus entry and infection assays. Signaling molecules previously shown to be activated by gp120 are shown in bold, and confirmed pathways are shown with a solid line (12, 87, 134, 141, 144). Suspected factors that may be involved are shown in normal font, and potential pathways are shown with dotted lines. Ras is shown in black because it was shown to be activated by Env in this study. Open boxes represent siRNA or selective small molecule inhibitors used to inhibit Gαq and molecules downstream of Gαq.
CCR5

siRNA

General: Bis I, Calphostin C, Ch Ch
Specific: Ro-32-0432, Go-6976 PKC-I (20-28)

gp120

Gq

PLC

PKC

Ca^{2+}

Pyk2

Ras

Rac-GEF-I

Tiam1

Rac

N

C

U73122

Dantrolene, CPA, TG, XC

siRNA

FTS
Fig. 2. Env-mediated Rac activation and cell-cell fusion are PTX-insensitive and PKA independent. (A) U87.CD4.CCR5 cells were treated with 200ng/ml PTX for 18h prior to and during 30 min incubation with BSC40 cells expressing no Env (grey) or Env from HIV-1 strain ADA (black). Whole cell lysates were then analyzed by Rac specific G-LISA activation assay (average A490 of duplicate wells ± standard deviation; data are representative of results from three similar experiments). (B) Actin-dependent cell fusion. Average fusion compared to untreated control reactions and detected by β-galactosidase activity ± standard deviation is shown. U87.CD4.CCR5 cells were infected with vaccinia virus expressing β-galactosidase (vCB21R) overnight, then these cells were pretreated with DMSO alone (black) and the PKA inhibitors PKA 14-22 (grey) and H89 (black) at indicated concentrations (uM) for 1h. A portion of these cells was mixed 1:1 with HIVADA or HIVUNC Env-expressing cells (subtracted as background) for 3h in the presence of inhibitors. Data are representative of results from three similar experiments performed in triplicate. Cell fusion was normalized using untreated cells as incubated with HIVADA Env as 100%. The rest of the cells were lysed and whole cell lysates were analyzed for PKA activity in the presence of inhibitors. PKA (ng) based on standard curve of A570nm of varying amounts of PKA. Results representative of three independent experiments performed in duplicate. (C) U87.CD4.CCR5 cells were treated for 1h with 1uM TAK-779 or 10uM H89 prior to and during 30 min incubation with BSC40 cells expressing no Env (subtracted as background), HIVADA Env (open bars) or HIVHXB2 Env (solid bars). Whole cell lysates were then analyzed by Rac specific G-LISA activation assay. Average A490 of duplicate wells ± standard deviation; data are representative of results from three similar experiments (*, P < 0.01).
Fig. 3. Gaq down-regulation with siRNA reduces Env-mediated cell-cell fusion. (A) Average fusion compared to untreated control reactions and detected by β-galactosidase activity ± standard deviation are shown. U87.CD4.CCR5 cells were transfected with 100nM control siRNA (control) or 100nM siRNA targeted against Gaq, Gi or Gs. 24h post-transfection U87.CD4.CCR5 cells were serum starved. 48h post transfection the cells were infected with vCB21R alone or with vaccinia virus encoding the constitutive-active point mutant RacV12 (vRacV12) in complete media. 72 h post-transfection the transfected U87.CD4.CCR5 were incubated with HIV_{UNC} (subtracted as background) HIV_{ADA}, HIV_{YU2}, HIV_{89.6} or HIV_{HXB2} Env-expressing cells for 3h. Data are representative of results from three similar experiments performed in triplicate (**, P < 0.05). Cell fusion was normalized using control siRNA transfected cells incubated with HIV_{ADA} Env as 100%. (B) 2X10^5 of each population of transfected cells were lysed and whole cell lysates were analyzed by western blot. There are three immunoblots, the lysate from cells transfected with 100nM control siRNA (lane1) or 100nM siRNA targeted to Gaq (lane 2), Gi (lane 3), or Gs (lane 4), were loaded on each immunoblot and were initially probed with anti-Gaq (left), anti-Gi (middle), or anti-Gs (right), then all blots were stripped and probed with anti-actin (bottom). The relative reduction index (RI) was calculated as the quotient of the densitometry signal for the Gaq, Gi or Gs band and that for actin (shown below), and then normalized by the ratio obtained with control siRNA (defined as 1). Data represent results from 1of 3 experiments with similar results. (C) U87.CD4.CCR5 cells were transfected with 100nM control siRNA (solid bars), 100nM Gaq siRNA, Gi siRNA, or Gs siRNA (open bars) as described above. 72h post-transfection cells were incubated for 30 min with BSC40 cells expressing no Env
(subtracted as background), or HIV_{ADA} Env. Whole cell lysates were then analyzed by Rac specific G-LISA activation assay. Average A490 of duplicate wells ± standard deviation; data are representative of results from three similar experiments (**, P < 0.05).
Fig. 4. PLC-β is required for Env-mediated fusion upstream of Rac. Average fusion compared to untreated control reactions and detected by β-galactosidase activity ± standard deviation are shown. (A) Serum starved U87.CD4.CCR5 cells were infected with vCB21R alone, or with vRacV12 in complete media overnight. These cells were then pretreated with DMSO alone, or 3uM of PLC-β inhibitor U73122 and its negative analog U73343 for 1h and the inhibitors were also present during the incubation with HIV_{UNC} (subtracted as background) HIV_{ADA}, HIV_{YU2}, HIV_{89.6} or HIV_{HXB2} Env-expressing cells for 3h. Data are representative of results from three similar experiments performed in triplicate (*, P < 0.01, **, P < 0.05). Cell fusion was normalized using DMSO treated cells incubated with HIV_{ADA} Env as 100%. (B) U87.CD4.CCR5 cells were treated for 1h with 1uM TAK-779, 3uM U73122, 3uM U73343 (open bars), or DMSO alone (solid bars) prior to and during incubation for 30 min with BSC40 cells expressing no Env (subtracted as background), or HIV_{ADA} Env. Whole cell lysates were then analyzed by Rac specific G-LISA activation assay. Average A490 of duplicate wells ± standard deviation; data are representative of results from three similar experiments (*, P< 0.01, **, P < 0.05). (C) Serum starved PBLs were infected with vCB21R in complete media overnight. These cells were then pretreated with DMSO alone, 1uM TAK-779 or 3uM of PLC-β inhibitor U73122 and its negative analog U73343 for 1h and the inhibitors were also present during the incubation with HIV_{UNC} (subtracted as background) HIV_{ADA}, HIV_{YU2}, HIV_{89.6} or HIV_{HXB2} Env-expressing cells for 3h. Data are representative of results from three similar experiments performed in triplicate (*, P < 0.01, **, P < 0.05). Cell fusion was normalized using DMSO treated cells incubated with HIV_{ADA} Env as 100%.
Fig. 5. Intracellular Ca^{2+} release is required for Env-mediated fusion upstream & downstream of Rac. Average fusion compared to untreated control reactions and detected by β-galactosidase activity ± standard deviation are shown. (A) Serum starved U87.CD4.CCR5 cells were infected with vCB21R alone, or with vRacV12 in complete media overnight. These cells were then pretreated with DMSO alone or 100uM dantrolene, 10uM cyclopiazonic acid (CPA), 2uM thapsigargin (TG), or 5uM Xestospongin C (XC) for 1h and the inhibitors were also present during the incubation with HIV\textsubscript{UNC} (subtracted as background) HIV\textsubscript{ADA}, HIV\textsubscript{YU2}, HIV\textsubscript{89.6} or HIV\textsubscript{HXB2} Env-expressing cells for 3h. Data are representative of results from three similar experiments performed in triplicate (*, P < 0.01). Cell fusion was normalized using DMSO treated cells incubated with HIV\textsubscript{ADA} Env as 100%. (B) U87.CD4.CCR5 cells were treated for 1h with DMSO alone (solid bars), 1uM TAK-779, 100uM dantrolene, 10uM CPA, 2uM TG, 5uM XC, or 100uM RacGEF inhibitor (open bars) prior to and during incubation for 30 min with BSC40 cells expressing no Env (subtracted as background), or Env from HIV\textsubscript{ADA} Env. Whole cell lysates were then analyzed by Rac specific G-LISA activation assay. Average \textit{A}_{490} of duplicate wells ± standard deviation; data are representative of results from three similar experiments (*, P < 0.01). (C) Serum starved PBLs were infected with vCB21R in complete media overnight. These cells were then pretreated with DMSO alone or 100uM dantrolene, 10uM cyclopiazonic acid (CPA), 2uM thapsigargin (TG), and 5uM Xestospongin C (XC) for 1h and the inhibitors were also present during the incubation with HIV\textsubscript{UNC} (subtracted as background) HIV\textsubscript{ADA}, HIV\textsubscript{YU2}, HIV\textsubscript{89.6} or HIV\textsubscript{HXB2} Env-expressing cells for 3h. Data are representative of results from
three similar experiments performed in triplicate (*, P < 0.01). Cell fusion was normalized using DMSO treated cells incubated with HIV\textsubscript{ADA} Env as 100%. 

![Graph A](image)

![Graph B](image)

![Graph C](image)
Fig. 6. PKC is required for Env-mediated fusion upstream of Rac. Average fusion compared to untreated control reactions and detected by β-galactosidase activity ± standard deviation are shown. (A, B) Serum starved U87.CD4.CCR5 cells were infected with vCB21R alone or with vRacV12 overnight in complete media. These cells were then pretreated with DMSO alone or the PKC inhibitors Bisindolylmaleimide I (Bis I, 10uM), Calphostin C (0.5uM), and Chelerythrine Chloride (Ch Ch, 50uM) for 1h (A) or pretreated with the specific PKC inhibitors Go-6976 (Ca^{2+}-dependent, 1uM), Ro-32-0432 (selective for α & β, 10uM), and PKC-I (20-28) (myristilated peptide inhibitor specific for α & β, 100uM) for 1h (B). The inhibitors were also present during the incubation with HIV_{UNC} (subtracted as background) HIV_{ADA}, HIV_{YU2}, HIV_{89.6} or HIV_{HXB2} Env-expressing cells for 3h. Data are representative of results from three similar experiments performed in triplicate (*, P<0.01, **, P < 0.05). Cell fusion was normalized using DMSO treated cells incubated with HIV_{ADA} Env as 100%. (C) U87.CD4.CCR5 cells were treated for 1h with DMSO alone (solid bars), 1uM TAK-779, 0.5uM Calphostin C, 50uM Ch Ch, 1uM Go 6976, 100uM PKC-I (22-28) and 100uM RacGEF inhibitor (open bars) prior to and during incubation for 30 min with BSC40 cells expressing no Env (subtracted as background), or HIV_{ADA} Env. Whole cell lysates were then analyzed by Rac specific G-LISA activation assay. Average A490 of duplicate wells ± standard deviation; data are representative of results from three similar experiments (*, P < 0.01). (D) Serum starved PBLs were infected with vCB21R overnight in complete media. These cells were then pretreated with DMSO alone or the PKC inhibitors Ch Ch, (50uM) or Go-6976 for 1h. The inhibitors were also present during the incubation with HIV_{UNC} (subtracted as background) HIV_{ADA}, HIV_{YU2}, HIV_{89.6} or HIV_{HXB2} Env-expressing cells
for 3h. Data are representative of results from three similar experiments performed in triplicate (*, P<0.01). Cell fusion was normalized using DMSO treated cells incubated with HIV_{ADA Env} as 100%.

A

![Graph A](chart1.png)

B

![Graph B](chart2.png)

C

![Graph C](chart3.png)

D

![Graph D](chart4.png)
Fig. 7. Env-induced fusion and Rac activation are dependent on Pyk2. (A) Average fusion compared to untreated control reactions and detected by β-galactosidase activity ± standard deviation are shown. U87.CD4.CCR5 cells were transfected with 100nM control siRNA (control) or 100nM siRNA targeted against Pyk2 or Rac. 24h post-transfection U87.CD4.CCR5 cells were serum starved. 48h post transfection the cells were infected with vCB21R alone or with vaccinia virus encoding the constitutive -active point mutant RacV12 (vRacV12) in complete media. 72 h post-transfection the transfected U87.CD4.CCR5 were incubated with HIV_{UNC} (subtracted as background) HIV_{ADA}, HIV_{YU2}, HIV_{89.6} or HIV_{HXB2} Env-expressing cells for 3h. Data are representative of results from three similar experiments performed in triplicate (*, P < 0.01). Cell fusion was normalized using control siRNA transfected cells incubated with HIV_{ADA} Env as 100%. (B) 2X10^5 of the each population of transfected cells were lysed and whole cell lysates were analyzed by western blot. There are two immunoblots, the lysate from cells transfected with 100nM control siRNA (lane1) or 100nM siRNA targeted to Pyk2 (lane 2) or Rac (lane 3) were loaded on each immunoblot and were initially probed with anti-Pyk2 (left) or anti-Rac (right), then all blots were stripped and probed with anti-actin (bottom). The relative reduction index (RI) was calculated as the quotient of the densitometry signal for the Pyk2 or Rac band and that for actin (shown below), and then normalized by the ratio obtained with control siRNA (considered 1). Data represent results from 1of 3 experiments with similar results. (C) U87.CD4.CCR5 cells were transfected with 100nM control siRNA (solid bars), 100nM Pyk2 or 100nM Rac siRNA (open bars) as described above. 72h post-transfection cells were incubated for 30 min with BSC40 cells expressing no Env (subtracted as background), or HIV_{ADA} Env. Whole
cell lysates were then analyzed by Rac specific G-LISA activation assay. Average A490 of duplicate wells ± standard deviation; data are representative of results from 1 of 3 similar experiments (**, P < 0.05).
Fig. 8. Ras is required for Env-mediated fusion upstream & downstream of Rac.

Average fusion compared to untreated control reactions and detected by β-galactosidase activity ± standard deviation are shown. (A) Serum starved U87.CD4.CCR5 cells were infected with vCB21R alone, or with vRacV12 in complete media overnight. These cells were then pretreated with DMSO alone or indicated concentrations of Ras inhibitor FTS for 1h and inhibitor was also present during the incubation with HIV_{UNC} (subtracted as background) HIV_{ADA}, HIV_{YU2}, HIV_{89.6} or HIV_{HXB2} Env-expressing cells for 3h. Data are representative of results from three similar experiments performed in triplicate (*, P < 0.01). Cell fusion was normalized using DMSO treated cells incubated with HIV_{ADA} Env as 100%. (B) U87.CD4.CCR5 cells were treated for 1h with DMSO alone (solid bars), 1μM TAK-779, 50μM FTS, and 100μM RacGEF inhibitor (open bars) prior to and during incubation for 30 min with BSC40 cells expressing no Env (subtracted as background), or Env from HIV_{ADA} Env. Whole cell lysates were then analyzed by Rac specific G-LISA activation assay. Average A_{490} of duplicate wells ± standard deviation; data are representative of results from three similar experiments (*, P < 0.01, **, P <0.05). (C) Serum starved PBLs cells were infected with vCB21R in complete media overnight. These cells were then pretreated with DMSO alone, 50μM Ras inhibitor FTS, or 100μM RacGEF inhibitor for 1h and inhibitor was also present during the incubation with HIV_{UNC} (subtracted as background) HIV_{ADA}, HIV_{YU2}, HIV_{89.6} or HIV_{HXB2} Env-expressing cells for 3h. Data are representative of results from three similar experiments performed in triplicate (*, P < 0.01). Cell fusion was normalized using DMSO treated cells incubated with HIV_{ADA} Env as 100%. (D) Ras activation assay. Western blot analysis of Raf-1 binding fractions from lysates of U87.CD4.CCR5 cells mixed with
BSC40 cells expressing no Env (lane 3), HIV\textsubscript{HXB2} Env (lane 4) or HIV\textsubscript{ADA} Env (lanes 5-9). 1uM TAK-779 was included to inhibit CCR5-Env binding (lane 5). Cells were pretreated with DMSO alone (lane 6) and indicated concentrations of FTS to inhibit Ras (lane 7-9) for 1hr and during the 30 min incubation with Env-expressing cells. Positive (lane 1) and negative (lane 2) controls were generated by GTP\textsubscript{γ}S- and GDP-loading of reaction lysates, respectively. Increases (n-fold) in the amount of Ras-GTP compared to lane 3 were determined by densitometry (normalized to lysate loading control) and are indicated below the blot. Data represent results from 1 of 3 experiments with similar results.

![Graphs showing percent fusion and A590 measurements](image)

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Fig 9. Effect of inhibitors on virus-dependent cell fusion with HIV-1 R5 virus and infection of TZM-BL cells with HIV-1 R5 virus or A-MLV- pseudotyped and VSV-G pseudotyped HIV-1 virus. (A) Average fusion compared to untreated control reactions and detected by β-galactosidase activity ± standard deviation are shown. One population of serum starved U87.CD4.CCR5 cells was infected with vCB21R and the other population of U87.CD4.CCR5 cells was infected with vPT7-3 in complete media overnight. These cells were then mixed (1:1) in triplicate wells of 96 well plate and pretreated with DMSO alone, TAK-779 (1uM), U73122 and U73343 (3uM), Ch Ch (50uM), Go-6976 (1uM), PKC-I (20-28) (100uM), dantrolene (100uM), CPA (10uM), TG (2uM), XC (5uM) and FTS (50uM) for 1h. Inhibitors were also present during incubation with 100ng of HIV\text{YU2} per well for 3h at 37°C. Data are representative of results from three similar experiments (*, P < 0.01). Cell fusion was normalized using DMSO treated cells mixed with HIV\text{YU2} as 100%. (B) JC53BL cells were pre-incubated with DMSO alone, TAK-779 (1uM), U73122 and U73343 (3uM), Ch Ch (50uM), Go-6976 (1uM), dantrolene (100uM), CPA (10uM), TG (2uM), XC (5uM), FTS (50uM), NH\textsubscript{4}Cl (50mM), and OA (100nM) for 1h and inhibitors were also present during 3h infection period with 150ng of HIV\text{YU2}, A-MLV-pseudotyped HIV-1 or VSV-G-pseudotyped HIV-1 per well. Virus and inhibitor were then washed off the cells and the cells were incubated in the same concentration of inhibitor at 37°C overnight. Luciferase activities in the infected cell lysates were measured at 24h post-infection and were used to calculate virus infectivity relative to that of the control. Data are representative of results from 1 of 3 similar experiments performed in triplicate (**, P < 0.05). Cell infection was normalized using DMSO treated cells as 100%.
Fig 10. Gaq inhibitors do not affect surface expression and localization of CD4 and CCR5.GFP. (A) Confocal micrographs of U87.CD4.CCR5 cells untreated, treated with DMSO alone, TAK-779 (1uM), U73122 (3uM), U73343 (3uM), Calphostin C (0.5uM), Chelerythrine Chloride (50uM), PKC-I 20-28 (100uM), dantrolene (100uM), FTS (50uM) and RacGEF Inhibitor (100uM) for 3h, and CD (1uM) for 15 min, fixed and stained with anti-CD4-PE antibodies (red) and counterstained with TO-PRO3 (blue). The green GFP signal and red PE signal have been merged to show areas of colocalization (yellow). Data are representative of results from 3 experiments. Images were collected using an oil objective (magnification X63). (B) U87.CD4.CCR5 cells were incubated with all the inhibitors listed in (A) except CD for 3h and detached by treatment with 5mM EDTA. U87 cells and untreated and treated U87.CD4.CCR5 cells were labeled for surface expression of CD4 and CCR5 and analyzed by flow cytometry. Unlabeled U87.CD4.CCR5 cells were used to compensate for GFP. Data are expressed as percentage of surface expression based on DMSO treated cells as 100%.
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B

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Fig 11. Pretreatment with inhibitors results in specific effects on target molecules.

(A) U87.CD4.CCR5 cells were treated with the PKA inhibitor H89 (10uM), PLC inhibitor U73122 and its negative analog U73343 (10uM), PKC inhibitor Ch Ch (50uM), Intracellular Ca2+ inhibitor dantrolene (100uM) and Ras inhibitor FTS (50uM) for 1h. Whole cell lysates were then analyzed for PKA activity in the presence of inhibitors. PKA (ng) based on standard curve of densitometry of varying amounts of PKA. Results representative of two independent experiments performed in duplicate (*, P < 0.01, **, P <0.05). (B) Western blot analysis of phosphorylated Pyk2 from lysates of U87.CD4.CCR5 cells mixed with BSC40 cells expressing no Env (lane 1) or Env from HIV-1 strains HXB2 (lane 3), or ADA (lanes 2, 4-12) at 37°C for 5 min. Cells were pretreated with DMSO alone (NI, lane 1-3), 1uM TAK-779 (lane 4), 3uM U73122 and U73343 (lane 5-6), 0.5uM Calphostin C (lane 7), 50uM Ch Ch (lane 8), 1uM Go-6976 (lane 9), 100uM PKC-I (20-28) (lane 10), 100uM dantrolene (lane 11) and 50uM FTS (lane 12) for 1hr and during the 5 min incubation with Env-expressing cells. Cell lysates were resolved by 10% SDS-PAGE, transferred to a nitrocellulose membrane, and probed with anti-Pyk2 phosphospecific [pY579/580] antibody (upper panel, P-Pyk2). The same blot was then stripped and reprobed with an antibody specific for total Pyk2 (lower panel). Blots shown are representative of 3 independent experiments.
Chapter 4

Role of Abl Kinase and the Wave2 Signaling Complex in HIV-1 Entry at a Post-Hemifusion Step
Preface to Chapter 4

Chapter 4 has been prepared as a manuscript for submission to PNAS by Brooke Harmon and Lee Ratner. All experiments and data were performed and collected by Brooke Harmon.
Role of Abl Kinase and the Wave2 Signaling Complex in HIV-1 Entry at a Post-Hemifusion Step

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Abbreviations: DAS, dasatinib; Env, envelope; IMB, imatinib; NIL, nilotinib; PBL, peripheral blood lymphocyte; RNAi, RNA interference; si, small interfering
Abstract

Entry of human immunodeficiency virus type 1 (HIV-1) commences with binding of the envelope glycoprotein (Env) to the receptor CD4, and one of two coreceptors, CXCR4 or CCR5. Env-mediated signaling through coreceptor results in Gαq-mediated Rac activation and actin cytoskeleton rearrangements necessary for fusion. In this study, we show that the mechanism of Env-induced Rac activation is mediated by the Rac guanine nucleotide exchange factor Tiam-1, which associates with the adaptor protein IRSp53 to link Rac to the Wave2 complex and activates the actin polymerization complex Arp2/3. Env-mediated fusion and HIV-1 infection are dependent on Tiam-1, IRSp53, Wave2, and Arp3 as shown by attenuation of fusion and infection in cells expressing siRNA targeted to these signaling components. Env-dependent cell fusion and HIV-1 infection were also inhibited by siRNA to Abl and Abl kinase activity inhibitors, imatinib, nilotinib, and dasatinib. Fusion was blocked by these inhibitors without altering cell viability or surface expression of CD4 and CCR5. Similar results were obtained when cell-cell fusion was induced by Env expressed on viral or cellular membranes and when cell lines or primary cells were the target. Using membrane curving agents and fluorescence microscopy, we showed that inhibition of Abl kinase activity arrests fusion at the hemifusion step, suggesting a role for actin remodeling in pore formation and expansion. These results also suggest a potential use of Abl kinase inhibitors to treat HIV-1 infected patients.
Introduction

HIV-1 enters cells by fusion at the plasma membrane and endocytosis (1-3). HIV-1 entry requires multiple conformational changes in the HIV-1 glycoprotein, and rearrangement of the actin cytoskeleton. These events are triggered by binding of the viral envelope (Env) surface subunit gp120 to the primary receptor, CD4, and one of two chemokine coreceptors, CCR5 or CXCR4 (2,12). This interaction activates signaling events similar to those initiated by natural ligands, such as Ca\(^{2+}\) mobilization, activation of RhoGTPases, and phosphorylation of tyrosine kinases, pyk2, Zap70 and p56lck (12,49,73). The Rho GTPase sub-family stimulates myosin based contractility and drives the formation of stress fibers and focal adhesions. The Rac GTPase sub-family stimulates lamellipodia and membrane ruffles, and the Cdc42 subfamily stimulates the formation of filopodia (26,27,157). We showed that HIV-1 Env binding to target cells activates Rac, stimulates membrane ruffles and lamellipodia, and fusion is inhibited by dominant negative Rac (12,45). Furthermore, HIV-1 Env-induced Rac activation depends on activation of G\(\alpha\)q, phospholipase C (PLC), Ca\(^{2+}\) mobilization, protein kinase C (PKC), pyk2 and Ras (73). In the current study we identified the fusion-specific effectors of Rac required for actin cytoskeleton rearrangements that mediate membrane fusion and entry.

Rac GTPase is regulated by guanine nucleotide exchange factors (GEFs) (26,27,157). HIV-Env induced Rac activation is mediated by a Rac-specific GEF, either Tiam-1 or Trio (44,45). GEFs not only facilitate the GDP to GTP switch for their targeted GTPase, but they also select downstream effector proteins by participating in scaffold protein complexes that organize components of a specific signaling pathway.
There are multiple effectors of Rac, including serine/threonine kinases, lipid kinases, actin-binding proteins, and adaptor/scaffold molecules (27, 158). PAK is a downstream effector of Rac and Cdc42 that promotes stabilization of actin networks. Another downstream effector of Rac that nucleates actin polymerization is the Arp2/3 complex. The Arp2/3 complex is activated by the Wave2 complex through IRSp53, an adaptor protein that binds Rac and Wave2 (27). The Wave2 complex includes Rac-associated protein 1, Nck-associated protein, Abl-interacting protein 2, and heat shock protein C300. Wave2 also associates with Abl, which promotes Wave2 phosphorylation and activation (159). In addition to determining which Rac effectors are critical for membrane fusion, we studied the steps in the membrane fusion process affected by these signaling molecules. These data demonstrate that the Wave2 signaling complex and Abl are required for Env-mediated membrane fusion, entry, and infection and that inhibition of Abl kinase arrests the fusion process at hemifusion.

**Results**

**HIV-1 Env-mediated fusion depends on the Wave2 signaling complex.**

To determine whether Abl, Trio, or Tiam-1 are required for HIV-1 Env-mediated cell fusion, expression of these proteins was downregulated by RNA interference (RNAi) in U87.CD4.CCR5 cells. Cells expressing siRNAs were mixed with BSC40 cells expressing different Env subtypes and Env-dependent fusion was measured. Transfection of target cells with siRNA to Tiam-1 and Abl decreased levels of Env-mediated cell-cell fusion by an average of 79 ± 5% and 74 ± 5% respectively for both HIV-1 R5 and dual-tropic Env-subtypes (Fig 1A, left). There was no significant fusion observed with CCR5 expressing target cells and X4 Env expressing cells, as expected. The decrease in the levels of fusion
correlated well with the decreased steady-state level of Tiam-1, and Abl as detected by immunoblot (Fig. 1C). The siRNA directed against Trio had no effect on Env-induced cell-cell fusion despite a 70% reduction in expression of the Trio protein (Fig 1A, C). To determine whether Tiam-1 and Abl are acting exclusively upstream of Rac, a constitutively active Rac mutant, RacV12 was expressed in siRNA transfected cells. Expression of RacV12 reversed the effects on fusion of siRNA to Tiam-1, suggesting that Tiam-1 is functioning upstream of Rac (Fig 1A, right). In contrast, levels of fusion in cells expressing RacV12 and siRNA to Abl were only 53 ± 1% that of cells expressing RacV12 and control siRNA, suggesting a role for Abl upstream and downstream of Rac.

Tiam1 binds to the Rac and Cdc42 effector IRSp53, enhancing IRSp53 binding to Rac and the Wave2 scaffolding complex (150). To determine the role of these Rac effectors in Env-mediated membrane fusion, their expression was downregulated by RNAi in U87.CD4.CCR5 cells. The siRNA expressing cells were mixed with Env-expressing cells and fusion measured. Expression of siRNAs to Irsp53, Wave2, and Arp3 decreased fusion by 74 ± 5%, 77 ± 4%, and 78 ± 4%, respectively, and this decrease was not overcome by expression of RacV12, suggesting that these proteins are required downstream of Rac (Fig 1B). The decrease in fusion correlated with the downregulation in protein expression as seen by immunoblot (Fig. 1C), and each siRNA was specific for its target protein (Fig S1A). Treatment of cells stably expressing siRNA resistant Arp3, with Arp3 targeted siRNA had no effect on Env-mediated cell-cell fusion (Fig. 1D). However, treatment of untransfected cells and cells stably expressing siRNA resistant Arp3 with siRac decreased fusion by 75 ± 5% and 76 ± 3% respectively (Fig. 1D). These results show that the effect of RNAi on fusion is specific to inhibition of target molecules.
Small molecule inhibitors of Abl kinase activity inhibit HIV-1 entry.

Since treatment of cells with Abl targeted siRNA led to a decrease in fusion, we determined whether treatment with Abl kinase inhibitors, imatinib (IMB), nilotinib (NIL), and dasatinib (DAS), block fusion and infection. IMB is a relatively specific inhibitor of Bcr-Abl, Abl, Arg, and class III receptor tyrosine kinases. NIL is a kinase inhibitor 20-50 fold more potent than IMB at inhibiting Abl. DAS, originally designed as a Src family kinase inhibitor, also inhibits ephrin and platelet-derived growth factor receptor kinases, and kit. DAS is 300 fold more potent than IMB at inhibiting Abl (160, 161). To determine the concentrations of these Abl kinase inhibitors that inhibit Env-mediated fusion without non-specific effects, trypan blue analysis, vaccinia virus infection, and T7 polymerase activity were measured in addition to Env-dependent cell fusion (Fig. S2). Treatment of U87.CD4.CCR5 cells with 10uM IMB, 500nM NIL, and 300nM DAS for 1h prior to and during 3h incubation with Env-expressing cells decreased Env-mediated fusion by an average of 95 ± 2%, 92 ± 5%, and 92 ± 6%, respectively, and Abl kinase activity by 85-87% (Fig. 2A, S1B). There was no decrease in T7 polymerase activity, or localization of CD4 and CCR5 on the cell surface (Fig. S2, S3). Expression of RacV12 in cells treated with IMB, NIL and DAS increased the level of fusion by an average of 3.5-fold (**, P < 0.05) compared to treated cells without RacV12, suggesting a role of Abl kinase activity upstream of Rac (Fig. 2B). To determine the effect of these Abl kinase inhibitors on Env-induced Rac activation, U87.CD4.CCR5 cells were treated with inhibitors for 1h prior to mixing with BSC40 cells expressing no HIV-1 Env, HIV-1 X4 Env, or HIV-1 R5 Env for 30 minutes in the presence of inhibitor. The mismatched X4 Env, that does not induce Rac activation in
CCR5 expressing cells, and the CCR5 inhibitor TAK-779, which completely blocks Env-mediated Rac activation in CCR5 expressing cells, were included as controls. Env-induced Rac activation was abolished in cells treated with TAK-779 and all three of the Abl kinase inhibitors (Fig. 2C). To validate these effects in a relevant HIV-1 target cell, PBLs which express CD4, CCR5 and CXCR4, were used as the target cell in an Env-dependent cell fusion assay. Treatment of PBLs with IMB, NIL, and DAS decreased fusion by an average of 92 ± 1%, 92 ± 3%, and 99.5 ± 1%, respectively, for HIV-1 R5, dual-tropic and X4 Env subtypes (Fig. 2D). In addition, infection of CD4, CCR5 and CXCR4-expressing cells with R5 and X4 HIV-1 was inhibited by treatment with IMB in a dose dependent manner (Fig. S2C). These results suggest that Abl is required for Env-induced fusion mediated by both CCR5 and CXCR4. The CCR5 inhibitor TAK-779, as expected, completely blocked fusion and infection mediated by R5 Env-expressing cells, inhibited fusion mediated by dual-tropic Env by 56 ± 2%, and had no effect on fusion or infection mediated by X4 Env (Fig. 2D, S2C).

To confirm these results using virus particles with relevant levels of virus-associated glycoprotein, we used a virus-dependent cell fusion assay based on the ability of virus particles to bridge two cells and allow transfer of cytoplasmic contents (12, 45). For this assay we used two populations of U87.CD4.CCR5 cells, one expressing the T7 polymerase and the other expressing the β-galactosidase (β-gal) gene under the T7 promoter. Both populations were incubated with inhibitors for 1h prior to 3h incubation with R5 virus HIV\text{YU2}. In this assay controls included untreated and inhibitor treated cells that were not incubated with virus, TAK-779, and T-20, which blocks entry by inhibiting the conformational change in HIV-1 gp41 required for fusion (135). Virus-dependent cell
fusion was reduced by an average of 94 ± 3% in cells treated with IMB, DAS, and NIL compared to cells treated with DMSO alone, and treatment with TAK-779 and T-20 completely inhibited fusion (Fig. 2E). To confirm the specificity of these effects, we performed an Env-dependent fusion assay with cells stably expressing two different drug resistant Bcr-Abl mutants (Y253F and T315I), or expressing wildtype (WT) Bcr-Abl (162). Expression of the drug resistant Bcr-Abl mutants but not WT Bcr-Abl resulted in recovery of fusion (Fig. 2F), demonstrating that the effects of these inhibitors on Env-dependent fusion are specific to inhibition of Abl.

**Infection of TZM-BL cells with HIV-1 particles but not particles pseudotyped with Amphotropic Murine Leukemia Virus (A-MLV) Env or Vesicular Stomatitis Virus Glycoprotein (VSV-G) depend on Abl and the Wave2 signaling complex**

To determine whether the Wave2 signaling complex and Abl are required exclusively for HIV-1 entry, or virus-induced fusion and infection in general, we examined infection with HIV-1 versus A-MLV Env (A-MLV-HIV-1) or VSV-G pseudotyped HIV-1 (VSV-G HIV-1) using the TZM-BL assay. HIV-1 Env induces virus-cell fusion at the cell surface to facilitate entry, whereas viruses pseudotyped with VSV-G or A-MLV Env induce clathrin- or caveola-mediated endocytosis, respectively (145). TZM-BL cells, a derivative of HeLa cells that express physiological levels of CD4, CCR5, CXCR4, and luciferase (luc) under the control of the HIV-1 LTR, were pretreated with the Abl kinase inhibitors for 1h prior to incubation with virus for 3h, and a subsequent 24h incubation with inhibitor only (117, 135). Ammonium chloride (NH₄Cl) which inhibits clathrin mediated endocytosis, and okadaic acid (OA), which inhibits caveola-mediated endocytosis, were included as controls. Fig. 3A shows that treatment with IMB, NIL, and
DAS decreased infection with HIV\textsubscript{YU2} virus by 93 ± 10%, 94 ± 0.5%, and 92 ± 14%, respectively, comparable to the reductions in Env-dependent and virus-dependent cell fusion. The Abl kinase inhibitors had no effect on infection with A-MLV HIV-1 or VSV-G HIV-1, but treatment with NH\textsubscript{4}Cl blocked infection with VSV-G HIV-1, and treatment with OA blocked infection with A-MLV HIV-1 (Fig. 3A, middle and bottom). These data show that Abl kinase inhibitors were able to block HIV-1 Env-mediated fusion specifically, had no effect on infection via clathrin-mediated or caveola-mediated endocytosis, and do not affect post-entry steps.

To test the effect of Wave2 complex targeted siRNAs on infection, TZM-BL cells were transfected with 200nM control or siRNA directed towards Tiam-1, Trio, Abl, Irsp53, Wave2 and Arp3. These cells were incubated with virus for 3h, and media alone for 24h. The decreased levels of HIV-1\textsubscript{YU2} infection of TZM-BL cells expressing siRNA targeted to Tiam-1, Abl, Irsp53, Wave2, and Arp3 was comparable to levels of Env-mediated cell fusion with U87.CD4.CCR5 cells expressing these siRNAs, whereas the siRNA to Trio had no effect (Fig. 3B, 1A, 1B). Steady state levels of target proteins in cells expressing targeted siRNAs were decreased to similar levels as in U87 cells (Fig. 1C). Infection of TZM-BL cells with A-MLV HIV-1 or VSV-G HIV-1 was not affected by expression of the targeted siRNAs (Fig 3B), suggesting that Tiam-1, Abl, Irsp53, Wave2, and Arp3 are not necessary for post-fusion steps in the virus life cycle.

The Abl kinase inhibitors block fusion and infection at a post-hemifusion step

HIV-1 Env-induced fusion and release of the viral capsid into the cytosol is a multistep process. 1) The surface subunit gp120 binds CD4, and undergoes a conformational change, and actin cytoskeletal rearrangements in the target membrane
bring the coreceptor CCR5 or CXCR4 into close proximity. 2) Coreceptor binding to gp120 triggers conformational changes in gp41 to produce a prebundle conformation that inserts into the target cell membrane, allowing lipid mixing or hemifusion, and pore formation. 3) Additional conformational changes induce formation of the gp41 6-helix-bundle which prevents pore closure and facilitates pore enlargement and full fusion (4, 7, 163). To determine which step(s) in the membrane fusion process are blocked by the Abl kinase inhibitors, we examined the effect of membrane curving agents on fusion and infection. Oleic acid (OLA), chlorpromazine (CPZ), dibucaine (DB), and trifluoperazine (TFP) are lipid analogs that insert into the inner leaflet of the cell membrane. OLA induces curvature in the membrane that promotes formation of a hemifusion intermediate but cannot induce pore formation if there is a block at hemifusion. CPZ, DB, and TFP are membrane-permeable weak bases that partition into inner leaflets of cell membranes and induce curvature and relieve a block at hemifusion (164, 165). To determine the effect of inhibitors and lipid analogs on virus-dependent cell fusion, U87.CD4.CCR5 cells were treated with TAK-779, IMB, NIL, and DAS for 1h, prior to 30 minute incubation with HIV_{YU2}, and treatment with CPZ, DB, and TFP for 1 min or OLA for 5 min. The lipid analogs were washed off, inhibitor and virus were added back, and fusion was measured after 3h. Cells were also incubated with the lipid analogs in the absence of HIV_{YU2} to account for the effects of these agents on the cells and on T7 polymerase activity. TAK-779 mediated inhibition of fusion was not affected by these lipid analogs. The average level of fusion for CPZ treated cells, treated with IMB, NIL, and DAS, versus DMSO alone was 78 ± 3% versus 5 ± 3% in the absence of any lipid analog (Fig. 4A). Addition of the lipid analogs DB and TFP increased levels of fusion in cells treated with Abl
kinase inhibitors to 52 ± 3% and 55 ± 2% respectively, in comparison to cells treated with DMSO alone. Addition of OLA did not increase fusion in cells treated with any of the inhibitors.

To confirm these results with another cell type and to show the effect of lipid analogs on HIV-1 infection, TZM-BL cells were similarly treated with inhibitors and lipid analogs but after the 3h incubation with HIV-1_{YU2}, virus was washed off, inhibitors were added back and cells incubated for 24h. Addition of CPZ and TFP to TZM-BL cells treated with Abl kinase inhibitors increased average infection to 56 ± 5% and 59 ± 4% respectively, versus DMSO treated cells (Fig. 4B). Infection of TZM-BL cells treated with Abl kinase inhibitors in the absence of lipid analogs was only 11 ± 5% that of DMSO treated cells. OLA had no effect on infection (Fig. 4B). These results suggest that inhibition of Abl kinase arrests fusion at a hemifusion step, preventing pore formation, pore enlargement and content mixing.

To confirm that Abl kinase inhibitors cause arrest at hemifusion, we used a modification of a fusion assay described previously (166). CHO-K1 cells, that lack expression of the lipid ganglioside GM1, were engineered to express GFP and the HIV-1_{ADA} (R5) Env protein. U87.CD4.CCR5 cells were used as the target cell, and lipid mixing was detected when GM1, detected by a TRITC-conjugated form of cholera toxin β-subunit (CTX-β), was transferred from the target cell to CHO-K1-GFP cells. Complete fusion is detected when cells express GM1, GFP, and are multinucleated. Quantification was performed for three independent experiments and percentage of hemifused GFP+, GM1+ cells with single nuclei and the percentage of multinucleated fully fused cells was enumerated for 68 cells from each condition (Fig. 4C, S4, Table S1). There were 83.1
±10.9% hemifused cells with IMB-treated cells mixed with HIV_{ADA}-expressing CHO-K1 cells, compared to DMSO treated cells with 22.3 ± 4.9% hemifused cells and 75.5 ± 6.2% fully fused cells (Fig 4C). With no HIV-1 Env or with the addition of TAK-779 there was little or no hemifusion or full fusion. These results confirm that Abl kinase activity is required at a post-hemifusion step.

**Discussion**

Dynamic regulation of the actin cytoskeleton is required for fusion of biological membranes. For Env-mediated membrane fusion actin rearrangements in the target cell are critical for formation of the gp120-CD4-coreceptor complex, as well as post-binding steps (11, 12, 25, 33, 44, 45, 73). Our previous data demonstrated that Env-induced Rac activation is mediated by Gαq and its downstream effectors, including Ras. We showed that a small molecule inhibitor of Rac GEFs, Trio and Tiam-1 eliminated Env-dependent fusion (44, 45). Other studies showed that Ras promotes Rac activation via direct interaction with Tiam1, or by phosphatidylinositol 3-kinase (PI3K)-mediated activation of Tiam-1 (144). Env-dependent Rac activation likely occurs through the first mechanism, since treatment of target cells with PI3K inhibitors had no effect on Env-dependent cell fusion (167).

The non-receptor tyrosine kinase, Abl, modulates actin upstream and downstream of Rac (168, 169). In the current study, we show that the activity of Abl kinases is required both upstream and downstream of Rac for Env-induced membrane fusion. Env-mediated fusion and Rac activation were attenuated in cells treated with siRNA targeted to Abl, or Abl kinase inhibitors, IMB, NIL, and DAS, which was only partially overcome by expression of RacV12. This inhibition was observed with Env expressed on viral or
cellular membranes, and when cell lines or primary cells were the target. The inhibitor concentrations employed did not alter levels of CD4 or CCR5 expression or localization on the surface of cells nor did they result in nonspecific effects on vaccinia virus infection or T7 polymerase activity. The effects of these inhibitors on Env-mediated fusion were specific to inhibition of Abl as demonstrated by reversal of inhibition in cells expressing drug resistant Abl mutants.

Abl kinase has been shown to modulate actin reorganization downstream of Rac by phosphorylation and activation of the Wave2 complex. Abl interactor adaptors associate with Wave2, and promote membrane translocation and phosphorylation of Wave2 (159, 170). Wave2 mediates signaling downstream of Rac, through Arp2/3-mediated actin polymerization. Tiam1 binds to the adaptor protein IRSp53, and promotes the interaction of activated Rac and Wave2. In the current study we demonstrated that IRSp53, Wave2, and Arp3 are required downstream of Rac for HIV-1 entry and infection. Env-mediated fusion was attenuated in cells expressing siRNA targeted to IRSp53, Wave2 and Arp3 and this inhibition was not overcome by expression of RacV12. The decrease in fusion observed with Arp3 targeted siRNA was overcome by expression of siRNA resistant Arp3 suggesting that the effects observed with RNAi were specific. Infection of TZM-BL cells with HIV-1 was similarly inhibited in cells expressing Tiam-1, Abl, IRSp53, Wave2 and Arp3 directed siRNA. HIV-1 infection was also inhibited in cells treated with Abl kinase inhibitors. The inhibition of HIV-1 infection by targeted siRNA or Abl kinase inhibitors was overcome when virus was pseudotyped with VSV-G or A-MLV Env. These results suggest that these signaling mediators are important for HIV-1 Env
mediated entry and are not necessary for clathrin or caveola-mediated endocytosis, and they are not required at post-entry steps in the virus life cycle.

The current study also showed that the block in fusion caused by inhibition of Abl kinase occurs after hemifusion and before cytoplasmic mixing. This conclusion was based on the 1) confocal microscopy demonstration that addition of IMB to the fusion reaction allowed membrane but not cytoplasmic mixing, and 2) observation that lipid analogs that overcome a block at hemifusion overcame Abl kinase inhibition of HIV-1 virus dependent cell fusion and infection. These results support a model whereby HIV-1 Env binding to CCR5 stimulates activation of Goq resulting in activation of Ras, via pyk2 and Abl phosphorylation of the Ras GEF complex. Activated Ras then mediates Rac activation via the Rac GEF Tiam-1, and activated Rac interacts with IRSp53. IRSp53 promotes Rac activation of the Wave2 complex, which is also activated by Abl, and activated Wave2 induces subsequent activation of Arp2/3-mediated actin rearrangements which facilitate pore formation, pore enlargement, and entry of HIV-1.

Many microbial pathogens depend on Abl family kinases to mediate efficient infection of their targeted host, including Shigella flexneri, enteropathogenic Escherichia coli, Heliobacter pylori, Anaplama phagocytophilum, coxsackievirus, poxvirus, and murine AIDS virus. Abl kinases are involved in pathogen entry, intracellular movement, and exit from target cells; proliferation of target cells; and phosphorylation of microbial effectors. Many of these processes involve reorganization of the target cell actin cytoskeleton and depend on the same signaling pathways as HIV-1 (12, 73, 171). Discovery of these signaling mediators as fundamental components of microbial pathogenesis provides new targets for therapeutic intervention. The clinical application of IMB, NIL, and DAS,
which block deregulated Abl kinases in leukemia patients, demonstrate that inhibition in vivo is possible with manageable side effects (160, 161, 172). In addition IMB has been shown to be an effective inhibitor of anti-apoptotic pathways induced by HIV-1 in macrophages (173). Most current antiviral therapies target viral proteins and mutation of the virus leads to therapy resistance. Therefore, using inhibitors that target host signaling proteins essential for HIV-1 entry may be an efficient new strategy for treatment of infected patients.

Materials and Methods

Reagents & Cell Lines. U87.CD4.CCR5 cells are astrogloma cells expressing CD4, CCR5-GFP or HA-CCR5. CHO-K1 cells (ATCC) were grown in F-12K media with 10% serum and other cells maintained as described (116), pMSCVneo-WT, Y253F, and T315I Bcr-Abl were gifts from Dr. R. Van Etten (162). The siRNA resistant mutations were generated in Arp3 based on sequences obtained from Santa Cruz Biotechnology Inc (SCBT), by PCR-mediated mutagenesis. WT and mutant cDNAs were cloned in pcDNA3.1+zeo. IMB, NIL, and DAS were from LC Laboratories and were used at 10uM, 500nM, and 300nM respectively unless indicated; CPZ (0.5mM), DB (5mM), TFP (0.3mM), OA (100nM), OLA (50uM) and NH₄Cl (50mM) were from Sigma; TAK-779 (1uM), and T-20 (10ug/ml) were from the AIDS Research and Reference Reagent Program; and siRNA constructs were from SCBT and used at 200nM (73).

Viruses. WT vaccinia (WR strain) and recombinant vaccinia viruses expressing β-gal (vCB21R), T7 polymerase (vPT7-3), constitutively active Rac GTPase (vRacV12), or HIV-1 Env proteins were described (116). HIV with R5 YU2 or X4 HXB2 Env in
HIV_{NL4-3} backbone were generated from 293T cells; some were pseudotyped with A-MLV) or VSV glycoproteins (73). TZM-BL assays were performed as described (73).

**Fusion and hemifusion assays.** Envelope-mediated and virus-dependent fusion assays were described. Average fusion compared to untreated control reactions were detected by β-gal activity ± standard deviation (73). Hemifusion assays were performed with 2x10^6 CHO-K1 cells nucleofected with a GFP expression plasmid, and after 24h infected with vaccinia virus expressing HIV_{ADA} Env or no Env. After 16h, 4x10^5 U87.CD4.CCR5.HA cells were added for 3h, fixed with paraformaldehyde, stained with TRITC-conjugated CTX-β (CTX-555, EMD), and analyzed on a 510 Meta LSM confocal microscope.

**Statistical Analysis.** Fusion and infectivity results were compared using a two-tailed t-test. All p values, unless indicated, were <0.03.

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Figures

Fig. 1. Downregulation of Wave2 signaling complex with siRNA reduces HIV-1 Env-mediated cell fusion and infection. U87.CD4.CCR5 cells were transfected with control siRNA (control) or siRNA targeted against (A) Trio, Tiam-1, Abl, Rac, (B) Irsp53, Wave2, and Arp3. Cells were serum starved 24h post-transfection (pt), infected with vCB21R alone or with vRacV12 48h pt, and 72h pt cells were incubated for 3h with HIV_{UNC} (subtracted as background), HIV_{ADA}, HIV_{YU2}, HIV_{89.6} or HIV_{HXB2} Env-expressing cells and β-gal activity was measured (C) Each population of transfected cells were analyzed by western blot with antibodies to the designated protein or actin. The relative reduction index (RI) is the quotient of the densitometry signal for the target band and that for actin, normalized by the ratio obtained with control siRNA. (D) U87.CD4.CCR5 cells engineered to express a siRNA resistant clone of Arp3 were transfected with control siRNA, or siRNA targeted against Arp3 or Rac. Cell fusion was measured by β-gal activity, and was normalized using control siRNA transfected cells incubated with HIV_{ADA} Env as 100%. All data are representative of results from three similar experiments performed in triplicate.
**Fig 2. Abl kinase is required upstream and downstream of Rac.** (A) Serum starved U87.CD4.CCR5 cells were infected with vCB21R alone, or with (B) vRacV12 overnight, then treated with DMSO alone, TAK-779, IMB, NIL, or DAS for 1h and the inhibitors were also present during 3h incubation with HIV-1 Env-expressing cells and β-gal activity as measured. (C) U87.CD4.CCR5 cells were treated for 1h with TAK-779, IMB, NIL, or DAS and during 30 min incubation with BSC40 cells expressing no Env (subtracted as background), HIV<sub>ADA</sub> Env or HIV<sub>HXB2</sub> Env. Whole cell lysates were analyzed by Rac specific G-LISA activation assay. Average A490 of triplicate wells ± standard deviation are shown. (D) Serum starved PBLs were infected with vCB21R in complete media overnight, treated with DMSO, TAK-779, IMB, NIL, or DAS for 1h prior to addition of HIV-1 Env-expressing cells and β-gal activity was measured. (E) U87.CD4.CCR5 cells were infected overnight with vCB21R or vPT7-3, then mixed (1:1) in triplicate wells, treated for 1h with DMSO, TAK-779, T20, IMB, NIL, DAS, and with 100ng of HIV<sub>YU2</sub> for 3h at 37°C. β-gal activity was measured and cell fusion was normalized using DMSO treated cells mixed with HIV<sub>YU2</sub> as 100%.(F) U87.CD4.CCR5 cells engineered to express indicated clones of Bcr-Abl were treated with Abl inhibitors and HIV<sub>UNC</sub> or HIV<sub>ADA</sub> Env as described above or analyzed by western blot with anti-Abl or anti-actin antibody (inset). All data are representative of results from three similar experiments performed in triplicate.
Fig. 3. Infection with HIV-1 particles but not particles pseudotyped with MLV Env or VSV-G is dependent on Abl and the Wave2 signaling complex. (A) TZM-BL cells were incubated for 1h with DMSO, NH$_4$Cl, OA, IMB, NIL, or DAS and 150ng of HIV$_{YU2}$, A-MLV-or VSV-G-HIV-1 per well was added for 3h, washed, and cells were incubated with inhibitors overnight and luc activity was measured. (B) TZM-BL cells were transfected with control siRNA or siRNA directed against indicated target proteins and 48h later infected with 150ng of HIV$_{YU2}$, A-MLV- or VSV-G-HIV-1 for 3h, cells were washed and incubated overnight, and luc activity measured. Cell infection was normalized using (A) DMSO treated cells or (B) cells transfected with control siRNA as 100%. All data are representative of results from three similar experiments performed in triplicate.
Fig. 4. Abl kinase inhibitors block virus-dependent fusion and infection at a post-hemifusion step. (A) Serum starved U87.CD4.CCR5 cells were infected with vCB21R or vPT7-3 overnight, then mixed (1:1) in triplicate wells of 96 well plate, and these cells or (B) TZM-BL cells were treated with DMSO, TAK-779, IMB, NIL, or DAS for 1h, (A) 100ng or (B) or 150ng of HIV\textsubscript{YU2} added for 30 min with indicated lipid analogs for 1-5 min (A) Cells were washed, and virus and inhibitors were added back for 3h and β-gal activity was measured or (B) cells were then washed and incubated in inhibitor overnight and luc activities were measured. Data are representative of results from three similar experiments. Cell fusion and infection were normalized using DMSO treated cells infected with HIV\textsubscript{YU2} as 100%. (C) CHO-K1 cells that do not express GM1 were transfected with a GFP expressing plasmid (green), and 24h later infected with WT vaccinia virus or vaccinia virus expressing HIV\textsubscript{ADA} Env. After another 24h, CHO-K1 cells were overlayed for 3h with U87.CD4.CCR5 cells pre-treated for 1h with DMSO, TAK-779, or IMB. Cells were fixed and stained with CTX-555 (red), and counterstained with TO-PRO 3 (blue). Images were collected using an oil objective (magnification X63). The upper panels are a consolidation of three different images and the lower panels are a consolidation of two different images. Images were cropped but relative cell size was maintained. The percentage of hemifused cells is listed.
Supporting Information

Fig. S1. Abl is activated by HIV-1 Env and Pretreatment with inhibitors and siRNA results in specific effects on target molecule. (A) U87.CD4.CCR5 cells were transfected with 200nM control siRNA or siRNA directed against Trio, Tiam-1, Abl, IRSp53, Wave2, Arp3 and Rac and 48 h later each population of transfected cells was lysed and analyzed by western blot with antibodies to the designated protein or actin. The relative reduction index (RI) is the quotient of the densitometry signal for the target band and that for actin, normalized by the ratio obtained with control siRNA. Data are from 1 of 3 experiments with similar results. The bottom blot depicting Rac levels in cells transfected with the labeled siRNA demonstrates that the siRNAs have no effect on Rac expression. (B) Abl kinase activity was measured using PAthScan Bcr/Abl activity assay from Cell Signaling. Depicted is western blot analysis of a downstream target of activated Abl kinase, phosphorylated CrkL, and loading control eIF4E from lysates of U87.CD4.CCR5 cells mixed 1:1 with BSC40 cells expressing no Env (lane 1) or Env from HIV-1 strain ADA (lanes 2-5) at 37°C for 20 min. Cells were pretreated with DMSO alone (NI, lane 1-2), 1μM TAK-779 (lane 3), 10μM IMB (lane 4), 500nM NIL (lane 5), 300nM DAS (lane 6), 100μM Rac GEF Inhibitor (lane 8), for 1hr and during the 20min incubation with Env-expressing cells. Cell lysates were resolved by 10% SDS-PAGE, transferred to a nitrocellulose membrane, and probed with phosphospecific primary antibody cocktail and anti-rabbit or anti-biotin secondary. Blots shown are representative of 3 independent experiments.
Fig. S2. Abl kinase inhibitors decrease Env-induced fusion, virus-dependent fusion and infection of TZM-BL cells with R5 and X4 virus in a concentration dependent manner. Average fusion compared to untreated control reactions was detected by β-gal activity ± standard deviation. (A) Serum starved U87.CD4.CCR5 cells were infected with vCB21R alone, or with vRacV12 overnight, then treated with DMSO alone, 1uM TAK-779 or 1uM, 5uM and 10uM IMB, for 1h and the inhibitors were also present during the 3h incubation with HIV_{UNC} (subtracted as background) HIV_{ADA}, HIV_{YU2}, HIV_{89,6} or HIV_{HXB2} Env-expressing cells. (B) U87.CD4.CCR5 cells were infected overnight with vCB21R or vPT7-3, then mixed (1:1) in triplicate wells, treated for 1h with DMSO, 1uM TAK-779, or 1uM, 5uM and 10uM IMB, and 100ng of HIV_{YU2} added for 3h at 37°C. Cell fusion was normalized using DMSO treated cells mixed with HIV_{YU2} as 100%. (C) TZM-BL cells were incubated for 1 h with DMSO, 1uM TAK-779, 1, 5 and 10uM IMB, and 150ng of HIV_{YU2} or 150ng HIV_{HXB2} per well was added for 3h, washed, and cells were incubated with inhibitors at 37°C overnight. (D) Serum starved U87.CD4.CCR5 cells were infected with vCB21R alone, or with vRacV12 overnight, then treated with DMSO alone, 125, 250 and 500nM NIL, or (E) DMSO alone, 75, 150 and 300nM DAS, for 1h and the inhibitors were also present during the 3h incubation with HIV_{UNC} (subtracted as background) HIV_{ADA}, HIV_{YU2}, HIV_{89,6} or HIV_{HXB2} Env-expressing cells. Data are representative of results from three similar experiments.
Fig. S3. Abl kinase inhibitors do not affect surface expression and localization of CD4 and CCR5.GFP. (A) Confocal micrographs of U87.CD4.CCR5 cells treated with DMSO alone, TAK-779 (1uM), IMB (10uM), NIL (500nM), Dasatinib (300nM) and RacGEF Inhibitor (100uM) for 3h, and CD (1uM) for 15 min, fixed and stained with anti-CD4-PE antibodies (Sigma, red) and counterstained with TO-PRO3 (blue). The green GFP signal and red PE signal have been merged to show areas of colocalization (yellow). Data are representative of results from 3 experiments. Images were collected using an oil objective (magnification X63). The NIL panel is a consolidation of 2 separate images. (B) U87.CD4.CCR5 cells were incubated with no inhibitor, DMSO alone, TAK-779 (1μM), IMB (10μM), NIL (500nM), Dasatinib (300nM) and RacGEF Inhibitor (100μM) for 3h and detached by treatment with 5mM EDTA. U87 cells, and untreated and treated U87.CD4.CCR5 cells, were stained with anti-CCR5 (R&D) or anti-CD4 antibodies (Sigma), and goat anti-mouse PE conjugated antibody. Cells were analyzed on a FACS Calibur flow cytometer. Unlabeled U87.CD4.CCR5 cells were used to compensate for GFP. Data are expressed as percentage of surface expression based on DMSO treated cells as 100%.
**Fig. S4. Abl kinase inhibitors block fusion at a post-hemifusion step.** (A) CHO-K1 cells that do not express GM1 were transfected with a GFP expressing plasmid, and 24h later infected with wildtype vaccinia virus or vaccinia virus expressing HIV\textsubscript{ADA}. After another 24h, CHO-K1 cells were overlayed for 3h with U87.CD4.CCR5.HA cells pretreated for 1h with DMSO, 1\mu M TAK-779, or 10\mu M IMB. Cells were fixed and stained with TRITC-conjugated CTX (CTX-555), and counterstained with TO-PRO 3 (blue). Images were collected using an oil objective (magnification X63). The upper right middle panel (ADA DMSO) is a consolidation of two different images. Images were cropped but relative cell size was maintained.
SI Table 1. Quantification of cell-cell hemifusion assay

<table>
<thead>
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<th>HIV Env</th>
<th>Inhibitor</th>
<th>% hemifusion ± SD</th>
<th>% fusion ± SD</th>
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<tr>
<td>None</td>
<td>DMSO</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>ADA (R5)</td>
<td>DMSO</td>
<td>22.3 ± 4.9</td>
<td>75.5 ± 6.2</td>
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<tr>
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<td>TAK-779</td>
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<tr>
<td>ADA (R5)</td>
<td>Imatinib</td>
<td>83.1 ± 10.9</td>
<td>14.3 ± 5.1</td>
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Chapter 5

Discussion and Conclusions
Introduction:

Substantial insights into the mechanism used by HIV-1 viral proteins to promote fusion have come from numerous studies using cell fusion assays, techniques that monitor viral core-associated enzymes into the host cell, and time-resolved imaging of single viral particles. The current model for viral protein-mediated fusion has emerged from extensive biochemical and functional data using these systems (7, 174-182). Fusion commences with binding of trimeric gp120 to CD4 which induces a structural change in gp120 that creates the binding site for coreceptor. Upon binding to coreceptor the gp120/gp41 complex undergoes additional conformational changes that results in dissociation of gp120 and gp41 and insertion of the hydrophobic TM domain of gp41 into the target cell membrane (4, 7, 183). In addition to anchoring the viral proteins to the target membrane, the fusion peptide also destabilizes lipid bilayers, by reducing the free energy barrier required to pass through curved fusion intermediates (182, 184). Next the extended trimeric conformation of gp41 that is bridging the viral and target membrane folds back on itself forming a hairpin structure and driving membrane merger (hemifusion) (7). The energy from the final folding of gp41 into the 6HB conformation is thought to facilitate pore formation and enlargement and the 6HB, which forms after pore formation, is thought to prevent pore closure (7).

Dilation of fusion pores to a size that is large enough for nucleocapsid delivery (~50nm) is required for infection but the mechanism of pore enlargement in not well understood. However evidence from our lab demonstrates a role for actin remodeling in HIV-1 entry post binding that would support the idea of HIV-1 hijacking cellular machinery to create and expand the fusion pore (7, 12). In Chapters 2-4 we sought to
determine the mechanism used by Env to mediate actin cytoskeleton rearrangements and to establish what role these rearrangements are playing in fusion.

A previous study from our lab looked at the role of the actin filament network during HIV-1 Env-dependent entry and virus dependent syncytium formation, utilizing the actin cytoskeleton disrupting drugs JP, LA, and CD (7, 12). Previous studies showed that formation of the Env-CD4-coreceptor complexes was inhibited by treatment of target cells with the actin filament capping drug CD but the effect of other actin-targeted drugs and the role of actin at later stages in entry was not investigated (25, 185). The study from our lab went on to show that the actin filament stabilizing drug, JP and the actin monomer sequestering drug, LA inhibited cell fusion mediated by X4 and R5 Env by 80 to 100% whether added before or after incubation at 25°C. As was shown previously CD was only inhibitory when the drug was added before the 25°C incubation (12, 25, 185). These results indicate that modulation of the actin cytoskeleton is necessary for HIV-1 entry both prior to and after formation of Env-CD4-coreceptor complexes.

This study then went on to support the role for actin-based structures in HIV-1 Env-mediated syncytium formation by confocal imaging. Membrane ruffles, lamellipodia, and filopodial extensions were observed in target cells mixed with Env-expressing cells by time-lapse confocal microscopy (12). These actin structures are mediated by Rac and Cdc42, while Rho stimulates formation of stress fibers which were not observed in the target cell (12, 31). This study went on to show that Rac was activated in target cells exposed to Env and that this activation was required for fusion and dependent on Env ligation of CCR5. The results from this study indicate that HIV-1 Env-coreceptor interactions stimulate an intracellular signaling cascade that promotes
Rac mediated reorganization of the actin filament network necessary to facilitate membrane fusion (12, 25, 185).

Results

The data presented in Chapter 2 further substantiated a role for Rac activation in membrane fusion. Treatment of target cells with a Rac GEF inhibitor blocked HIV-1 fusion as measured by the Env-dependent fusion assay, infection, or the virus-dependent fusion assay (45). The concentration of Rac GEF inhibitor that blocked HIV fusion was similar to that required to block Env-induced Rac activation. The specificity of the inhibitor was examined by expressing a constitutively active Rac mutant, RacV12 in target cells treated with inhibitor. Expression of RacV12 overcame the effects of the Rac GEF inhibitor on Env-dependent fusion and on Env-dependent Rac activation (45). In addition the Rac GEF inhibitor had no effect on Env-dependent Rho activation (data not shown). This study also gave insight into the Rac GEF(s) required for Env-mediated Rac activation, as Tiam-1 and Trio are the only Rac GEFs known to be sensitive to this inhibitor (44). Additional studies looking at the role of downstream mediators of Rac in HIV Env-mediated fusion showed no effects of chemical inhibitors of NADPH oxidase, MEK, or myosin light chain. Expression of DN PAK-2 and PI3-K in target cells also had no effect on Env-mediated fusion (unpublished findings). In addition, a study looking at IL-17-mediated HIV permissiveness in quiescent T cells showed that DN PI3Kp110 and the PI3-K inhibitor wortmannin had no effect on fusion or infection of cells with HIV (186).

In an effort to identify the role of CCR5-mediated signaling in Env-induced Rac activation and membrane fusion we made various mutations in the domains of the
intracellular loops (ICLs) and cytoplasmic tail of CCR5 thought to associate with
different signaling effectors. It has been shown previously that certain intracellular
domains of CCR5 and CXCR4 can be mutated without affecting HIV-induced membrane
fusion (78-85, 187-189). It has also been shown that specific domains of CCR5 associate
with different Gα proteins and scaffolding molecules such as β-arrestin (40, 49, 80, 132).
To determine which CCR5 domains and subsequently which signaling molecules were
required for Env signaling to Rac, U87 CD4 cell lines were established that expressed
mutations in the ICL2, ICL3 or the cytoplasmic tail of CCR5. The ICL3 mutations made
in CCR5 were previously made in CCR1, which has 100% sequence homology to CCR5
in this region. These mutations in ICL3 of CCR1 disrupted CCR1 coupling to Gαq (80).
The mutations made in ICL2 replaced the arginine (R) and tyrosine (Y), from the
conserved DRY motif thought to associate with Gai and β-arrestin, with glycine (G) and
alanine (A) (85). The mutation in the C terminus of CCR5 was a truncation of the
cytoplasmic tail engineered to end with amino acid (aa) 313. This mutation had been
shown previously to disrupt β-arrestin association and Ca²⁺ flux (83-85).

After these cell lines were established they were sorted by GFP for CCR5
expression equal to that of U87 CD4 cells expressing wildtype CCR5 (wt cells). Upon
examination of surface expression by flow cytometry with a CCR5 monoclonal antibody
the mutants were observed to have a drastic reduction in the amount of surface expression
compared to wt cells. These results were then confirmed by confocal microscope
analysis. Next these cells were sorted for CCR5 surface expression similar to wt cells
using a monoclonal antibody to CCR5. However upon examining surface expression of
CCR5 on these newly sorted cell lines by flow cytometry and confocal microscopy, it
became apparent that there was still little to no surface expression of mutant CCR5 (figure 1). The sequences for all of the mutants were double checked and are the same as the CCR5 expressed in wt cells except for the targeted mutations. These results together with the confocal analysis lead to the conclusion that these mutations prevent proper surface trafficking of CCR5, as similar levels of CCR5 are synthesized (figure 1B). Although these mutations were all made previously in other studies, further investigation of the literature confirmed that the mutations do in fact affect surface expression of CCR5. (84, 85, 190, 191)
Fig 1. Mutations in the ICL2, ICL3, and a deletion in the C-terminus of CCR5.GFP prevent its surface expression. (A) Confocal micrographs of U87.CD4.CCR5.GFP cells (wildtype CCR5, far left), U87.CD4.CCR5.ICL3.GFP (middle left), U87.CD4.CCR5.ICL2.GFP (right center), and U87.CD4.CCR5∆C.GFP (far right), fixed and stained with anti-CD4-PE antibodies (red). The green GFP signal and red PE signal have been merged to show areas of colocalization (yellow). (B) The mutant CCR5 constructs were transiently transfected into 293T cells and 48hr post transfection untransfected (not shown but used to determine R1) and transfected 293T cells were labeled for surface expression of CCR5 (anti-CCR5 primary and anti-mouse PE-conjugated secondary) and analyzed by flow cytometry. The lower left quadrant are cells that do not express CCR5 (R1), the lower right quadrant are cells that express CCR5 as determined by expression of GFP but it is not on the surface (R2), and the upper right quadrant are cells that express CCR5 at the surface (R4, PE staining).

Green: CCR5-GFP  Red: CD4  Yellow: Colocalization

A

B

U87.CD4.CCR5 (wildtype)  U87.CD4.CCR5.ICL3 (ICL3 mutations)  U87.CD4.CCR5.ICL2 (RY → GA)  U87.CD4.CCR5∆C (Stop codon at a.a.313)
Since generation of CCR5 molecules unable to couple with signaling intermediates was not possible, we looked for alternative methods to determine the signaling pathway that regulates Env-mediated Rac activation and membrane fusion. In chapter 3 we utilized small interfering RNA (siRNA) and various small molecule inhibitors to determine the Env-induced signaling pathway required for Rac activation and fusion. The results from this study demonstrate that HIV-1 Env mediates activation of the Gαq pathway via CCR5 and this activation is critical for HIV-1 induced membrane fusion. Expression of Gαq, Gαi, and Gαs was downregulated by siRNA, and Env-mediated Rac activation and fusion were measured. Env-induced Rac activation and cell fusion were attenuated in cells expressing siRNA targeted to Gαq but not in cells expressing siRNA targeted to Gαi or Gαs (73). Treatment of cells with inhibitors to the Gαq downstream effectors, PLCβ, PKC, intracellular Ca^{2+} release, and Ras, also blocked Env-mediated Rac activation and membrane fusion. Whereas inhibitors specific for PKA, the downstream effector of Gαs, and PTX which blocks signaling via Gαi had no effect on Env-dependent Rac activation and cell fusion (73). In addition, Env-dependent Rac activation and cell fusion was also attenuated in cells expressing siRNA targeted to pyk2 which is activated downstream of PKC and intracellular Ca^{2+} release, and is a mediator of Ras activation. Expression of RacV12 overcame the effects of Gαq and pyk2 siRNA as well as the effects of inhibitors of PLCβ and PKC on Env-dependent cell fusion, suggesting that these signaling mediators are acting upstream of Rac. Whereas levels of Env-induced fusion were only partially recovered by expression of RacV12 in cells treated with inhibitors to intracellular Ca^{2+} release and Ras, suggesting they play a role both upstream and downstream of Rac (73).
Similar results were obtained when cell fusion was induced by Env-expressed on viral or cellular membranes and when cell lines or primary cells were the target. Furthermore, single cycle infection of HIV-1 was also attenuated when Gαq and its downstream signaling mediators were blocked. On the other hand, infection of cells with A-MLV Env or VSV-G pseudotyped HIV-1 was not blocked by inhibitors to PKC, intracellular Ca\(^{2+}\) release and Ras, suggesting that these signaling mediators are specifically required for HIV-1 mediated fusion (73). The PLC inhibitor had a greater effect on HIV-1 infection than on Env-dependent fusion and also slightly decreased infection of cells with A-MLV-HIV-1 and VSV-G-HIV-1, suggesting that PLC is required at a post-fusion step in the virus life cycle (73).

In summary, chapter 3 provides evidence that HIV-1 Env interaction with CCR5 stimulates a signaling cascade involving Gαq, PLC, PKC, intracellular Ca\(^{2+}\) release, Pyk2 and Ras that allows activation of Rac and subsequent actin cytoskeleton rearrangements necessary for fusion. These results confirm and extend the implication of Rac in HIV-1 infection, and elucidate new potential target molecules of HIV-1 inhibitory drugs.

The data presented in chapter 4 builds upon this pathway and demonstrates the role of the Rac GEF Tiam-1, the src tyrosine kinase Abl, and the Wave2 complex in mediating Env-induced reorganization of the actin cytoskeleton necessary for membrane fusion. This chapter also demonstrates that Abl kinase is required for membrane fusion after the hemifusion step, supporting the hypothesis that the virus hijacks cellular machinery to facilitate pore formation and enlargement (7). In this study we used siRNA to downregulate expression of the Rac GEFs Tiam-1 and Trio. We also used siRNA to downregulate expression of the src tyrosine kinase Abl. Abl has been shown previously
to modulate the actin cytoskeleton both upstream and downstream of Rac (157, 168-170, 192-199). Env-mediated cell fusion and HIV-1 infection of TZM-BL cells was attenuated in cells expressing siRNA targeted to Tiam-1 and Abl but not in cells expressing siRNA targeted to Trio, despite a 70% reduction in protein levels of Trio. Expression of RacV12 in cells completely overcame the effect of Tiam-1 directed siRNA, but only partially recovered levels of fusion in cells expressing Abl directed siRNA. These results support the idea that Tiam-1 is required upstream of Rac to facilitate its activation and Abl is functioning upstream and downstream of Rac in Env-mediated cell fusion. As mentioned previously, different GEFs are induced by specific signaling components and act not only as activators of Rac but also control the effectors activated. GEFs participate in subsequent signaling by directing localization of the activated Rac GDP to GTP exchange and by interacting in scaffold complexes that organize specific downstream signaling mediators (27, 28, 31, 42, 44, 144, 150).

These results demonstrate a role for Tiam-1 and Abl, but not Trio, in Env-mediated Rac activation and membrane fusion suggesting that the downstream effector of Rac is the Arp2/3 actin nucleating complex. Arp2/3 is activated downstream of Rac by the Wave2 complex which includes five main proteins, Wave2, Sra-1, Nap-1, Abi2, and HSP C300. The tyrosine kinase Abl has been shown to associate with the Wave2 complex via the Abi2 protein and and has been shown to phosphorylate Wave2 enhancing the ability of Wave2 to activate the Arp2/3 complex. In addition, the adaptor protein IRSp53 has been shown to interact with Tiam-1, and this interaction links Rac to Wave2 mediated Arp2/3 activation (150). In this study we observed a decrease in Env-mediated fusion and HIV-1 infection with cells expressing Irsp53, Wave2 and Arp3.
directed siRNA. This decrease was not overcome by expression of RacV12, demonstrating a role for these proteins downstream of Rac in Env-mediated fusion. However expression of siRNA resistant Arp3 did overcome the decrease in fusion in cells expressing siRNA to Arp3 but not siRNA to Rac, suggesting that the effects observed with RNAi were specific. The decrease in fusion and infection observed with siRNA targeted to Abl, Tiam-1, IRSp53, Wave2 and Arp3 directly correlated with the decrease in protein expression as seen by immunoblot.

Since treatment of cells with Abl targeted siRNA attenuated Env-dependent fusion and HIV-1 infection, we next sought to determine the effects of commercially available Abl kinase inhibitors, IMB, NIL and DAS, on fusion and infection. The inhibitor concentrations employed did not alter levels of CD4 or CCR5 expression or localization on the surface of cells nor did they result in nonspecific effects on vaccinia virus infection or T7 polymerase activity. These inhibitors were used in multiple assays including Env-dependent cell fusion with cell lines and primary cells as the target, virus-dependent cell fusion, Infection of TZM-BL cells with both R5 and X4 virus and Rac activation assays. The Abl kinase inhibitors completely abolished fusion and infection mediated by both R5 and X4 Env. Env-mediated Rac activation and virus dependent cell fusion were similarly abolished. Expression of RacV12 partially overcame the inhibitor effects on Env-mediated fusion similar to the effects of RacV12 expression seen in cells expressing Abl targeted siRNA. Treatment of cells expressing drug resistant clones of Bcr-Abl with Abl kinase inhibitors had no effect on Env-mediated fusion suggesting that the effects of these drugs on fusion were specific to inhibition of Abl. The inhibition of infection of TZM-BL cells expressing Abl, Tiam-1, IRSp53, Wave2 and Arp3 directed
siRNA or treated with Abl kinase inhibitors was overcome when HIV-1 virions were pseudotyped with VSV-G or A-MLV Env. These results suggest that these signaling mediators are necessary for HIV-1 Env mediated fusion and entry but are not necessary for clathrin or caveloa-mediated endocytosis, and they are not required at post-entry steps in the virus life cycle. An Abl kinase assay was also performed and demonstrated that incubation of target cells with HIV-1 Env-expressing cells resulted in a 2 fold increase in Abl kinase activity (measured by an increase in phosphorylated CrkL, a downstream target of active Abl kinase) and Abl kinase activity is inhibited by IMB, NIL, and DAS.

To determine what step in the fusion process is dependent on Abl kinase-mediated actin cytoskeleton rearrangements we used the membrane-permeable, cationic amphipaths, CPZ, DB, and TFP that induce positive spontaneous curvature of the plasma membrane, which in turn disrupts the hemifusion intermediate and promotes pore formation (176, 200, 201). We also tested the effect of the exogenous cone shaped lipid OLA that promotes negative spontaneous curvature of the membrane and lipid mixing (165, 202). Brief exposure of Abl kinase inhibitor treated cells to CPZ, DB, and TFP 30 min after incubation with virus increased fusion by ten to fifteen fold above the fusion observed with inhibitor treated cells not treated with membrane curving agents. Brief exposure of TAK-779 treated cells to CPZ, DB, and TFP did not lead to an increase in fusion as was expected. Infection of TZM-BL cells was similarly increased for Abl kinase inhibitor treated cells upon exposure to CPZ and TFP. OLA did not increase virus-dependent membrane fusion or infection of HIV-1 in cells treated with Abl kinase inhibitors. No increase was observed in cells lacking exposure to virus, or in CCR5 cells exposed to X4 virus, indicating that the increase in fusion and infection was dependent on
Env. The block was not overcome by the exogenous lipid OLA, which promotes lipid mixing and hemifusion suggesting that the inhibitor treated cells are not arrested prior to hemifusion. Another assay utilizing confocal microscopy demonstrated that addition of IMB to the fusion reaction allowed lipid but not cytoplasmic mixing, confirming that inhibition of Abl kinase arrests fusion at the hemifusion stage and that Abl kinases are required for pore formation.

In conclusion the data from chapters 2, 3, and 4 support the role of Env-mediated signaling to the actin cytoskeleton in membrane fusion and virus entry. Chapter 2 confirmed the requirement for Rac activation in membrane fusion and gave clues to the Rac GEF mediating Env-dependent Rac activation (45). Chapter 3 demonstrated that the signaling pathway activated by Env binding to coreceptor required for Rac activation and membrane fusion was the \( \alpha_q \) pathway. This pathway involves multiple signaling components including PLC\( \beta \), PKC, pyk2 and Ras. Chapter 4 established a role for the Rac GEF Tiam-1 and Abl in Env-dependent Rac activation and membrane fusion and showed that signaling from Rac to the actin cytoskeleton is dependent on Abl, IRSp53, Wave2 and Arp3. Data present in chapter 4 also suggested that Abl kinase-mediated actin cytoskeleton rearrangements are required at a post-hemifusion step in the fusion process and that HIV-1 membrane fusion and infection can be inhibited by IMB, NIL and DAS.

**Future Directions**

The Rac-Wave2-Arp2/3 pathway described in Chapter 4 mediates actin polymerization, however actin rearrangement is a dynamic process that not only requires actin nucleation but also requires release of monomeric actin, and exposure of barbed
ends. In addition to activating Arp2/3, Rac has been shown to regulate actin dynamics by activating ADF/cofilin to accelerate actin turnover, and by regulating proteins such as gelsolin that promote the uncapping of actin at filament ends, leading to exposure of barbed ends (30, 35). The signaling mediators necessary for release of capping proteins and actin monomers in Env-mediated actin cytoskeletal rearrangements are unknown, and could provide additional targets for therapeutic intervention.

A recent study demonstrated a requirement for phosphatidylinositol 4-phosphate 5-kinase (PI4P5-K) activity, which mediates production of phosphatidylinositol (4,5)-bisphosphate (PIP$_2$), in HIV-1 membrane fusion and entry (203). The production of PIP$_2$ is triggered by Env-CD4 interactions and localizes at sites of Env-target cell contact along with F-actin. PI4P5-K is a downstream effector of Rac where it mediates focal complex assembly and is involved in release of actin capping proteins from barbed ends of actin filaments. PI4P5-K is also a downstream effector of Rho and is involved in focal adhesion assembly (30). Another protein implicated in cell motility and Rac-mediated actin structures is profilin. When actin barbed ends are free to polymerize, profilin promotes actin filament dynamics by catalyzing nucleotide exchange on actin from ADP-to ATP, thereby contributing to actin polymerization at the leading edge of lamellipodia (204). A role for profilin in HIV-1 cell fusion has recently been demonstrated in macrophages (204). Another protein that regulates actin cytoskeletal dynamics downstream of Rho GTPases is actin depolymerizing factor ADF/cofilin. ADF/cofilins bind to actin-GDP at the ends of aged filaments and promote actin monomer dissociation. ADF/cofilin can also bind to actin monomers and prevent GDP to GTP exchange. The role of ADF/cofilin in HIV-1 entry is slightly ambiguous since some studies suggest that...
it is phosphorylated and inactivated by Env-mediated ROCK activation (11), and other studies suggest that ADF/cofilin is dephosphorylated and activated by Env signaling through CXCR4 (205). The role of various downstream effectors of Rho and Rac that mediate actin turnover and uncapping of barbed ends in Env-dependent fusion should be tested with available siRNA and small molecule inhibitors.

Future studies should also address the role of CD4 in Env-induced actin cytoskeleton rearrangements necessary for fusion. It is clear based on many published articles that gp120 mediates actin-dependent recruitment of CD4 and CXCR4 to sites of gp120 binding (10, 11, 25, 77, 104, 206, 207). However it is unclear whether Env signaling through CD4 alone or signaling through CD4 and coreceptor is required for Env, CD4 and coreceptor clustering. In addition the mechanism of actin-mediated clustering of receptors is unclear. Some studies have shown a requirement for CD4 mediated activation of Ezrin-radixin-moesin (ERM) proteins that is not dependent on the Rho effector ROCK or PKC (77). However, Rho could still play a role in ERM activation through PIP$_2$ and another unknown mediator (27). Other studies have shown a role for CD4 and coreceptor-mediated filamin-A interactions in receptor clustering that is dependent on RhoA and ROCK mediated phosphorylation of ADF/cofilin (11).

In a previous study our lab demonstrated that expression of DN Rac completely attenuated Env-dependent fusion whereas expression of DN RhoA decreased fusion by 40% (18). Based on results showing a role for Rho activation in HIV-1 entry (11, 12, 33), I hypothesize that CD4-mediated RhoA activation is responsible for Env-CD4-coreceptor clustering required for binding, and subsequent signaling through coreceptor to Rac is necessary for pore formation and enlargement. (35). Rac and Rho have been shown to
actively antagonize one another and mediate different processes (27, 36, 37). The C terminus of gp41 interacts with and inhibits p115 Rho GEF, suggesting that Rho is turned off and unnecessary for fusion events after gp41 insertion (38). Therefore it seems likely that Rho is only involved in the initial steps of Env-induced fusion prior to hemifusion and is subsequently inactivated by Rac and/or the C terminus of gp41.

On the other hand, Env-mediated signaling to Rac and the actin cytoskeleton could occur through CD4 or coreceptor or both resulting in enhanced signaling (43, 49, 64, 89). No studies have been performed in cells expressing mutant CD4 and mutant coreceptor incapable of signaling. A signaling pathway similar to the Ga\(_q\) pathway is elicited by activation of CD4 suggesting that signaling necessary for fusion could occur through either receptor. The presence of CD4 could also play role in determining the signaling pathway elicited and the amount of activation (101, 102).

We are currently testing these hypotheses by expressing multiple CD4 mutants incapable of signaling in target cells and performing assays with lipid analogs and the hemifusion assay described in chapter 4.

**Preliminary Results:**

U87 cell lines expressing CCR5.GFP alone (U87 CCR5) and U87 cell lines expressing CCR5.GFP and tailless CD4 (U87 CD4.401 CCR5) have been established. U87 cell lines expressing CD4 mutants that cannot bind to filamin A or p56lck are being established. The cell lines expressing CCR5 alone were used in an Env-dependent cell fusion assay where they were infected with a vaccinia virus expressing CD4. These cells showed similar amounts of fusion to U87 cells expressing wt CD4 and wt CCR5.GFP (wt
cells). In addition the surface expression of CCR5 was determined to be equal to expression in wt cells via FACS. When this cell line was used in a Rac activation assay a significant decrease in the amount of Rac activation was observed in comparison to wt cells. These results suggest that CD4 is required for total Env-induced Rac activation. Based on these experiments it is still unknown whether CD4 is only required to induce the conformational change required for efficient binding to CCR5 or whether its cytoplasmic domain is involved in the signaling pathway leading to optimal Rac activation.

To address this question U87 CD4.401.CCR5 cells were engineered and sorted for surface expression of CD4 and CCR5 that is similar to wt cells. These cells as well as U87.CD4.CCR5, U87.CCR5 and U87.CD4 cells were studied in a quantitative Env- and virus-dependent cell fusion assay and Rac activation assay. Although these cells express similar levels of receptor at the surface, cell fusion and Rac activation with U87.CD4.401 CCR5 cells were reduced by 61 ± 7% and 64 ± 2% respectively compared to cells expressing wt CD4 and CCR5 (Fig. 2).
Fig. 2. Deletion of CD4 C-terminus attenuates Env-dependent fusion and Rac activation and virus-dependent fusion. (A) Average fusion compared to untreated control reactions and detected by β-galactosidase activity ± standard deviation are shown. U87.CD4.CCR5, U87.CD4, U87.CCR5 and U87.CD4.401.CCR5 were infected with vCB21R overnight then incubated with HIV$_{UNC}$ (subtracted as background) HIV$_{ADA}$, HIV$_{YU2}$, HIV$_{89.6}$ or HIV$_{HXB2}$ Env-expressing cells for 3h. Data are representative of results from three similar experiments performed in triplicate. Cell fusion was normalized using U87.CD4.CCR5 cells incubated with HIV$_{ADA}$ Env as 100%. (B) Cells were incubated for 30 min with BSC40 cells expressing no Env (subtracted as background), or HIV$_{ADA}$ Env. Whole cell lysates were then analyzed by Rac specific G-LISA activation assay. Average A$_{490}$ of duplicate wells ± standard deviation; data are representative of results from three similar experiments. (C) One population of U87.CD4.CCR5 or U87.CD4.401.CCR5 cells was infected with vCB21R and the other population of cells was infected with vPT7-3 in complete media overnight. These cells were then mixed (1:1) in triplicate wells of 96 well plate and incubated with 75, 100, and 150ng of HIV$_{YU2}$ per well for 3h at 37°C. Data are representative of results from three similar experiments (*, P < 0.01). Cell fusion was normalized using U87.CD4.CCR5 cells mixed with 150ng HIV$_{YU2}$ as 100%.
A

Env-Dependent Fusion Assay

Percent fusion

CD4.R5  CD4  R5  CD4.401.R5

YU2  (89.6 Dual)  HXB2 (X4)

B

Rac Activation Assay

A490

CD4 R5  CD4  R5  CD4.401 R5

C

Virus-Dependent Fusion Assay

Percent fusion

CD4 R5  CD4.401 R5

p24 (ng/ml)  75  100  150
Future studies will include Env- and virus-dependent fusion assays, and Rac and Rho activation assays with mutant CD4 cell lines. Kinetic assays and temperature assays will also be performed to determine whether the observed attenuation in fusion is at the binding step and if allowing increasing amounts of time for binding before raising the temperature will increase the levels of fusion with these mutants. Mutant CD4 and CCR5 localization should also be determined before and after treatment with Env to see if disrupting signaling through CD4 is effecting the recruitment of coreceptor to the site of Env-CD4 complexes. This can be done by confocal microscopy analysis and by co-immunoprecipitation assays with antibodies to CD4, coreceptor, and gp120 (Env) (208). Cells will also be treated with the small molecule inhibitors damnacanthal (blocks lck activity) and piceatannol (blocks syk kinase ZAP-70) and the effects on Env-dependent fusion will be measured (205). We will also test Env-dependent fusion with Jurkat cells, which like T cells have Lck, or Jurkat derivatives deficient in Lck, ZAP70, or LAT. Finally, assays to determine the fusion steps dependent on CD4 will be performed, using mutants and inhibitor treated cells in hemifusion assays with lipid analogs and with CHO-K1 cells.

**Therapeutic Implications and Conclusions**

As mentioned previously the current anti-retroviral therapy, HAART, has important limitations, including rapid virus replication rebound after withdrawal, the increasing emergence and transmission of multi-resistant viral strains, difficulties in patient compliance, severe side effects and high cost that prevents delivery to undeveloped countries (2, 57). These considerations underscore the need for further
progress in drug discovery. Blocking HIV entry, versus blocking other steps in the virus
life cycle is an attractive strategy since this would lock HIV outside the cell and thereby
prevent the virus from replenishing its latent cellular reservoirs, the last hurdle in HIV
eradication (57). However there are caveats to targeting the Env glycoprotein, as it one of
the most variable HIV proteins, making evolution of drug resistance likely.

Drugs that antagonize CD4, CXCR4, or CCR5 are also risky because they could
interfere with their normal immune signaling functions in the cell. In addition, use of
inhibitors that block both CCR5 and CXCR4 would be necessary to prevent promiscuous
coreceptor usage. Although CCR5 and CXCR4 are the only chemokine receptors thought
to be targeted by HIV in vivo, multiple other chemokine receptors have been shown to be
functional coreceptors in vitro, suggesting that under selective pressure HIV-1 could
evolve to use other chemokine receptors (50). An additional concern with the use of
coreceptor inhibitors is the risk of interfering with the physiology of other GPCRs which
are widespread. Cross reaction of inhibitors with other GPCRs would cause side effects
unrelated to interference with the chemokine system (57).

The discovery of a role for Abl kinase in HIV-1 Env-mediated entry is a
particularly encouraging result as the development and clinical application of IMB, DAS,
and NIL that block deregulated Bcr-Abl in chronic myeloid leukemia patients clearly
shows that inhibition of Abl kinases is possible in vivo with clinically manageable side
effects (160, 171, 193). The next step will be to test IMB (Gleevec) in individuals
infected with HIV-1 that have failed HAART therapy. Patients should be treated and the
effect of the drug should be monitored by viral loads. In addition viral samples should be
taken to monitor resistance of virus to IMB. PBMC and CD4+ T cells should also be monitored for virus induced-changes in susceptibility to infection in the presence of IMB.

Studies should also been done prior to patient treatment to test for emergence of drug resistance. PBMCs should be infected with R5 and X4 viruses in the presence of 3 different concentrations of IMB, for example 0.1uM, 1uM, and 10uM. These cells should be cultured for several weeks and used in entry and infection assays in the presence of IMB to test for new susceptibility to infection and fusion. Virus will be retitered on MAGI-5 cells in the presence or absence of IMB to test for resistance.

The Env-mediated signaling pathway to Rac contains multiple factors that could be potential drug targets. There are multiple PKC inhibitors in clinical trials or approved for clinical use to treat cancer and diabetes (153). Studies with the Ras inhibitor FTS in mice have given encouraging results for the treatment of various types of cancer, neurofibromatosis, and kidney disease (154-156). In addition, FTS is currently in phase I clinical trials for hematologic malignancies (155). The ability of these inhibitors and the Abl kinase inhibitors to specifically down-regulate their target molecules without adverse side effects suggests that these inhibitors might be appropriate drugs for treatment of HIV-1 and other infectious microbes that manipulate this pathway. In addition, determining the other signaling effectors of Rho and Rac that mediate Env-dependent fusion could provide additional targets for therapeutic intervention. This strategy of using inhibitors that disable host signaling proteins rather than viral proteins, essential for pathogen survival, may have a general efficacy in developing drugs to combat HIV-1 and other pathogens that acquire drug resistance.
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