The Adaptor Protein SLP-76 Regulates HIV-1 Release and Cell-to-Cell Transmission in T Cells

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HIV-1 infection in T cells is regulated by TCR activation. However, the cellular proteins of the TCR pathway that regulate HIV-1 infection are poorly characterized. In this study, in HIV-1 infection, we observed a significant reduction of HIV-1 virus production in Src homology 2 domain-containing leukocyte protein of 76 kDa (SLP-76)–deficient Jurkat T cells compared with wild-type and SLP-76–reconstituted Jurkat T cells. We further confirmed the role of SLP-76 in HIV-1 infection by small interfering RNA-mediated knockdown in MT4 cells and PBMCs. Structural-functional analysis revealed that the N-terminal domain of SLP-76 was important for regulating HIV-1 infection. Further mechanistic studies revealed that lack of SLP-76 impaired virus release, but did not affect viral entry, integration, and transcription. We also showed that SLP-76 plays a critical role in cell-to-cell transmission of HIV-1. Signaling studies revealed that SLP-76 associated with viral negative regulatory factor protein and multiple signaling molecules during HIV-1 infection. Furthermore, SLP-76 facilitated the association of negative regulatory factor and F-actin, suggesting that SLP-76 mediates the formation of a signaling complex that may regulate viral release via cytoskeletal changes. Taken together, our studies demonstrate a novel role for the adaptor molecule SLP-76 in regulating HIV-1 infection in T cells with the potential to develop innovative strategies against HIV-1.

T cell receptor activation has been shown to modulate HIV-1 replication in CD4+ T lymphocytes (1, 2). Quiescent T cells do not support efficient transcription, expression, and integration of the HIV-1 genome (3, 4). TCR-mediated signaling has been shown to regulate both pre- and postintegration steps in the HIV-1 life cycle (1, 2). TCRs mediate signaling through various downstream signaling molecules, including the Src homology 2 (SH2) domain-containing leukocyte protein of 76 kDa (SLP-76). SLP-76 is a key adapter protein that is indispensable for TCR activation and expressed in all hematopoietic lineages except mature B cells (5). SLP-76 is a multifunctional protein and mediates its functions through three modular domains: an amino N-terminal acidic region with three tyrosine phosphorylation motifs, a central proline-rich domain, and a C-terminal SH2 domain (6). The tyrosine-phosphorylated residues on the N terminus of SLP-76 have been shown to associate with the SH2 domain of various proteins including Vav (7, 8), noncatalytic region of tyrosine kinase adaptor protein (Nck) (9), and IL-2–inducible T cell kinase (ITK) (10). Biochemical and genetic evidence has clearly established the importance of SLP-76 in signal transduction downstream of several surface receptors including TCR (11, 12). Furthermore, SLP-76–deficient (SLP-76−/−) mice demonstrate a complete block in thymic development in the absence of peripheral T cells (13, 14). Recent studies have also demonstrated the importance of SLP-76 in actin cytoskeleton remodeling downstream of the TCR (15, 16). Actin cytoskeletal reorganization has been shown to play an important role in HIV-1 entry and release from the target cells (17–19).

Several experimental pieces of evidence indicate that negative regulatory factor (Nef), an HIV-1 protein required for efficient viral replication in vivo and AIDS disease progression, plays an important role in the modulation of TCR signaling (20–22). Nef is an ∼27-kDa myristoylated protein expressed soon after infection that associates with cell membranes and downmodulates an array of cell-surface molecules including CD4, MHC class I/II, CD28, and chemokine receptors (23, 24). In addition, Nef has been shown to interact with various components of the TCR signaling pathway (20–22, 25, 26). Nef has also been shown to modulate actin cytoskeletal remodeling and viral release (17, 19, 20, 25–28).

In the current study, we have shown that SLP-76 plays an important role in HIV-1 infection. Further elucidation of the molecular mechanism revealed that SLP-76 does not interfere with HIV-1 binding, entry, and preintegrative steps, but mediates viral release and spread. Our studies indicate that SLP-76 associates with Nef and multiple signaling molecules that lead to actin reorganization, which in turn may regulate HIV-1 virus release from the cell.

Materials and Methods

Cell culture

MT-4 cells, a human T cell line bearing human T cell leukemia virus type 1, wild-type (WT) Jurkat cells (J6.1 clone; National Institutes of Health AIDS Research and Reference Reagent program), SLP-76–deficient (SLP-76−/−) cell line (J14), and SLP-76–deficient cell line stably reconstituted with SLP-76 (J14/7611, SLP-76+/−) (kindly provided by Gary Koretzky, University of Pennsylvania) were grown in RPMI 1640 medium (Mediatech) supplemented with 10% (v/v) heat-inactivated FCS (Invitrogen), 100 U penicillin/ml, and 100 μg streptomycin/ml. Expression of SLP-76 in reconstituted SLP-76 cells was maintained by using 500 μg G418/ml.
PBMCs were isolated from heparinized venous blood collected from healthy donors (American Red Cross, Columbus, OH). Because only cells are used and are without identifiers, this work is exempt from institutional review board review. The blood was subjected to Ficoll-Paque density gradient centrifugation at 2200 rpm for 35 min. For the primary lymphocyte culture, the cells were suspended in RPMI 1640 containing 10% FCS, 2 mM glutamine, 50 IU/ml penicillin, 50 μg/ml streptomycin, and PHA (5 μg/ml) for 3 d. Cells were then removed and placed in fresh medium supplemented with recombinant human IL-2 (Advanced Biotechnologies, Columbia, MD). The purity of the PBMCs was checked by flow cytometry using CD3 Ab.

Viral isolates
The HIV-1 CXCR4 tropic (HIV-1 IIIB strain) and CCR5 tropic isolates (HIV-1 Bal) used in this study were obtained from the National Institutes of Health AIDS Research and Reference Reagent Program.

HIV-1 infection assay
WT, SLP-76−/−, and SLP-76+/− Jurkat cells were infected with HIV-1 IIIB at 10 ng p24/10⁶ cells. The cells were washed extensively after 2 h of infection to remove the residual virus and incubated for different time points. In the case of small interfering RNA (siRNA) and mutant plasmid transfected cells, the cells were infected at 20 ng p24/10⁶ cells. HIV-1 p24 Ag levels in the culture supernatant were assessed with the HIV-1 p24 core profile ELISA kit (Advanced Biosciences Laboratories).

Abs
Nef Ab and FITC-conjugated p24 Ab was obtained from Millipore (Billerica, MA). PE-conjugated CXCX4, allophycocyanin-conjugated CD4 Abs, and isotype conjugate controls were obtained from BD PharMingen (San Jose, CA). SLP-76, Vav1, NCK1, p21-activated kinase 2 (PAK2), and GAPDH Abs were obtained from Cell Signaling Technology (Danvers, MA). HRP-conjugated anti–HIV-1 p24 Ab was obtained from Advanced Biosciences Laboratories. Alexa Fluor-conjugated secondary Abs and phallidin-conjugated Alexa Fluor 568 was obtained from Invitrogen. Anti-FLAG Ab was obtained from Sigma-Aldrich (St. Louis, MO).

Confocal microscopy
For the colocalization analysis of Nef and F-actin as well as Nef and SLP-76, cells were fixed and permeabilized using the Fix/Perm solution (BD Biosciences, San Jose, CA) for 20 min at 4°C. The cells were washed three times with BD Perm/Wash buffer (BD Biosciences) and blocked with Normal Goat Serum (Invitrogen) for 1 h at 4°C. The cells were then washed with Perm/Wash buffer and incubated with respective primary Abs for 1 h at 4°C. After the incubation, the cells were washed three times and incubated with respective Alexa Fluor conjugate (Alexa Fluor 488 or 568) Ab for 1 h at 4°C. For the above confocal studies, the slides were mounted using Vectashield mounting medium with DAPI (Vector Laboratories) and then examined under an Olympus FV1000 confocal microscope (63×/1.4 oil; Olympus). The pictures were analyzed using FV-10 ASW software.

Flow cytometry
For the analysis of surface expression of CXCX4 and CD4 receptors, cells were washed twice with PBS, resuspended in PE-conjugated CXCX4 and allophycocyanin-conjugated CD4 Abs or with respective isotype conjugates as a control (BD Pharmingen), and then incubated for 1 h at 4°C. The cells were washed three times with ice-cold PBS and then analyzed by flow cytometry. To determine intracellular p24, cells were fixed and permeabilized using the Fix/Perm solution (BD Biosciences). The cells were stained with anti-p24 mAb (KC57, Beckman Coulter) and analyzed by flow cytometry using a FACSCalibur cytometer (BD Biosciences). The data were analyzed by CellQuest software (BD Biosciences).

Virus binding and entry assay
For virus binding assays, WT, SLP-76−/−, and SLP-76+/− cells (1 × 10⁶ cells) were resuspended in complete RPMI 1640 medium, and the cells were infected with HIV-1 IIIB at a concentration of 40 ng p24/10⁶ cells. After 1 h incubation at 4°C, the cells were extensively washed with ice-cold PBS to remove unbound viruses, transferred to fresh tubes, and finally lysed in ice-cold lysis buffer (1% Triton X-100). Total cell protein was estimated using Bradford assay, and all samples were normalized for protein content. Virus binding was monitored by measuring the amount of p24 in the cell lysates by ELISA. For virus entry assay, the cells were incubated with HIV-1 IIIB for 3 h at 37°C. Following this incubation, the cells were washed five times with PBS, treated with trypsin for 5 min at 37°C to remove the uninternalized virus from the surface, and washed once with complete RPMI 1640 medium and five times with PBS. The cells were lysed using lysis buffer (1% Triton X-100), and viral entry was monitored by measuring the p24 levels in the cell lysates by ELISA. Total cell protein was calculated using Bradford assay, and all samples were normalized for protein content.

Virus integration
Host cell nuclear integration of HIV in T cell lines and PBMCs was analyzed by PCR as described previously (29). DNA was prepared by using a DNA extraction kit (Qiagen, Valencia, CA) according to the manufacturer’s protocol. DNA samples were used for the amplification of integrated DNA with Alu-LTR–specific primers (Table I) using the following cycling conditions: 95°C for 10 min, then 40 cycles at 95°C for 15 s, 60°C for 1 min, and 72°C for 1 min. GAPDH was used as an internal control.

Determining proviral transcription of full-length and multiple-spliced HIV mRNA
RNA was prepared from 2 × 10⁶ HIV-1 IIIB-infected WT and SLP-76−/− cells using the QiAamp RNAeasy Mini Kit protocol (Qiagen). Full-length (FL) and multiple-spliced (MS) HIV mRNA transcript levels were determined by a modification of a protocol described by Brussels et al. (30). Quantitative RT-PCR (qRT-PCR) was performed using primers (200 nM) specific for FL and for MS (Table I) using 400 ng sample RNA in a SYBR
Green assay system using a Realplex Cycler (Eppendorf, Westbury, NY). PCR conditions included 95°C for 3 min (1 cycle), 94°C for 1 min, 56°C for 1 min, and 72°C for 1 min (40 cycles), followed by 4°C for 10 min.

**Cell–cell transmission**

The cell-to-cell transmission assay was carried out as previously described (31), with some modifications. Briefly, WT and SLP-76+/− cells were infected with HIV-1 IIIB at 20 ng/10^6 cells for 3 d. After 3 d of infection, when the cells showed 60–75% HIV-1 p24 positivity, these donor cells were counted and cocultured with target TZM-bl cells at different ratios (donor/target ratios: 1:1; 1:5, and 1:10). After 24 h, the donor cells were removed by aspiration of the medium containing the Jurkat cell clones. The TZM-bl cells were then incubated with fresh medium for 3 d, after which they were lysed. An equal amount of protein was used for luciferase activity using the Luciferase Reporter Kit (Promega). Efficiency of cell–cell transmission was measured in terms of relative luciferase units.

**Transfections**

To analyze the SLP-76 domains important for the HIV-1 infection, we transfected the respective domain mutated plasmids (Δ1–156, Δ224–244, and R448K) into SLP-76−/− cells using the Nucleofection Kit V (Program S018; Amrax Biosystems). WT and SLP-76−/− cells transfected with control plasmid were used as controls. After 24 h transfection, equal numbers of cells were used for the HIV-1 infection. The expression of different fragments in Jurkat cells was analyzed after 48 h transfection by Western blot. SLP-76−/−mutated plasmids were kindly provided by Gary Koretzky (University of Pennsylvania).

**Small interfering RNA-mediated knockdown**

siRNA-mediated knockdown of SLP-76 was performed using ON-TARGETplus SMARTpool SLP-76 siRNA (Dharmacon, Boulder, CO), according to the manufacturer’s protocol. Briefly, MT4 cells or PBMCs were transfected with 100 or 200 nM siRNA using the Amaxa system (Amaza Biosystems), as mentioned above. The respective, Non-Targeting siRNA SMARTpool was used as a control. SLP-76−/−mediated knockdown was estimated by Western blot analysis after 48 h of the initial transfection.

**Western blot analysis**

Equivalent amounts of protein extracts were run on a 4–12% gradient acrylamide gel (NuPAGE Bis-Tris gel; Invitrogen) and transferred onto nitrocellulose membranes. Immunodetection involved specific primary Abs, appropriate secondary Abs conjugated to HRP, and the ECL Western blotting detection system (GE Healthcare).

**Statistical analysis**

Reported data are the means ± SD of at least three independent experiments performed in duplicate or triplicate. The statistical significance was determined by the Student t test.

**Results**

**Differential susceptibility of the SLP76−/− and WT T cell clones to HIV-1 infection**

We first determined the role of SLP-76 in HIV-1 infection using SLP-76+/-, WT, and SLP-76+/+ Jurkat cell lines. The SLP-76−/− Jurkat cell variant expressed minimal amounts of SLP-76 in comparison with the WT and the SLP76+/+ Jurkat cells (Fig. 1A). We also analyzed the expression of HIV-1 receptor, CD4, and coreceptor CXCR4 in these cells. The SLP76−/− cells expressed CXCR4 and CD4 at levels similar to those observed in the WT and SLP-76+/+ Jurkat cells (Fig. 1B). We then studied the role of SLP-76 in HIV-1 virus production by infecting the WT and SLP-76+/− Jurkat cells with the laboratory-adapted X4-tropic virus HIV-1 IIIB. The supernatant was collected at various time points and analyzed for p24 Ag levels by ELISA. The SLP-76−/− Jurkat cells showed reduced HIV-1 virus production in comparison with the control WT Jurkat cells (Fig. 1C). We observed an increasing reduction in HIV-1 virus production in SLP-76−/− cells over various time periods. To determine whether the observed differ-

![Figure 2](http://www.jimmunol.org/)
ence in HIV-1 virus production between the WT and SLP-76−/− cells was due to the lack of SLP-76 molecule. HIV-1 virus production was evaluated in SLP-76−/− cells that were reconstituted with normal human SLP-76 (SLP-76+/+). Susceptibility to HIV-1 infection in the SLP-76+/+ Jurkat cells was restored to the levels observed in the WT Jurkat cells (Fig. 1C), indicating that SLP-76 is important for HIV-1 virus production.

**Knockdown of SLP-76 in T cells leads to a decrease in HIV-1 virus production**

To further confirm by an independent method the effect of SLP-76 downregulation on HIV-1 virus production in T cell lines and primary cells, we used siRNA-driven SLP-76 knockdown in MT4 cells and PBMCs. As shown in Fig. 2A, left panel, there was significant knockdown of SLP-76 in MT4 cells transfected with the SLP-76 siRNA compared with the nontargeting (NT) siRNA. When these transfected cells were infected with HIV-1 IIIB (Fig. 2A, right panel), there was ~50% reduction in virus production in SLP-76 siRNA-transfected cells in comparison with NT siRNA-transfected cells, thus corroborating the results observed with the SLP-76−/− Jurkat T cell line. Furthermore, we also analyzed the effect of SLP-76 in PBMCs by siRNA-mediated downregulation of SLP-76. We observed significant knockdown of SLP-76 in PBMCs transfected with SLP-76 siRNA compared with the NT control (Fig. 2B). There was no difference in viability between NT siRNA and SLP-76 siRNA-transfected PBMCs as demonstrated by trypan blue staining (Fig. 2C, left panel). We also determined that there was no difference in activation status of the PHA-stimulated PBMCs upon siRNA transfection, using CD38 as a marker. (Fig. 2C, right panel).

When the SLP-76 siRNA-transfected PBMCs were infected with HIV-1 IIIB (X4 virus), the cells showed reduced virus production in comparison with the NT siRNA-transfected cells (Fig. 2D, left panel). Furthermore, the SLP-76 knockdown cells also showed a reduced virus production with the R5 tropic HIV-1 virus (Bal strain) (Fig. 2D, right panel), indicating that SLP-76 modulates both X4 and R5 tropic virus infection in T cells. The above results underscore the role of SLP-76 in HIV-1 infection.

**The NH2 acidic domain of SLP-76 regulates HIV-1 infection**

Three distinct domains of SLP-76—the amino terminal acidic domain, the central proline-rich domain, and the C-terminal SH2 domain—mediate protein interactions that are potentially important for its functions (6). A series of SLP-76 mutants schematically represented in Fig. 3A has been generated previously (32) in which each mutant was designed to disrupt one of the three SLP-76 domains described. The first mutant, Δ1–156, consists of an in-frame deletion of the first 156 aa in the N-terminal region. The second mutant, Δ224–244, contains a deletion of 20 aa in the central region of SLP-76. The third mutant, R448K, contains a point mutation of a key arginine responsible for binding of phosphorytrosines to the SH2 domain of SLP-76 (Fig. 3A).

To study the region of SLP-76 important for HIV-1 virus production, we transfected these SLP-76 constructs (Δ1–156, Δ224–244, and R448K) into SLP-76−/− Jurkat T cells using the Amaxa Nucleofection Kit (Amaxa Biosystems). The transient expression of different mutant constructs in SLP-76−/− cells was confirmed by Western blot (Fig. 3B). The ability of HIV-1 IIIB to infect these transfected cells in comparison with the WT and SLP-76−/− Jurkat cells was evaluated. As shown in Fig. 3C, transfection with Δ1–156 mutant showed ~50% reduction in HIV-1 virus production, whereas the other mutants did not show any significant inhibition of HIV-1 virus production in comparison with WT-infected cells. These studies suggest the importance of the NH2 terminal domain of SLP-76 in regulating HIV-1 infection.

**SLP-76 does not regulate early steps of viral life cycle**

We further studied the mechanism by which SLP-76 regulates HIV-1 infection. For this, we first determined the steps in the HIV-1 life cycle that were regulated by SLP-76. The first step in the viral life cycle is the binding of the viral envelope protein to the primary receptor, CD4, and coreceptors, CXCR4 or CCR5. We have already demonstrated that SLP-76 expression does not regulate CD4 or CXCR4 expression in Jurkat T cells (Fig. 1B). We tested whether the absence of SLP-76 expression specifically inhibited the binding of HIV-1 virus as assessed by p24 Ag assay of the cell lysates. There was no change in viral binding to SLP-76−/− cells compared with SLP-76+/+ and WT cells (Fig. 4A). Next, we specifically tested whether the absence of SLP-76 expression specifically inhibited the binding of HIV-1 virus to the primary receptor, CD4, and coreceptors, CXCR4 or CCR5. There was no difference in viral binding to SLP-76−/− cells compared with SLP-76+/+ and WT cells (Fig. 4A). Next, we specifically tested whether the absence of SLP-76 blocked cellular entry of HIV-1 in the WT, SLP-76+/+, and SLP-76−/− cells using a viral binding assay. Again, we observed no change in the amount of virus that enters the cell in SLP-76−/− cells as compared with the WT and SLP-76+/+ control cells (Fig. 4B).

We analyzed the role of SLP-76 in postentry events including first-strand cDNA synthesis and integration by PCR using primers as shown in Table I. We did not observe any difference between HIV-1–infected WT and SLP-76−/− cells in both first-strand cDNA synthesis (data not shown) and integration (Fig. 4C). Uninfected WT and SLP76−/− cells were used as controls (Fig. 4C). We further verified the role of SLP-76 in HIV-1 entry and integration using an siRNA knockdown strategy in PBMCs. There was no change in viral entry and integration in SLP-76 siRNA-transfected PBMCs compared with NT siRNA-transfected PBMCs (Fig. 4D, left panel). There was no difference in activation status of the PHA-stimulated PBMCs upon siRNA transfection, using CD38 as a marker. (Fig. 2C, right panel).

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SLP-76 does not inhibit viral binding, entry, integration, and transcription of HIV-1 in the T cells. (A) To analyze the effect of SLP-76 on virus binding, the various Jurkat T cell clones were infected with HIV-1 IIIB (40 ng/10^6 cells) and incubated for 1 h at 4°C, and the cell lysates were analyzed for HIV-1 p24 levels. Virus level in the WT control was 263 ± 20 pg/ml of p24 Ag. (B) To analyze the HIV-1 internalization, various Jurkat T cell clones were infected with HIV-1 IIIB (40 ng/10^6 cells) and incubated for 3 h at 37°C, and cell lysates were then analyzed for the HIV-1 p24 levels by ELISA. Virus level in the WT control was 63 ± 10 pg/ml of p24 Ag. (C) To analyze the effect of SLP-76 on integration, cells were infected with HIV-1 IIIB (20 ng/10^6 cells) for 24 h. DNA was extracted from the respective samples, and the integrated HIV-1 DNA was analyzed by PCR. Uninfected WT and SLP-76^−/− cells were used as controls. (D) To analyze the effect of SLP-76 on HIV transcription, WT and SLP-76^−/− cells were transfected with NT and SLP-76 siRNAs. The transfected cells were infected with HIV-1 IIIB (40 ng/10^6 cells) at 37°C for 3 h, and the cell lysates were analyzed for HIV-1 p24 levels. Virus level in the WT control was 230 ± 20 pg/ml of p24 Ag. (E) NT and SLP-76 siRNA knockdown PBMC samples were infected with HIV-1 IIIB (20 ng/10^6 cells) for 72 h, and DNA was used for the analysis of HIV integration. (F) To analyze the effect of SLP-76 on HIV transcription, WT and SLP-76^−/− cells were infected with HIV and FL (left panel), and spliced RNA (right panel) transcription was evaluated by qRT-PCR. Data are representative of three independent experiments.

**Table I. Primers used in the study**

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<th>Name</th>
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<th>Sequence</th>
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<td>HIV-FL F</td>
<td>HIV-FL gene</td>
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<td>HIV-MS gene</td>
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<tr>
<td>HIV-MS R</td>
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<td>5'-GTCTCTAACAGCGCTGATG-3'</td>
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**FIGURE 4.** SLP-76 does not inhibit viral binding, entry, integration, and transcription of HIV-1 in the T cells. (A) To analyze the effect of SLP-76 on virus binding, the various Jurkat T cell clones were infected with HIV-1 IIIB (40 ng/10^6 cells) and incubated for 1 h at 4°C, and the cell lysates were analyzed for HIV-1 p24 levels. Virus level in the WT control was 263 ± 20 pg/ml of p24 Ag. (B) To analyze the HIV-1 internalization, various Jurkat T cell clones were infected with HIV-1 IIIB (40 ng/10^6 cells) and incubated for 3 h at 37°C, and cell lysates were then analyzed for the HIV-1 p24 levels by ELISA. Virus level in the WT control was 63 ± 10 pg/ml of p24 Ag. (C) To analyze the effect of SLP-76 on integration, cells were infected with HIV-1 IIIB (20 ng/10^6 cells) for 24 h. DNA was extracted from the respective samples, and the integrated HIV-1 DNA was analyzed by PCR. Uninfected WT and SLP-76^−/− cells were used as controls. (D) To analyze the effect of SLP-76 on HIV transcription, WT and SLP-76^−/− cells were transfected with NT and SLP-76 siRNAs. The transfected cells were infected with HIV-1 IIIB (40 ng/10^6 cells) at 37°C for 3 h, and the cell lysates were analyzed for HIV-1 p24 levels. Virus level in the WT control was 230 ± 20 pg/ml of p24 Ag. (E) NT and SLP-76 siRNA knockdown PBMC samples were infected with HIV-1 IIIB (20 ng/10^6 cells) for 72 h, and DNA was used for the analysis of HIV integration. (F) To analyze the effect of SLP-76 on HIV transcription, WT and SLP-76^−/− cells were infected with HIV and FL (left panel), and spliced RNA (right panel) transcription was evaluated by qRT-PCR. Data are representative of three independent experiments.

SLP-76 does not regulate HIV-1 transcription

We further evaluated the role of SLP-76 in regulating the post-integration steps prior to viral release. We quantitated the number of FL and spliced viral transcripts in WT and SLP-76^−/− cells infected with HIV by qRT-PCR using primers as shown in Table I. There was no difference in FL (Fig. 4F, left panel) and spliced mRNA levels (Fig. 4F, right panel) between HIV-1 infected WT and SLP-76^−/− cells, suggesting that SLP-76 does not regulate HIV transcription in T cells.

SLP-76 regulates release of HIV-1 from T cells

We next analyzed whether SLP-76 modulates the release of virus using WT and SLP-76^−/− Jurkat cells. WT and SLP-76^−/− Jurkat T cells were infected with HIV-1 IIIB, and 72 h later, cells were centrifuged, and the supernatant was collected and assayed for virus release by HIV-1 p24 ELISA. Supernatant from SLP-76^−/− cells showed ~60% reduction in extracellular p24 compared with the supernatants from WT cells (Fig. 5A). In addition, supernatants were also ultracentrifuged and the viral pellets immunoblotted for p24. We observed significantly decreased expression (5-fold reduction) of HIV-1 p24 in the supernatants of SLP-76^−/− cells in comparison with the supernatants from WT Jurkat cells (Fig. 5B). We also monitored the intracellular p24 levels in these cells postinfection with HIV-1 IIIB by flow cytometry after 72 h of infection. Interestingly, we did not observe any significant difference in intracellular p24 levels in SLP-76^−/− cells compared with WT cells (Fig. 5C). The above results suggest that virus release is blocked in the SLP-76^−/− cells.

SLP-76 is important for the cell–cell transmission of virus

In addition to infection with cell-free virions, the importance of cell-associated spread across connecting membrane bridges and close cell–cell contacts referred to as virological synapse (VS) for HIV-1 propagation is increasingly recognized and thought to
SLP-76 plays a key role in facilitating cell–cell transmission of virus.

SLP-76 associates with Nef and modulates actin cytoskeletal reorganization

We further studied the molecular mechanisms by which SLP-76 modulates HIV-1 infection. SLP-76, being an adaptin protein, associates with several molecules to mediate its functions. HIV-1 Nef is an important accessory protein in the virus that influences T cell activation and is known to interact with various molecules that mediate T cell activation (20, 21, 25, 26, 35). We therefore determined whether SLP-76 may mediate HIV-1 release by binding to Nef. The association of Nef with SLP-76 was first shown by overexpressing FLAG-tagged SLP-76 and Nef in 293 T cells (Fig. 6A). Next, we demonstrated the association of SLP-76 and Nef in Nef-overexpressing Jurkat T cells by confocal microscopy (Fig. 6B, top panel). In addition, we also showed that SLP-76 associates with Nef in HIV-1–infected Jurkat T cells (Fig. 6B, middle panel), but not in HIV-1–infected SLP-76–/– cells (Fig. 6B, bottom panel). Furthermore, we demonstrated the association between SLP-76 and Nef by immunoprecipitation in Nef-overexpressing Jurkat T cells (Fig. 6C) and HIV-1–infected Jurkat T cells (Fig. 6D). These results suggest that HIV-1 Nef may associate directly with SLP-76.

Given that Nef and SLP-76 colocalize in T cells upon HIV-1 infection, it is reasonable to hypothesize that adapter protein SLP-76 may facilitate Nef’s function by modulating its association with other signaling molecules. A recent study has shown that a cooperative interaction of SLP-76 with Vav and Nck, both of which bind to the N-terminal domain of SLP-76 and are important for actin polymerization downstream of TCR (15). In addition, PAK2 has been shown to be a major Nef-associating protein that mediates its pathogenic effects. Therefore, we also determined the association of the SLP-76/Nef complex with PAK2, Vav1, and Nck1 in Nef-overexpressing Jurkat T cells. As shown in Fig. 7A, immunoprecipitation with Nef in Nef-overexpressing Jurkat cells revealed the association of Nef with PAK2, Vav1, and Nck1. In addition, immunoprecipitation with SLP-76 in Nef-overexpressing Jurkat cells indicated that, in addition to Nef, SLP-76 also associated with other signaling molecules including PAK2 and Nck1 (Fig. 7B). We further evaluated the association of Nef with Nck1, Pak2, and Vav1 in HIV-1–infected SLP-76–/– cells in comparison with WT Jurkat cells. We observed that the association of Nef with Vav1, Pak2, and Nck1 was greatly reduced in SLP-76–/– cells (Fig. 7C), suggesting that the association of Nef with these molecules is likely to be dependent on SLP-76. The above studies clearly suggest that the formation of a multimeric complex consisting of SLP-76, Nef, PAK2, Vav1, and Nck1 in HIV-1–infected cells may regulate HIV-1 virus production.

HIV-1 transmission across the VS depends on cell polarization, including reorganization of the actin cytoskeleton and recruitment of viral components to cell–cell contacts (33, 36). Because both SLP-76 and Nef have been shown to modulate actin polymerization and cytoskeletal remodeling, we compared Nef-actin association in WT and SLP-76–/– cells during HIV-1 infection by confocal microscopy. As shown in Fig. 8, top panel, we observed an association of Nef and F-actin in HIV-1–infected WT cells that was absent in HIV-1–infected SLP-76–/– cells. Pearson’s coefficient values calculated using FV-10-ASW software (Olympus) indicated a strong Nef–F-actin association in WT cells that was absent in SLP-76–/– cells (Fig. 8, bottom panel). Based on our results, we hypothesize that SLP-76, through the formation of a multimeric complex consisting of Nef and various host proteins, constitutes the predominant mechanism of HIV-1 propagation in T lymphocyte cultures (33, 34). We further asked whether SLP-76 may affect direct cell-to-cell transmission of HIV-1. Briefly, WT and SLP-76–/– Jurkat cells were first productively infected with HIV-1. After 3 d, ∼60–75% of the cells were HIV-1 p24 positive in both SLP-76–/– and WT cells (data not shown); these cells were used as donors and cocultivated with TZM-bl cells (target cells) in various ratios: 1:1, 1:5, and 1:10. We observed that in comparison with WT cells, SLP-76–/– cells (used at ratio 1:1) showed ∼30-fold reduction in cell–cell transfer to TZM-bl cells (Fig. 5D). Similar kinetics were observed with other donor/target ratios (data not shown). Altogether, these results indicate that SLP-76 may facilitate cell–cell transmission of virus.
including Vav1, Nck1, and Pak2, may modulate actin cytoskeleton remodeling, which is ultimately important for the HIV-1 release.

**Discussion**

Growing evidence suggests that TCR signaling complex plays a major role in regulating HIV-1 infection (1, 2). SLP-76 is a major adaptor molecule that links proximal and distal TCR signaling events through its function as a molecular scaffold in the assembly of multiprotein signaling complexes (37). In the current study, we demonstrate for the first time, to our knowledge, the prominent role played by SLP-76 in HIV-1 infection. Several lines of evidence presented in the study support this conclusion. In our studies using SLP-76−/−, WT, and SLP-76+/+ (reconstituted) Jurkat T cells, we found that the SLP-76−/− cell line was significantly resistant to HIV-1 infection. The role of SLP-76 was confirmed in MT4 cells and PBMCs using a SLP-76–specific siRNA, suggesting that our findings are not cell line-specific or due to off-target effects.

Our studies using mutants in various SLP-76 domains indicated that the N-terminal domain of SLP-76 was responsible for its modulation of HIV-1 infection in T cells. Structure-function studies on SLP-76 have previously shown the importance of the N-terminal domain in TCR signaling (38, 39). The phosphotyrosines at aa 112 and 128 in the N-terminal acidic region mediate binding to Vav1, Nck1, and the p85 subunit of PI3K, whereas Tyr145 binds ITK (7–10). Other studies suggest that this region is a substrate of ZAP-70 (11). From our results, it appears that the binding of N-terminal domain of SLP-76 to various other molecules may be important in positively regulating HIV-1 infection. In previous studies, ITK and ZAP-70 have been shown to play a role in regulating HIV-1 in-
Infection in T cells (40, 41). ITK is a Tec family tyrosine kinase that binds to SLP-76 and functions as its downstream mediator in TCR signaling. A recent study demonstrating that ITK regulates viral entry, transcription, and assembly/release during HIV infection (40) may suggest that SLP-76 could be one of the upstream mediators of ITK regulating virus replication.

We also demonstrated that SLP-76 is required for cell–cell transmission of HIV-1 using coculture of HIV-1–infected and noninfected cells. Cell–cell transmission of HIV-1, through the formation of a VS, is the predominant mode of HIV-1 transfer in vivo and probably occurs in secondary lymphoid organs, where most lymphocytes are present and cells are in close contact with each other and with APCs (42). In addition to lymphocytes, studies have shown that SLP-76 is expressed in other hematopoietic cells, including monocytes/macrophages and dendritic cells that are important APCs for HIV-1 (5, 43). Our results with T cells suggest that SLP-76 may also play an important role in transmission of HIV from these APCs to T cells. Though the role of SLP-76 has been studied in the formation of an immunological synapse (44), our results suggest for the first time, to our knowledge, its role in the formation of a VS.

SLP-76, being an adaptor protein, lacks intrinsic enzymatic activity, serves as an essential scaffold for the construction of multimeric protein complexes, and is indispensable for signal transduction in several pathways including TCR activation (37). Our next aim was to evaluate whether SLP-76 mediates HIV-1 infection by associating with viral proteins known to influence T cell activation such as Nef. Using 293 T cells overexpressing Nef and SLP-76, Nef-overexpressing Jurkat T cells, and HIV-1–infected cells, we could demonstrate an association between SLP-76 and Nef. Though the associations of Nef with several other molecules in the TCR signal transduction pathway have been shown (20, 21, 25, 26), this is the first study, to our knowledge, demonstrating an association between SLP-76 and Nef. A large array of Nef-regulated effector functions are mediated by interactions of this viral adaptor protein with several host cell factors in the TCR pathway (20, 21, 25, 26). We demonstrated that SLP-76 is involved in the formation of a multimeric complex consisting of Nef and other host binding partners, including Vav1, Nck1, and Pak2.

The manipulation of the actin cytoskeleton allows for efficient cell–cell spread of the virus. An SLP-76 complex downstream of TCR has recently been shown to modulate actin cytoskeletal reorganization in T cells (15). Furthermore, Nef has also been reported to mediate actin microfilament reorganization, which in turn mediates viral release (17, 19, 20, 25–28). In the current study, we showed reduced Nef–actin association in HIV-1–infected SLP-76/−/− cells in comparison with HIV-1–infected WT cells. Furthermore, we have shown that SLP-76 associates with Nef and other signaling molecules. These data suggest that SLP-76 may form a multimeric complex consisting of Nef and other host cell factors that modulate actin cytoskeletal changes, which in turn mediate viral release.

Taken together, our studies, for the first time to our knowledge, demonstrate a novel function of the adaptor molecule SLP-76 in regulating HIV-1 virus production. These findings also provide new information on the modulation of the TCR signaling pathway by HIV-1. Specifically, this study demonstrates the association of SLP-76 with the viral protein Nef and its role in modulating actin cytoskeletal remodeling. Our findings suggest that the formation of the immunological synapse and VS may involve common...
signaling molecules such as SLP-76. Therefore, targeting SLP-76 or its interaction with Nef may represent an attractive target for the future therapeutic interventions against HIV-1.

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Disclosures

The authors have no financial conflicts of interest.

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