Induction of Phosphorylation and Intracellular Association of CC Chemokine Receptor 5 and Focal Adhesion Kinase in Primary Human CD4+ T Cells by Macrophage-Tropic HIV Envelope

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Induction of Phosphorylation and Intracellular Association of CC Chemokine Receptor 5 and Focal Adhesion Kinase in Primary Human CD4⁺ T Cells by Macrophage-Tropic HIV Envelope

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Binding of HIV-1 envelope glycoproteins to the surface of a CD4⁺ cell transduces intracellular signals through the primary envelope receptor, CD4, and/or the envelope coreceptor, a seven-transmembrane chemokine receptor. Macrophage-tropic strains of HIV-1 preferentially use CCR5 as an entry coreceptor, whereas T cell-tropic strains use CXC chemokine receptor-4 for entry. Intracellular signals transduced by HIV-1 envelope may have immunopathogenic consequences, including anergy, syncytium formation, apoptosis, and inappropriate cell trafficking. We demonstrate here that a recombinant envelope protein derived from an M-tropic isolate of HIV-1 can transduce CD4-dependent as well as CCR5-dependent intracellular signals in primary human CD4⁺ T cells. Novel HIV-induced intracellular signals that were identified include tyrosine phosphorylation of focal adhesion kinase (FAK) and CCR5, which are involved in cell adhesion and chemotaxis, respectively. HIV envelope-induced cellular association of FAK and CCR5 was also demonstrated, suggesting that ligation of CD4 and CCR5 leads to the formation of an activation complex composed of FAK and CCR5. Activation of this signaling pathway by HIV-1 envelope may be an important pathogenic mechanism of dysregulated cellular activation and trafficking during HIV infection. The Journal of Immunology, 1999, 163: 420–426.

The relatively small fraction of T cells infected with HIV in seropositive subjects argues against direct infection as the sole mediator of the immunopathogenesis of HIV infection (9). A role for HIV envelope in the immunopathogenesis of HIV disease has been suggested by a number of studies (reviewed in Ref. 10). The presence of measurable levels of circulating soluble gp120 in HIV-infected subjects (11, 12) and the accumulation of high concentrations of virions (both infectious and defective) in lymphoid tissue (13–15) underscore the potential for envelope to contribute to T cell dysfunction in a process distinct from infection of CD4⁺ T cells.

The discovery of CD4 as the major receptor for HIV entry prompted Ascher and Sheppard (16) to hypothesize that intracellular signals that are transduced by the HIV envelope binding to CD4 might be important mediators of HIV-induced immune dysfunction. The more recent discovery that certain chemokine receptors function as coreceptors that are necessary for HIV entry has highlighted the possibility that intracellular signals that are transduced by ligation of chemokine receptors may also be involved in the immunopathogenesis of HIV infection (17, 18). Ligation of chemokine receptors by RANTES results in phosphorylation of focal adhesion kinase (FAK) (19), a 125-kDa protein that is also involved in signal transduction following TCR engagement (20); FAK is also a critical mediator of signal transduction from extracellular matrix proteins through cell surface integrin receptors (reviewed in Ref. 21). Recruitment of FAK by integrin receptors leads to autophosphorylation of FAK followed by its association with cytoskeletal proteins such as paxillin in focal adhesion complexes. Phosphorylation of proteins in these focal adhesion complexes by FAK appears to be an important signal responsible for the regulation of cellular locomotion and adhesion.

Intracellular signals transduced by HIV-1 envelope have been implicated in a number of immunopathogenic processes (reviewed in Ref. 22). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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5 Abbreviations used in this paper: M-tropic, macrophage-tropic; CXCR4, CXC chemokine receptor 4; T-tropic, T cell-tropic; FAK, focal adhesion kinase; sCD4, soluble CD4; TBS-T, Tris-buffered saline containing 0.1% Tween-20.
in Ref. 10) including anergy (22–25), syncytium formation (26), apoptosis (27), and inappropriate cell trafficking (18, 27). Delineation of intracellular signaling pathways that are transduced by HIV envelope glycoproteins and determination of the dependence of these pathways on CD4 vs chemokine receptor ligation should provide important insights into mechanisms of HIV-mediated immunomodulation and may suggest new avenues for therapeutic intervention.

Materials and Methods

Cells and reagents

Cells were used from normal donors who were confirmed to be CCR5 wild-type homozygotes by PCR as previously described (28). Cells obtained by leukapheresis from these individuals were subjected to Ficol-Hypaque centrifugation, followed by rosetting with neuraminidase (Sigma, St. Louis, MO)-treated SRBC. The rosepositive T cell-enriched fraction was incubated with mouse Abs to CD8 (PharMingen, San Diego, CA), CD14 (PharMingen), CD16 (PharMingen), CD19 (PharMingen), and CD56 (PharMingen), followed by selection with magnetic beads coated with goat anti-mouse IgG (PerSeptive Biosystems, Watertown, MA). The remaining cells were >98% CD3 CD4 by FACS analysis. CD4 T cells were cultured in RPMI 1640 (Life Technologies, Grand Island, NY) containing 10% heat-inactivated FBS (Life Technologies). Additions to the medium were cultured in RPMI 1640 (Life Technologies, Grand Island, NY) containing 1% Triton X-100, 1 mM PMSF, 10 mM sodium fluoride. The protein concentration of the lysates was determined using a colorimetric assay according to the manufacturer’s instructions (Bio-Rad, Hercules, CA). Protein (500 μg) was immunoprecipitated from the R5 HIV-1 strain JR-FL (6) or 25 ng/ml anti-CD4 Ab (Leu 3A, Becton Dickinson Immunocytometry Systems, San Jose, CA). Cross-linking was achieved with anti-gp41 Ab (clone 3D6) (6) conjugated to magnetic tosyl-activated Dynabeads (Dynal, Lake Success, NY) or goat anti-mouse Ab-coated Dynabeads (Dynal) at a ratio of three beads per cell. Untreated controls were exposed to secondary Abs conjugated to Dynabeads alone.

Abs used for immunoprecipitation and/or Western blot analysis were used according to the manufacturer’s recommendation and included antiphosphotyrosine (clone 4G10, Upstate Biotechnology, Lake Placid, NY), anti-Lck (clone 28, Transduction Laboratories, Lexington, KY), anti-ZAP-70 (clone 29, Transduction Laboratories), anti-psyk2 (clone 11, Transduction Laboratories), anti-CCR5 (clone 2D7, PharMingen; or C903, Santa Cruz Biotechnology). Anti-V3 loop Ab (clone F19B) (29) was used to interface with the interaction between HIV-1 envelope and its coreceptor (CCR5); pertussis toxin (List Biological Laboratories; or C903, Santa Cruz Biotechnology). Anti-CD4 Ab alone, and an autofluorescence control. Microscopy was performed using a Zeiss LSM 410 scanning laser confocal microscopy system (Carl Zeiss, Thornwood, NY) built around a Zeiss 135 Axiovert inverted microscope fitted with an Omnicron argon/krypton dual gas laser set to emit at 488, 568, and 647 nm.

Results

Homotypic aggregation of CD4 T cells induced by HIV envelope

Primary CD4 T cells were purified from normal CCR5 wild-type donors and cross-linked with recombinant oligomeric transmembrane-deleted HIV-1 R5 strain (JR-FL) gp120/41 envelope (6). Cross-linking was enhanced by the addition of magnetic beads bearing 3D6 anti-gp41 Ab (6). Homotypic aggregation of the CD4 T cells was observed as early as 10 min following HIV envelope cross-linking and was inhibited in the presence of sCD4 (data not shown). These results were similar to those reported for the effects of the CC chemokine RANTES on T cells (19).

In vitro protein kinase assay

Lysates from CD4 T cells treated with HIV-1 envelope or control supernatant were immunoprecipitated with anti-Flag Ab conjugated to protein A agarose. Immunoprecipitates were washed and resuspended in kinase buffer (20 mM HEPES (pH 7.5), 10 mM MgCl2, 1 mM DTT, 1 mM sodium orthovanadate, and 25 mM β-glycerophosphate) and incubated for 30 min at 30°C with 10 μCi [γ-32P]ATP (6000 Ci/mmol; Amerham) and 1 μM unlabeled ATP. Laemmli buffer was then added, and SDS-PAGE was performed as described above.

CD4-dependent tyrosine phosphorylation of proteins induced by HIV envelope

Lysates from HIV envelope-treated CD4 T cells were subjected to immunoprecipitation and immunoblotting with antiphosphotyrosine Ab. Tyrosine phosphorylation of several proteins ranging between 30–200 kDa was observed as early as 5 min following HIV envelope treatment (Fig. 1). The pattern of protein phosphorylation was similar to that induced by RANTES (19) and by CD4 cross-linking (Fig. 1). Proteins were identified by immunoblotting with specific mAbs after antiphosphotyrosine immunoprecipitation (Fig. 2). Cross-linking CD4 T cells with either HIV envelope or anti-CD4 Abs led to phosphorylation of psyk2, paxillin, Lck, and a protein of approximately 35 kDa; this latter protein was identified as CCR5. The specificity of the anti-CCR5 Abs used in these Western blots was confirmed in control experiments using cell lines transfected with CCR5 (Materials and Methods). The HIV envelope-induced phosphorylation of the above-mentioned proteins was strongly inhibited by sCD4 (Fig. 3). At later time points (20–90 min) following HIV envelope cross-linking, a band appeared, indicating tyrosine phosphorylation of a protein of approximately 125 kDa. This protein was identified by immunoblotting as FAK (Fig. 3). Considerable donor-to-donor variability was seen with regard to the kinetics and magnitude of FAK phosphorylation. The HIV envelope-induced phosphorylation of FAK also appeared to be a CD4-dependent signal, since it was inhibited in the presence of sCD4 (Fig. 3). In addition, the HIV envelope-induced phosphorylation of FAK and CCR5 was not inhibited by pertussis toxin, which interferes with signaling through Gs proteins (data not shown).

Identification of CCR5 by Western blot

293 cells (American Type Culture Collection, Manassas, VA) were transfected with the pcDNA3 plasmid (Invitrogen, Carlsbad, CA) expressing CCR5 or empty pcDNA3. Cells subjected to SDS-PAGE Western blot was performed with C20 Ab. 293 cells transfected with CCR5/FLAG were subjected to immunoprecipitation with anti-FLAG Abs (Eastman Kodak) followed by Western blot with C20 Ab.
CCR5-dependent signals transduced by HIV envelope

As noted, the pattern of protein phosphorylation induced by HIV-1 envelope was similar to that induced by CD4 cross-linking (Fig. 1), suggesting that signals transduced by HIV envelope are largely CD4 dependent (22, 25). Phosphorylation of ZAP70, however, was induced by treatment with HIV envelope, but not with CD4 Abs (Fig. 2), suggesting that this activation event was primarily CCR5 dependent (19). The inhibition of ZAP70 phosphorylation by sCD4 (Fig. 3) suggests that although this activation signal is transmitted primarily through CCR5, CD4 appears to play an important role in allowing HIV envelope to bind to CCR5 in this process (4–8). In addition to the apparent CCR5-dependent phosphorylation of ZAP70, other evidence suggested that a subset of the phosphorylation events induced by HIV envelope were chemokine receptor dependent. For example, we used a mAb directed against the V3 loop of M-tropic HIV envelope, which is a critical determinant of interaction with CCR5 (30, 31); this Ab inhibits entry of R5 strains of HIV into T cells (32) and abrogates the determinant of interaction with CCR5 (30, 31); this Ab inhibits against the V3 loop of M-tropic HIV envelope, which is a critical mokine receptor dependent. For example, we used a mAb directed the phosphorylation events induced by HIV envelope were che-

Redistribution of FAK to focal adhesion complexes induced by HIV envelope

We used confocal microscopy to visualize FAK in orthogonal sections at serial time points to determine its cellular localization following treatment of CD4+ T cells with HIV envelope. FAK was initially visualized only along the basal cellular axis, at points of contact with the laminin substratum (Fig. 4). Concentration of FAK in medial sections of the cells was seen 15 min following envelope treatment (Fig. 4); by 60 min after envelope treatment, FAK was visualized in a rim-like pattern in medial sections (Fig. 4), and it was heavily concentrated in patches in apical sections (i.e., sections in contact with the anti-envelope Ab-coated cover-slip where envelope cross-linking occurred), consistent with the formation of focal adhesion complexes. Soluble CD4 prevented these envelope-mediated changes in FAK localization (Fig. 4). These data indicate that CD4-dependent HIV envelope-mediated signaling events induce changes in the intracellular localization of FAK and association of FAK with focal adhesion complexes.

Association of FAK and CCR5 following HIV envelope treatment

We performed an in vitro kinase assay in which lysates from CD4+ T cells treated with HIV-1 envelope were immunoprecipitated with anti-FAK Ab, followed by 32P incorporation into kinase substrates (Fig. 5a). A faint band was visualized at 125 kDa, consistent with phosphorylation of FAK itself. Among other substrates, a 35-kDa protein coimmunoprecipitated with FAK; this latter band corresponded to CCR5, as shown by immunoblotting (Fig. 5b), confirming a physical association between phosphorylated FAK and CCR5. Treatment with sCD4 prevented the envelope-induced phosphorylation and association of these proteins (Fig. 5, a and b). Further evidence supporting the HIV envelope-induced association between CCR5 and FAK was obtained by identifying FAK by Western blot following immunoprecipitation of HIV envelope-treated CD4+ T cells lysates with anti-CCR5 Abs (Fig. 5c); tyrosine phosphorylation of FAK that coimmunoprecipitated with CCR5 was demonstrated by antiphosphotyrosine immunoblotting (data not shown). Finally, we used confocal microscopy to demonstrate that HIV envelope treatment of CD4+ T cells results in an activation complex consisting of FAK and CCR5. In
unstimulated conditions, CCR5 and FAK were not associated; however, following HIV envelope treatment, significant colocalization of CCR5 and FAK was observed (Fig. 6).

Discussion

We have identified novel intracellular signaling events in primary human CD4+ T cells that are initiated by HIV envelope-mediated ligation of CD4 and CCR5; these signaling events included, among others, phosphorylation and intracellular association of CCR5 and FAK in focal adhesion complexes. HIV envelope glycoproteins are known to induce a number of biological responses in primary T cells, including induction of the secretion of proinflammatory cytokines and enhancement of apoptosis (reviewed in Ref. 10). The underlying basis for these responses was presumed to involve the interaction of the HIV envelope with the CD4 molecule, but was otherwise poorly understood. Upon the discovery of CCR5 as a primary coreceptor for HIV, our laboratory and others demonstrated that the HIV envelope can transduce intracellular signals through CCR5 in a manner analogous to that of β-chemokines (17, 18). The signaling events transduced by the interaction of HIV envelope with CCR5 induced chemotaxis of CD4+ T cells, raising the possibility that such a response may promote the recruitment of uninfected cells to sites of active viral replication (18). Clearly, HIV envelope can induce a wide range of functional responses in CD4+ T cells; identification of these intracellular signaling pathways should elucidate the molecular mechanisms responsible for envelope-induced immunopathogenic events.

Most of the envelope-induced phosphorylation events that we observed were CD4 dependent. Of note, phosphorylation of ZAP70 and CCR5 appeared to be at least partially dependent on signaling through CCR5; however, even these phosphorylation events were inhibitable by sCD4, suggesting a critical role for CD4 in allowing the HIV envelope to interact efficiently with CCR5 in the initiation of signal transduction (4–8). Our data are consistent with a model in which HIV envelope signaling through CD4 results in the phosphorylation and association of proteins, including CCR5 and FAK. As previously reported, the interaction of the HIV

FIGURE 3. Inhibition of HIV envelope-induced phosphorylation of multiple substrates by sCD4.

FIGURE 4. Redistribution of FAK to focal adhesion complexes following treatment of CD4+ T cells with HIV envelope, as demonstrated by immunohistochemistry and confocal microscopy. Concentration of FAK in envelope-treated cells was pronounced in apical sections of the cells, where HIV envelope cross-linking occurred; this process was inhibited by sCD4.
envelope with CD4 results in a conformational change within the envelope, which then allows it to interact with CCR5 (4–8).

The experimental conditions that were employed were chosen to recapitulate the physiologic environment in which CD4+ T cells may encounter HIV envelope glycoproteins in vivo. In this regard, oligomeric complexes of the HIV envelope are representative of the envelope structure in vivo (34, 35), and cross-linking might occur due to the high density of envelope glycoproteins associated with accumulation of virions in lymphoid tissue (14) (i.e., virions entrapped in the follicular dendritic cell network) or on the surface of an infected cell.

Some of the envelope-induced activation events that we observed confirm previous observations, including phosphorylation of Lck (22), ZAP70 (25), and pyk2 (17). Phosphorylation of CCR2B and CCR5 in response to chemokine ligands has been reported in transformed cell lines (36–39); however, the present study describes CCR5 phosphorylation in primary human CD4+ T cells. The data suggest that phosphorylation of CCR5 following HIV envelope interaction with CD4+ T cells is dependent on signals transduced through CD4 as well as through CCR5 itself. The dependence of this phosphorylation event on CCR5 is suggested by partial inhibition by an anti-V3 loop Ab, which blocks the interaction between the HIV envelope and CCR5. The insensitivity of CCR5 phosphorylation to pertussis toxin was unexpected; however, this result does not exclude the possibility that this intracellular signal may be transduced through a pertussis toxin-insensitive G protein (40). In fact, Bacon et al. have shown that some signals transduced by RANTES are pertussis toxin sensitive, while others are pertussis toxin insensitive (41). Envelope-induced phosphorylation of FAK, ZAP70, and pyk2 was anticipated by the report of β-chemokine-induced phosphorylation of these proteins (19, 42), presumably via signaling through CCR5. In transformed cell lines, envelope-induced phosphorylation of pyk2 was demon-

![Figure 5](http://www.jimmunol.org/covers/5-6424.jpg)

**FIGURE 5.** Association of FAK and CCR5 in an activation complex induced by treatment of CD4+ T cells with HIV envelope. *a*, Immunoprecipitation of cell lysates with anti-FAK Abs followed by incubation of immunoprecipitated proteins in kinase buffer and 32P-labeled ATP revealed HIV envelope-induced phosphorylation of several proteins, including those of approximately 35 (arrow) and 125 kDa. Soluble CD4 inhibited these phosphorylation events. *b*, Immunoblotting with anti-CCR5 Ab revealed that the 35-kDa protein (arrow) that coimmunoprecipitated with FAK was CCR5. Soluble CD4 inhibited the HIV envelope-induced association of CCR5 with FAK. *c*, Anti-FAK immunoblot after immunoprecipitation of HIV envelope-treated CD4+ T cell lysates with anti-CCR5 Ab. Untreated controls were as described in Fig. 1.

![Figure 6](http://www.jimmunol.org/covers/6-6424.jpg)

**FIGURE 6.** HIV envelope-induced colocalization of FAK and CCR5 in CD4+ T cells, as demonstrated by immunohistochemistry and confocal microscopy. In untreated cells no FAK was visible in the plane of focus; thus, no colocalization of FAK and CCR5 was seen. In contrast, HIV envelope-treated cells exhibited abundant expression of CCR5 (green, *lower left panel*) and FAK (red, *lower middle panel*) with colocalization of the two proteins (gold, *lower right panel*).
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strated to be entirely CCR5 dependent (17); our observation that pyk2 phosphorylation by HIV envelope was a CD4 dependent event may reflect our use of freshly isolated primary cells.

The relatively small fraction of T cells found to be HIV infected in HIV-seropositive subjects ex vivo argues against direct infection as the sole mediator of the immunopathogenesis of HIV infection (9). The presence of high concentrations of HIV envelope glycoproteins in anatomic sites that are important with regard to disease pathogenesis (i.e., lymphoid tissue) (13–15) highlights the likelihood of frequent contact between CD4+ T cells and HIV envelope glycoproteins in this setting. In this regard, the potential for envelope to contribute to CD4+ T cell dysfunction in a process distinct from direct infection has been suggested by a number of studies (reviewed in Ref. 10). HIV-envelope-mediated immune dysfunction may result not only from aberrant intracellular signals related to the ability of envelope to serve as a CD4 and chemokine receptor agonist, but also in some instances from the ability of HIV envelope to block the physiologic effects of chemokines by acting as a chemokine receptor antagonist (43).

HIV envelope-induced cellular aggregation and formation of focal adhesion complexes at points of contact with HIV envelope probably result from activation of proteins involved in cell adhesion and chemotaxis and may facilitate the recruitment of uninfected target cells to areas with high concentrations of virus (i.e., lymphoid tissue) (9, 14, 18). The ability of the HIV envelope glycoprotein to recruit uninfected target cells to anatomic sites that are favorable for viral replication is reflected in the dichotomy of viral burden between lymphoid tissue and peripheral blood compartments (9).

Intracellular signals transduced by HIV envelope may further contribute to the immunopathogenesis of HIV infection by priming cells to become optimal targets for infection. In this regard, our observation that HIV envelope can induce the formation of focal adhesion complexes complements a recent report that highlights the importance of the cytoskeleton in facilitating the receptor and membrane conditions necessary for HIV entry (44). Thus, the formation of focal adhesion complexes induced by HIV envelope may be a critical event that facilitates fusion and viral entry; this process may, in turn, enhance the efficiency of early events necessary for viral replication in primary cells. The previous demonstration that HIV coreceptor function and signal transduction are dissociable may be due to the use of transformed cell lines (45–47) in which membrane and cytoskeletal conditions optimal for the early stages of viral replication (i.e., fusion and entry) are constitutively present. It is also noteworthy that phosphorylation of pyk2 and FAK may occur after TCR engagement, suggesting a role for these proteins in T cell activation (20). In this regard, activation of these same proteins by CD4-dependent and CCR5-dependent signaling pathways transduced by HIV envelope may prime some cells for HIV infection, as activated T cells are most susceptible to HIV infection (16, 48). Signaling through CCR5 may not only promote susceptibility to HIV infection, but may also enhance the early stages of HIV replication. Of note, ligation of CCR5 with β-chemokines is known to inhibit cellular entry of R5 strains of HIV by a mechanism that is independent of intracellular signaling; how ever, the intracellular signals that are transduced through CCR5 can enhance replication of X4 strains of HIV in CD4+ T cells (49, 50).

HIV envelope-induced phosphorylation of FAK and pyk2 is also interesting in light of a recent report suggesting that activation of these two related proteins can have dichotomous effects on survival in certain cell types (i.e., induction of apoptosis by pyk2 as opposed to cell attachment and survival mediated by FAK) (51); thus, the differential tendency of these proteins to become phosphorylated in CD4+ T cells in response to a given stimulus may in part determine the fate of the cell upon encountering HIV. Of note, we have observed consistently higher HIV envelope-induced levels of phosphorylated pyk2 compared with FAK, an observation that may in part explain the ability of HIV envelope to induce apoptosis in vitro (27).

The identification of an HIV envelope-induced activation complex consisting of phosphorylated CCR5 and FAK identifies new and potentially important mechanisms of HIV envelope-mediated immunopathogenesis. Subversion by HIV of intracellular signaling pathways that regulate cell adhesion, trafficking, and activation allows envelope glycoproteins to prime potential target cells for HIV infection. Improvements in immune function that are observed following initiation of highly active antiretroviral therapy are thought to result from expansion of CD4+ T cells and possibly partial restoration of a disrupted TCR repertoire (52, 53); however, it seems likely that at least part of this immune reconstitution is due to the decreased availability of HIV envelope glycoproteins to transduce aberrant intracellular signals.

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due to HIV-1 envelope interactions with chemokine receptors CXCR4 or CCR5.


