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The Accessory Factor Nef Links HIV-1 to Tec/Btk Kinases in an Src Homology 3 Domain-dependent Manner*◆

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Background: HIV-1 replication in T cells requires the activity of the Tec family kinase Itk.
Results: Cell-based fluorescence complementation shows that HIV-1 Nef selectively interacts with Itk, Btk, and Bmx through their SH3 domains.
Conclusion: Nef links HIV-1 with the host cell kinase Itk during the viral life cycle.
Significance: Selective inhibition of Nef-Itk signaling may be of therapeutic benefit in HIV/AIDS.

The HIV-1 Nef virulence factor interacts with multiple host cell-signaling proteins. Nef binds to the Src homology 3 domains of Src family kinases, resulting in kinase activation important for viral infectivity, replication, and MHC-I down-regulation. Itk and other Tec family kinases are also present in HIV target cells, and Itk has been linked to HIV-1 infectivity and replication. However, the molecular mechanism linking Itk to HIV-1 is unknown. In this study, we explored the interaction of Nef with Tec family kinases using a cell-based bimolecular fluorescence complementation assay. In this approach, interaction of Nef with a partner kinase juxtaposes nonfluorescent YFP fragments fused to the C terminus of each protein, resulting in YFP complementation and a bright fluorescent signal. Using bimolecular fluorescence complementation, we observed that Nef interacts with the Tec family members Bmx, Btk, and Itk but not Tec or Txk. Interaction with Nef occurs through the kinase Src homology 3 domains and localizes to the plasma membrane. Allelic variants of Nef from all major HIV-1 subtypes interacted strongly with Itk in this assay, demonstrating the highly conserved nature of this interaction. A selective small molecule inhibitor of Itk kinase activity (BMS-509744) potently blocked wild-type HIV-1 infectivity and replication, but not that of a Nef-defective mutant. Nef induced constitutive Itk activation in transfected cells that was sensitive to inhibitor treatment. Taken together, these results provide the first evidence that Nef interacts with cytoplasmic tyrosine kinases of the Tec family and suggest that Nef provides a mechanistic link between HIV-1 and Itk signaling in the viral life cycle.

Unique to the primate lentiviruses HIV and simian immunodeficiency virus, Nef is expressed early in the viral life cycle (1, 2) and is required for high titer viral replication and disease progression *in vivo* (3–6). Previous studies have shown that nonhuman primates infected with Nef-deleted simian immunodeficiency virus failed to develop AIDS-like disease (5). Defective Nef alleles have also been detected in HIV sequences recovered from long term nonprogressors (7–10), individuals infected with HIV that do not or only very slowly develop AIDS despite many years without antiretroviral therapy. Furthermore, targeted expression of Nef in $CD4^+$ T cells and macrophages induces an AIDS-like syndrome in transgenic mice even in the absence of other HIV-1 gene expression (6). More recent studies with HIV-1-infected humanized mice show that viral load and $CD4^+$ T-cell loss are also dependent on Nef (10). Taken together, these studies support an essential role for Nef in HIV pathogenesis and AIDS progression.

Noncatalytic in nature, Nef functions by interacting with a multitude of host cell proteins involved in cellular activation, protein trafficking, immune recognition, and survival (11). Nef selectively binds to the Src homology 3 (SH3)[3] domains of several classes of host cell proteins (12), including members of the Src family of nonreceptor protein-tyrosine kinases. Of the Src-related kinases in the human kinome, Nef preferentially interacts with Hck, Lyn, and c-Src via their SH3 domains. Structural studies have shown that Nef interacts with Src family kinase SH3 domains through a highly conserved P*XX*P*X*R motif (13, 14), as well as a hydrophobic pocket formed by the three-dimensional fold of the Nef core (15). The Nef P*XX*P*X*R motif adopts a polyproline type II helix, which engages the hydrophobic grooves on the SH3 domain surface, whereas the hydrophobic pocket engages an isoleucine residue on the RT loop of the SH3 domain. Nef induces constitutive Src family kinase activation by displacing the SH3 domain from its regulatory position on the back of the kinase domain (16, 17).

Several lines of evidence support a critical role for the Nef P*XX*P*X*R motif in diverse Nef functions. Development of AIDS-like disease in Nef transgenic mice requires the P*XX*P*X*R motif and is delayed in mice that are homozygous-null for Hck (18).

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[3] The abbreviations used are: SH3, Src homology 3; BiFC, bimolecular fluorescence complementation; IF, immunofluorescence.

The P*XXPXR* motif is also required for Nef-mediated MHC-I (19) and CCR5/CXCR4 down-regulation (20), functions critical for the immune escape of HIV-infected cells. Recent work has established that selective inhibitors of Nef-induced SFK activation block Nef-dependent HIV infectivity and replication in cell culture (21–23). These studies support the idea that interaction of Nef with SH3-containing kinases and other proteins is a common event in HIV-infected cells that generates downstream signals essential for viral pathogenesis.

In addition to Src family kinases, members of the Tec kinase family are also expressed in HIV target cells. Like Src family members, Tec kinases also have SH2 and SH3 domains that control kinase activity and function (24, 25). The Tec family consists of five members as follows: Btk, which is expressed primarily in B cells; Itk, Tec, and Txk, which are expressed in T cells; and Bmx, which is expressed both in B cells and macrophages. Tec kinases modulate antigen receptor signaling and serve as critical mediators of immune responses in both B and T cells. In T cells, Tec kinases contribute to T helper cell differentiation and lineage commitment (24). Interestingly, Tec kinases are activated by direct phosphorylation by Src family kinases downstream of the T-cell receptor, leading to intracellular calcium mobilization as well as phospholipase Cγ and MAPK activation (25).

Recent studies have implicated the Tec family kinase Itk in the HIV-1 life cycle. Using selective pharmacological inhibitors and siRNA targeting Itk, Readinger *et al.* (26) showed that loss of Itk activity compromised viral transcription, particle assembly, and viral spread. However, the molecular mechanism linking HIV-1 to this T-cell kinase was not reported. The well known connection of HIV-1 Nef to Src family kinase activation, the close relationship of Src and Tec family kinases in T cells, and the requirement for Itk activity in HIV replication suggested a possible link between Nef and Tec family kinases in HIV target cells. In this study, we investigated the direct interaction of HIV-1 Nef with Tec family kinases using a cell-based bimolecular fluorescence complementation (BiFC) assay. We report here for the first time that Nef interacts directly with three members of this kinase family (Bmx, Btk, and Itk) through their SH3 domains. Allelic variants of Nef, representative of 10 distinct M-group HIV-1 subtypes, were all found to interact strongly with Itk in cells by the BiFC approach. Using a selective small molecule inhibitor of Itk (BMS-509744), we also show that Itk kinase activity is required for wild-type HIV infectivity and replication but not that of a Nef-defective mutant. Taken together, these results show that Nef provides a mechanistic link between HIV-1 and Itk signaling in the viral life cycle and support further exploration of this signaling pathway as a potential target for anti-retroviral drug development.

EXPERIMENTAL PROCEDURES

Cell Culture, Reagents, and Antibodies—Human 293T cells were purchased from the ATCC. TZM-bl indicator cells as well as the T lymphoblast cell lines CEM-T4 and Jurkat (clone E6-1) were obtained from the National Institutes of Health, AIDS Research and Reference Reagent Program. TZM-bl and 293T cells were cultured in Dulbecco's modified Eagle's medium/high glucose (DMEM) supplemented with 10% fetal bovine serum (Gemini Bio-Products). CEM-T4 cells were passaged in RPMI 1640 medium supplemented with 10% fetal bovine serum and L-glutamine. Cell culture media and supplements were purchased from Invitrogen. The Itk inhibitor BMS-509744 was the generous gift of Dr. Jian-Kang (Jack) Jiang of the National Institutes of Health, National Center for Advancing Translational Sciences.

Primary antibodies used in this study were obtained from Santa Cruz Biotechnology (Hck rabbit polyclonal, sc-72; Itk mouse monoclonal clone 2F12, sc-23902; Itk rabbit polyclonal M-109, sc-1697), Abcam (V5 tag mouse monoclonal, ab27671), Millipore (V5 tag rabbit polyclonal, ab3792; phosphotyrosine mouse monoclonal, clone 4G10, 05-321), National Institutes of Health, AIDS Research and Reference Reagent Program (HIV-1 JR-CSF Nef monoclonal, 1539; HIV-1 Nef monoclonal EH1, 3689; Nef Antiserum polyclonal, 2949), and the National Institutes of Health, NCI-Frederick AIDS and Cancer Virus Program (HIV-1 p24). Secondary antibodies used in this study were obtained from the following: Southern Biotech (goat anti-rabbit IgG (H+L) mouse/human ads-TXRD, 4050-07; goat anti-mouse IgG (H+L), human ads-TXRD, 1031-07; goat anti-mouse IgG (H+L), human ads-AP, 1031-04); LiCor (donkey anti-mouse IgG (H+L) IRDye 800CW, 926-32213); Invitrogen-Molecular Probes (Pacific Blue goat anti-mouse IgG (H+L), P31582; Pacific Blue goat anti-rabbit IgG (H+L), P10994), and National Institutes of Health, NCI-Frederick AIDS and Cancer Virus Program (goat anti-rabbit IgG (H+L)-HRP).

BiFC Assay Expression Vectors—The coding regions for wild-type, full-length Hck and Tec family kinases were amplified by PCR and fused in-frame with a V5 epitope tag at their C termini. The reverse primer for each kinase was designed to include the V5 tag sequence plus a unique KpnI or AccIII restriction site at the 3′-end. The C-terminal fragment of the Venus protein (encoding residues Ala-154 to Lys-238) was amplified by PCR with a unique KpnI or AccIII restriction site at the 5′-end. The PCR products were then subcloned into the mammalian expression vector pcDNA3.1(−) (Invitrogen) via these unique sites to create the final BiFC expression constructs. Truncated BiFC constructs were created with the Venus C-terminal fragment fused to the SH3 domain of each kinase. The vectors also encode the N-terminal myristoylation signal sequence and unique domain for Hck and the N-terminal pleckstrin and Tec homology domains for the Tec kinases. Tec family kinase cDNA clones were obtained from the Dana-Farber/Harvard Cancer Center PlasmID DNA Resource Core (Bmx, HsCD00327726; Btk, HsCD00346954; Itk, HsCD00021352; Tec, HsCD00341367; and Txk, HsCD00294897).

The BiFC expression construct, Nef-VN (SF2 allele; B clade), was created as described elsewhere (27). A similar BiFC construct with a P*XXPXR* mutant of Nef-SF2 was prepared for use as a negative control. The N-terminal sequence of the Venus protein (encoding residues Val-2–Asp-173) was PCR-amplified and subcloned via a unique BamHI restriction site to the C-terminal end of the coding sequences of representative Nef proteins from the HIV-1 M-group subtypes A1, A2, B, C, F1, F2, G, H, J, and K in the mammalian expression vector pcDNA3.1(−). The Nef-2PA, Hck-I96R, Btk-M96R, Itk-N96R and Tec-A96I mutants were created via site-directed mutagen-

esis (QuikChange II XL site-directed mutagenesis kit, Stratagene). The Venus template was a generous gift from Dr. Atsushi Miyawaki, RIKEN Brain Science Institute, Saitama, Japan.

BiFC Assay and Immunofluorescence—293T cells were plated on glass bottom microwell dishes (MatTek, P35G-1.5-14-C) and allowed to attach overnight. The cells were transfected with complementary pairs of BiFC expression vectors using standard calcium phosphate techniques. Thirty hours post-transfection, the cells were fixed with 4% paraformaldehyde, permeabilized with 0.2% Triton X-100, and blocked with 2% BSA in PBS overnight. Cells were stained with primary antibodies against Hck (1:1000), the V5 tag (1:750), or Nef (1:1000) for 1 h at room temperature. Immunostained cells were visualized with secondary antibodies conjugated to Texas Red or Pacific Blue at a dilution of 1:1000 and 1:500, respectively. Two-color imaging was done at constant exposure times for each channel using a Nikon Eclipse TE300 inverted microscope and a SPOT RT slider CCD high resolution digital camera and software (Diagnostic Instruments). Three-color images were recorded using confocal microscopy (Fluoview FV-1000, Olympus). Image analysis was performed with the Java-based image processing program, ImageJ (version 1.48s), to determine the BiFC and immunofluorescence (IF) intensities of individual cells. Mean pixel densities were calculated for the BiFC and IF channels for a minimum of 100 cells, and data were expressed as BiFC to IF signal ratios.

HIV Assays—Wild-type (WT) and Nef-defective (ΔNef) HIV-1 proviral genomes (NL4-3 strain) in the plasmid vector pUC18 were transfected into 293T cells with XtremeGENE 9 transfection reagent (Roche Applied Science) according to the manufacturer's protocol. The viral supernatant was collected 48 h post-transfection and amplified in MT2 T cells as described elsewhere (22). Titers of the amplified stocks were determined by HIV-1 p24 Antigen Capture ELISA kit (National Institutes of Health, NCI-Frederick) as per the manufacturer's protocol.

For HIV replication assays, CEM-T4 and Jurkat cells were incubated overnight in the absence or presence of the Itk inhibitor BMS-509744 in a final concentration of 0.5% DMSO as carrier solvent. Cells were then infected with the WT and ΔNef viruses, and replication was assessed 10 days later by p24 Gag ELISA of the culture supernatant.

For infectivity assays, the reporter cell line TZM-bl was preincubated with BMS-509744 overnight in a final concentration of 0.5% DMSO as carrier solvent. The WT and ΔNef viruses were also preincubated with the inhibitor for 4 h prior to infecting the cells. Viral infectivity was determined using the luciferase assay system (Promega) as described elsewhere (21).

Cytotoxicity Assay—CEM-T4, Jurkat, and TZM-bl cells were incubated at 37 °C in the absence or presence of the Itk inhibitor BMS-509744 with DMSO as a carrier solvent at a final concentration of 0.5%. Cytotoxicity was assessed for TZM-bl cells after 48 h and CEM-T4 and Jurkat cells after 10 days using the Cell Titer Blue reagent (Promega) and the manufacturer's protocol.

Immunoprecipitation and Blotting—Human 293T cells were transfected with wild-type full-length Itk and HIV-1 Nef (SF2 allele) either alone or in combination using the XtremeGENE 9 transfection reagent (Roche Applied Science) according to the manufacturer's protocol. For inhibitor experiments, 293T cells were preincubated with the Itk inhibitor BMS-509744 or the DMSO carrier solvent (0.5% final) overnight prior to transfection. Thirty hours post-transfection, the cells were lysed by sonication in lysis buffer (50 mM Tris-HCl, pH 7.4, 1% Nonidet P-40, 0.25% sodium deoxycholate, 150 mM NaCl, and 1 mM EDTA) supplemented with 1 mM sodium orthovanadate, 1 mM sodium fluoride, 5 units/ml Benzonase (Novagen), and Protease Inhibitor Set III (Calbiochem). Lysate protein concentrations were determined using Pierce Coomassie Plus assay reagent (Thermo Fisher). Itk was immunoprecipitated from 1 mg of each cell lysate with 1 μg of anti-Itk rabbit polyclonal antibody and protein G-Sepharose (Invitrogen) for 2 h at 4 °C, followed by washing with lysis buffer. Immunoprecipitated proteins were separated by SDS-PAGE, transferred to PVDF or nitrocellulose membranes, and probed with mouse monoclonal antibodies to phosphotyrosine (4G10), Itk, and Nef.

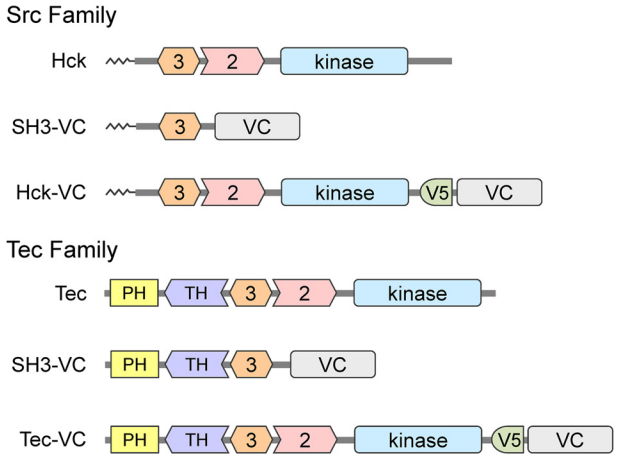


FIGURE 1. **Src and Tec family kinase domain organization and BiFC expression constructs.** The Src and Tec family kinases share a core of SH3 and SH2 domains followed by a kinase domain. Src family kinases are myristoylated at their N termini, which contributes to membrane targeting. Tec kinases have N-terminal pleckstrin homology (*PH*) and Tec homology (*TH*) domains that contribute to membrane association and kinase regulation, respectively. Truncated expression constructs were created for BiFC experiments (*SH3-VC*) in which sequences N-terminal to the SH2 domain from Hck and all five Tec family members were fused to the C-terminal fragment of the Venus form of YFP (*VC*). Full-length BiFC expression constructs were created in a similar manner, with a V5 epitope tag inserted between the C-terminal end of each kinase and the VC fragment to enable immunostaining for kinase expression.

RESULTS AND DISCUSSION

Src and Tec Kinase Families Share a Common Structural Domain Organization—Previous work has established that Nef binds to the SH3 domains of the Src family members Hck, Lyn, and c-Src, leading to kinase activation *in vitro* (16, 28), in defined cellular systems such as fibroblasts and yeast (17, 28, 29) and in HIV-1 target cells (21, 22). Members of the Tec family of nonreceptor protein-tyrosine kinases also possess SH3 domains important for their regulation (30). Fig. 1 illustrates the overall domain organization of these two kinase families, which share a core of SH3 and SH2 domains, followed by the kinase domain. In addition, Src family kinases have an N-terminal myristoylation signal sequence for membrane localization (31, 32), followed by an N-terminal sequence

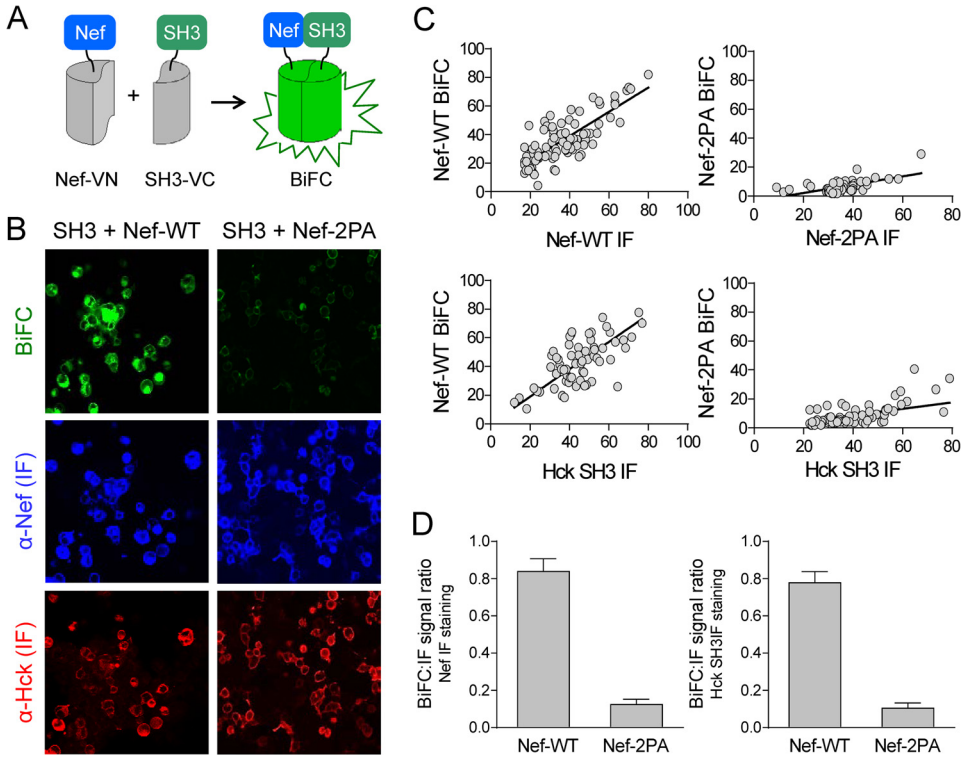


FIGURE 2. **Cell-based BiFC assay for Nef-SH3 domain complex formation.** *A,* BiFC principle. Nonfluorescent N- and C-terminal fragments of the YFP variant Venus are fused to Nef (*Nef-VN*) and the SH3 domain of Hck (*SH3-VC*). Co-expression and interaction of the two proteins inside cells juxtaposes the Venus fragments resulting in structural complementation and green fluorescence. *B,* Nef interaction with the Hck SH3 domain drives BiFC in transfected 293T cells. The Hck-SH3-VC fusion protein was co-expressed with the complementary wild-type Nef fusion partner (*Nef-VN*) or an interaction-defective mutant (*Nef-2PA-VN*) in 293T cells. Transfected cells were fixed and immunostained with anti-Nef and anti-Hck antibodies, followed by confocal microscopy. Representative BiFC and Nef IF images are shown. *C,* single-cell image analysis. Mean pixel intensities for the Nef/Hck-SH3 BiFC signal are plotted against the Nef and Hck IF signal intensities for ~100 cells. Results with WT and interaction-defective (*2PA*) forms of Nef are shown. The linear regression lines are also included on the plots. *D,* BiFC to Nef-IF and Hck SH3-IF signal ratios were determined from the average pixel intensities of whole image fields from three independent experiments and are presented as the mean ratio value ± S.E. Results with WT and interaction-defective (*2PA*) forms of Nef are shown. The mean BiFC/IF ratios obtained for the Nef-2PA mutant are significantly lower compared with wild-type Nef when normalized to either Nef or Hck SH3 immunofluorescence ($p < 0.0001$ in a two-tailed unpaired *t* test).

unique to each family member. Src family kinases also have a C-terminal tail with a phosphotyrosine residue critical for negative regulation of kinase activity (33). Structural studies have shown that the SH3 domain engages a polyproline type II helix formed by the SH2 kinase linker to hold the kinase in the down-regulated state (34–38). Binding of Nef to the SH3 domain displaces the linker, relieving its negative regulatory influence on the kinase domain (16, 17, 29). In contrast to Src family kinases, each Tec kinase possesses an N-terminal pleckstrin homology domain that binds to lipid phosphoinositides and acts as a membrane anchor (Fig. 1) (30). Tec kinases also possess a Tec homology domain N-terminal to the SH3 domain, consisting of a proline-rich motif engaged in intramolecular interaction with the SH3 domain that may also contribute to the regulation of kinase activity (39). The similarity in the domain architecture of these two kinases families led us to explore the possibility that Nef interacts with Tec kinases through their SH3 domains.

Development of a Cell-based BiFC Assay for Nef-SH3 Interaction—Previous studies of Nef interactions with the SH3 domains of Src family kinases have relied on solution-based approaches that do not account for the influence of membrane localization on both interacting partners. In addition, sequences N-terminal to the SH3 domain (Fig. 1) impact the spatial relationship between the SH3 domain and Nef at the membrane surface. To address these issues, we developed a cell-based BiFC assay to investigate the interaction of Nef with Tec and Src family kinases through their SH3 domains. In this assay, the two interacting proteins are fused to nonfluorescent fragments of the yellow fluorescent protein (YFP; yellow-shifted variant of GFP). When the two proteins of interest interact, juxtaposition of the YFP fragments results in structural complementation of the fluorophore, leading to a bright fluorescent signal (Fig. 2*A*) (40).

To develop a BiFC assay for Nef interactions with SH3 domains, we first examined the interaction of Nef with the SH3 domain of the Src family kinase, Hck, which has been studied in detail in several other systems (see Introduction). We first expressed the Hck SH3 domain as a fusion protein with the C-terminal fragment of Venus, a YFP variant that permits fluorophore maturation under cell culture conditions (41). The Hck BiFC fusion protein (Hck-SH3-VC) also retained the natural N-terminal myristoylation signal and unique domain to enable membrane targeting and proper spacing from the membrane (Fig. 1). Similarly, to preserve N-terminal myristoylation and membrane localization of Nef, the complementary N-terminal fragment of Venus (VN) was fused to the C-terminal end of HIV-1 Nef (SF2 allele) to make the Nef BiFC fusion protein (Nef-VN).

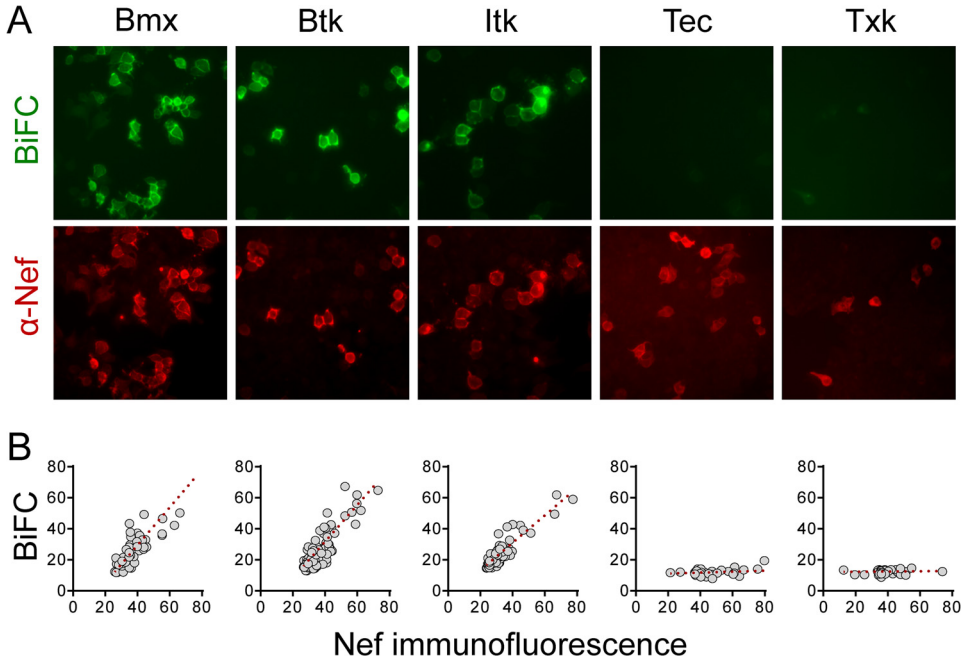


FIGURE 3. **BiFC reveals novel interactions between Nef and a subset of Tec family kinase N-terminal regions.** *A,* Tec family kinase N-terminal BiFC expression constructs (SH3-VC; see Fig. 1) were co-expressed with Nef-VN in 293T cells. Cells were fixed, immunostained with Nef antibodies, and imaged by epifluorescence microscopy for interaction (BiFC) and protein expression by IF 30 h later. *B,* mean pixel densities of the BiFC and Nef IF signal intensities were measured for ~100 cells and plotted as shown. The linear regression lines are also included on the plots.

To test for interaction of Nef with the Hck SH3 domain by BiFC, the Hck-SH3-VC and Nef-VN proteins were co-expressed in 293T cells, followed by immunostaining with anti-Nef and anti-Hck antibodies. As shown in Fig. 2*B*, co-expression resulted in a strong BiFC signal, consistent with interaction between Nef and the SH3 domain of Hck. To verify that the BiFC signal is dependent upon the interaction of Nef with SH3, the experiment was repeated with a Nef mutant in which the P*XX*P*X*R motif essential for SH3 binding was replaced with A*XX*A*X*R (Nef-2PA-VN). Co-expression of this Nef mutant with Hck-SH3-VC dramatically reduced the BiFC signal, consistent with the requirement for the P*XX*P*X*R motif in Nef engagement (Fig. 2*B*). IF staining with anti-Nef and anti-Hck antibodies showed a comparable level of protein expression in each transfected cell population.

Image analysis was performed to determine the relative fluorescence intensities for BiFC and IF from each of the transfected cell populations. Mean pixel intensities were determined for ~100 individual cells, and the BiFC and IF signals are plotted in Fig. 2*C*. This approach showed a strong linear correlation of Nef-SH3 interaction (BiFC signal) as a function of protein expression level relative to both Nef and the Hck SH3 domain. BiFC to IF ratios of ~1.0 were obtained for cells transfected with wild-type Nef, regardless of whether the Nef or SH3 IF signal was used as the normalizer. This ratio decreased by nearly 10-fold for cells expressing the Nef-2PA mutant, consistent with the essential role for the P*XX*P*X*R motif in SH3 binding by Nef. We also calculated BiFC to IF ratios from the mean pixel intensities of the entire image fields obtained from three independent transfected cell populations (Fig. 2*D*). This approach produced BiFC to IF ratios ~0.8 for wild-type Nef that was reduced to nearly 0.1 with the Nef-2PA mutant. These results validated the use of the BiFC approach to explore the interactions of Nef with Tec family kinases in a cell-based assay.

Nef Interacts with a Subset of Tec Family Kinases—Using the BiFC assay, we next investigated the interaction of HIV-1 Nef with Tec family kinases through their SH3 domains. First, we fused the SH3 domains of Btk, Itk, Tec, and Txk to the C-terminal fragment of Venus. These SH3-VC constructs also included the N-terminal pleckstrin homology and Tec homology domains to maintain proper membrane targeting and spacing (Fig. 1). Because Bmx lacks clear SH3 domain sequence homology, we created an analogous VC construct using the Bmx sequence encoding the N terminus through the beginning of the SH2 domain. Each of the Tec kinase SH3-VC constructs was then co-expressed with Nef-VN in 293T cells, followed by immunofluorescent staining for Nef as before. As shown in Fig. 3*A*, co-expression with Nef-VN yielded a strong, membrane-localized BiFC signal with the SH3-VC proteins from Bmx, Btk, and Itk but not for Tec or Txk. Single-cell image analysis yielded linear correlations for BiFC *versus* IF with the Bmx, Btk, and Itk SH3 domains, with BiFC to IF ratios of ~1.0. For the Tec and Txk SH3 domains, this ratio was less than 0.05, consistent with a lack of interaction (Fig. 3*B*). These results support the idea that HIV-1 Nef selectively interacts with Bmx, Btk, and Itk through their SH3 domains.

BiFC results presented in Fig. 3 show that Nef interacts with the N-terminal regions of Bmx, Btk, and Itk, most likely though their SH3 domains. However, the absence of the kinase domain in these constructs may expose an SH3 surface not normally available for interaction with Nef. To address this issue, we next performed BiFC experiments with full-length Bmx, Btk, and Itk as well as full-length Hck as a positive control. In each case, the C-terminal fragment of Venus was fused to the C-terminal end

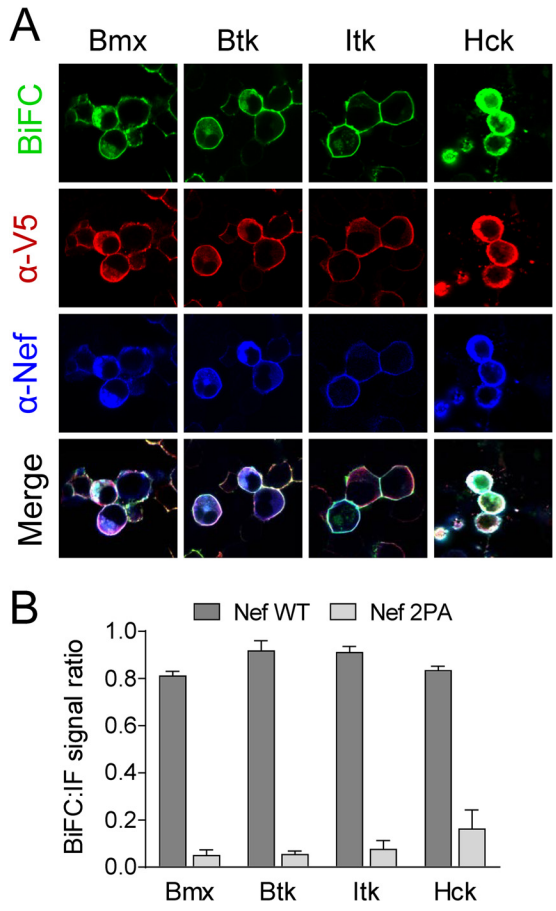


FIGURE 4. **Nef interacts with full-length Tec family kinases.** BiFC constructs for the full-length Tec family members Bmx, Btk, and Itk as well as the Src family kinase Hck (see Fig. 1) were co-expressed with wild-type Nef fused to the complementary BiFC fragment (Nef-VN) or a Nef mutant defective for SH3 binding (*Nef-2PA-VN*) in 293T cells. Cells were fixed, immunostained for Nef and kinase expression (V5 epitope) 30 h later, and analyzed by confocal microscopy. *A,* representative images of wild-type Nef interaction with each kinase by BiFC (*green*), Nef expression (*blue*), partner kinase expression (*red*), along with a merged image. *B,* quantitative comparison of the BiFC to IF ratios for interactions of the full-length kinases with wild-type *versus* mutant forms of Nef. The BiFC to Nef IF signal ratios were determined from the average pixel intensities of the whole image fields from three independent experiments using ImageJ. Data are plotted as the mean ratio ± S.E.

of the kinase (Fig. 1). A V5 epitope tag was inserted between the C terminus of the kinase and the VC fragment to enable immunofluorescent staining for kinase expression. Each of these kinase-VC proteins was co-expressed in 293T cells with Nef-VN as before, and the cells were fixed and immunostained for Nef as well as kinase expression. The cells were then imaged by three-color confocal microscopy for BiFC as well as Nef and kinase expression by immunofluorescence. As shown in Fig. 4*A*, a strong BiFC signal was observed with all four kinases, providing direct evidence that Nef interacts with full-length Bmx, Btk, and Itk as well as Hck in cells. The BiFC signal observed with each of the Tec family kinases showed striking localization to the cell periphery, consistent with the plasma membrane localization of both interacting partners. To verify that the BiFC signals observed with the full-length Tec kinases were dependent on the interaction of Nef with their SH3 domains, parallel experiments were performed with the Nef-2PA-VN construct that is defective for SH3 binding as described above. Co-expression of Nef-2PA with Bmx, Btk, and Itk significantly reduced the BiFC signals (Fig. 4*B*), supporting a requirement for the Nef P*XX*P*X*R motif for interaction with Tec family kinases through their SH3 domains.

Tec and Src Family Kinase SH3 Domains Interact with Nef in a Similar Manner—BiFC results presented above provide strong evidence for the interaction of Nef with a subset of Tec family kinases, and the results point to the SH3 domain as the site of Nef binding. Structural studies have shown that the Nef P*XX*P*X*R motif forms a polyproline type II helix that contacts the hydrophobic surface of Src family kinase SH3 domains (13, 42). This interaction is stabilized by a salt bridge between SH3 Asp-100 and the conserved arginine in the Nef P*XX*P*X*R sequence. Conserved residues in the Nef hydrophobic pocket (Tyr-120, Phe-90, and Trp-113) contact Ile-96 in the RT loop of the SH3 domain, and the importance of this interaction has been verified in mutagenesis studies (13, 15). A sequence alignment of the Tec family kinase SH3 domains with that of the Src family kinase Hck is presented in Fig. 5*A* and shows the clear amino acid sequence homology of the Btk, Itk, Tec, and Txk SH3 domains with that of Hck. Interestingly, the corresponding Bmx region is quite divergent in terms of sequence conservation (Fig. 5*B*). Nevertheless, Bmx interacts strongly with Nef in a P*XX*P-dependent manner (Fig. 4), suggesting that this region adopts an SH3-like fold that is competent for Nef binding.

To explore the structural basis of Tec family kinase SH3 domain interactions with Nef, we aligned the NMR structures of the Btk, Itk, and Tec SH3 domains with the x-ray crystal structure of Nef in complex with an Src family kinase SH3 domain (Protein Data Bank code 1EFN). As shown in Fig. 6*A*, an acidic residue is positioned in the Btk, Itk, and Tec SH3 domains for a possible ionic contact with Nef Arg-77 in a manner analogous to Asp-100 in the Src family kinase SH3 domain. The Txk SH3 domain, however, has an unfavorable asparagine in this position. Amino acid position 96 in the RT loops of the Btk and Itk SH3 domains is occupied by methionine and asparagine, respectively (Fig. 6*A*). These substitutions may be tolerated in place of the isoleucine found in the RT loop of the Src family kinase SH3 domain, as a strong BiFC signal was observed with each of these kinases and Nef (Figs. 3 and 4). In contrast, alanine and arginine are present in this position in the Tec and Txk SH3 domains, respectively. These differences may compromise interaction with the Nef hydrophobic pocket, as no BiFC signal was detected with these SH3 domains (Fig. 3). SH3 domain residues that occupy the SH3 domain RT loop positions 96 and 100 in the Tec *versus* Src family kinases are summarized in Fig. 6*B*; their positions in the overall SH3 sequences are highlighted in the sequence alignment presented in Fig. 5.

SH3 domain structural alignments described above suggest that the amino acid occupying the RT loop position analogous to Ile-96 in the Hck SH3 domain may be an important determinant of Nef binding to Tec family kinases via their SH3 domains. To test this possibility, we substituted RT loop residue 96 in the SH3 domains of full-length Hck, Btk, and Itk (all Nef binders) with arginine, which is present at this position in the Txk SH3 domain (a nonbinder). In all three cases, arginine substitution compromised interaction with Nef as visualized by BiFC (Fig. 6*C*). This reduction was stronger with Hck and Btk,

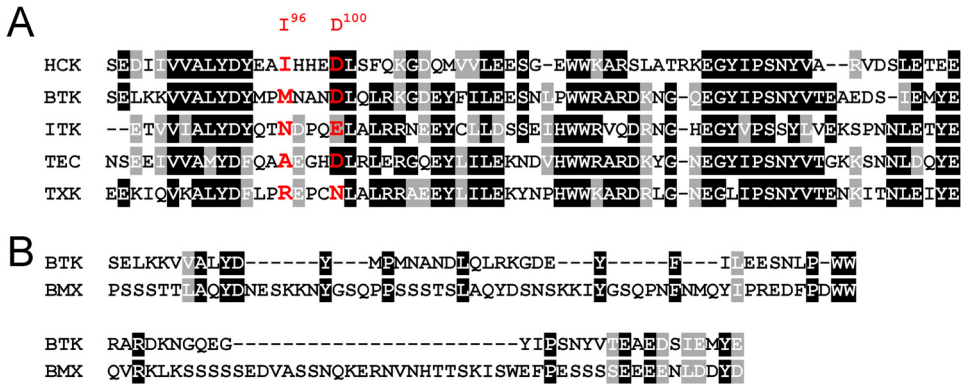
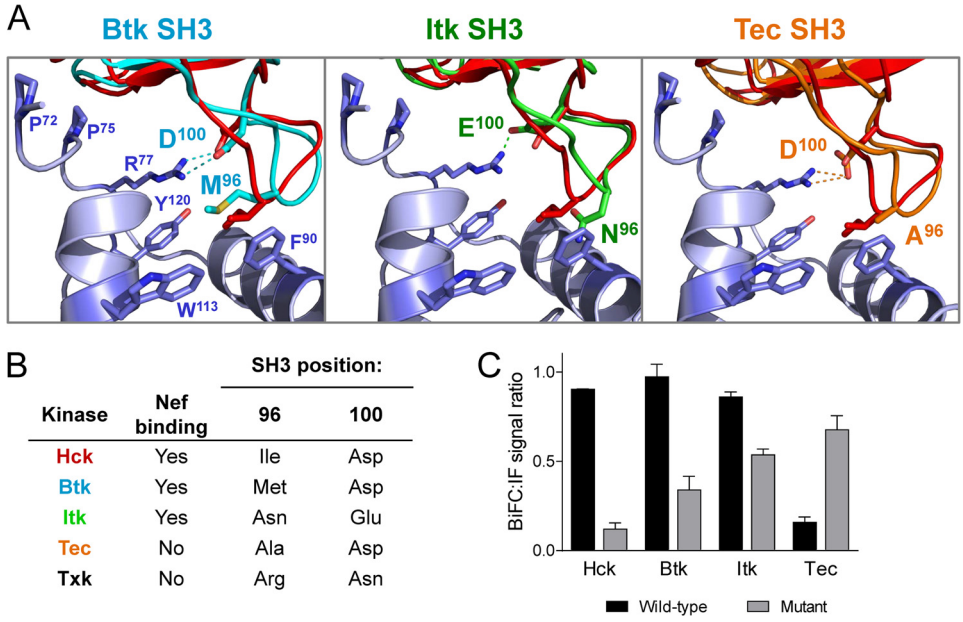


FIGURE 5. **SH3 domain sequence alignment.** *A,* SH3 domain amino acid sequence of the Src family kinase Hck is aligned with SH3 sequences of the Tec family members Btk, Itk, Tec, and Txk. The positions of Hck residues essential for Nef interaction are highlighted in *red* (Ile-96 and Asp-100). *B,* alignment of the Btk SH3 domain amino acid sequence with the corresponding region of Bmx. Despite low sequence homology, this region of Bmx is sufficient for Nef binding in a P*XX*P-dependent manner (see text).



Kinase	Nef binding	SH3 position: 96	100
Hck	Yes	Ile	Asp
Btk	Yes	Met	Asp
Itk	Yes	Asn	Glu
Tec	No	Ala	Asp
Txk	No	Arg	Asn

FIGURE 6. **Tec family kinase SH3 domain RT loops control interactions with Nef.** *A,* structural alignment of Tec family kinase SH3 domains with a Nef-SH3 complex. The x-ray crystal structure of Nef (rendered in *purple*) bound to an Src family kinase SH3 domain (*red*) was aligned with the NMR structures of the Btk (*cyan*), Itk (*green*), and Tec (*orange*) SH3 domains. An acidic residue is present at RT loop position 100 in all three Tec family SH3 domains for potential polar contact with Nef Arg-77 in a manner analogous to Asp-100 in the Hck SH3 domain. In Hck, RT loop Ile-96 makes hydrophobic contacts with conserved Nef residues Phe-90, Trp-113, and Tyr-120. Btk and Itk have methionine and asparagine at position 96, respectively, whereas human Tec has alanine at this position. The side chains of the conserved prolines in the Nef P*XX*P motif are also shown (Pro-72 and Pro-75). Note that in the mouse Tec SH3 structure used for the model, position 96 is occupied by Thr; this residue was replaced with alanine to match the human sequence for illustrative purposes. The Protein Data Bank codes for the structures used in this model are as follows: Nef-SH3 complex, 1EFN; Btk SH3, 1AWX; Itk SH3, 2RNA; and Tec SH3, 1GL5. Models of x-ray and NMR structures were produced using PyMOL. *B,* comparison of Hck SH3 domain residues essential for Nef binding and kinase activation with the analogous positions in Tec family kinases. These residues occupy RT loop positions 96 and 100 in the Hck SH3 domain. *C,* mutagenesis of RT loop residue 96 impacts Hck, Btk, Itk, and Tec interaction with Nef as assayed by BiFC. The BiFC to Nef-IF signal ratios were determined from whole field analyses of three independent transfected cultures as described in the legend to Fig. 2*D* and are plotted as the mean ratio value ± S.E.

although the effect was less pronounced with Itk, suggesting that other regions of Itk may contribute to Nef recruitment. In a complementary experiment, we substituted RT loop Ala-96 in the Tec SH3 domain with Ile, and we found that this change dramatically enhanced interaction with Nef by BiFC (Fig. 6*C*). These results support a key role for the SH3 domain in the interaction of Nef with Tec family kinases in cells and suggest that Nef interacts with Src and Tec family kinases through a similar mechanism.

Interaction with Itk Is a Conserved Property of all M-group HIV-1 Nef Alleles—Previous studies have reported that selective targeting of Itk with siRNA or kinase inhibitors blocks crucial steps in the HIV-1 life cycle (26, 43). Fluorescence complementation studies presented above show that Nef interacts with Itk via its SH3 domain, suggesting a mechanistic link between HIV-1 and Itk activity in the host cell. We therefore explored whether interaction with Itk is conserved across a wide variety of HIV-1 subtypes. For these studies, we created BiFC expression constructs for primary Nef alleles representative of nearly all M-group HIV-1 subtypes (A1, A2, B, C, F1, F2, G, H, J, and K) and co-expressed them with the complementary expression vector for full-length Itk. Sequence information for these alleles has been reported elsewhere (22). As shown in Fig. 7, co-expression of full-length Itk-VC in combination with the VN fusions

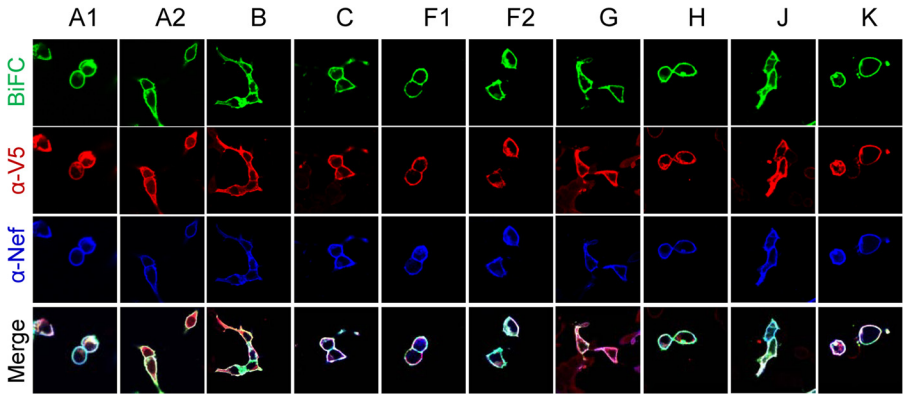


FIGURE 7. **Interaction with Itk is conserved across Nef alleles.** Nef clones representative of each of the M group HIV-1 clades shown (A1, A2, B, C, F1, F2, G, H, J, and K) were fused to the N-terminal fragment of Venus (*VN*) and co-expressed with the complementary full-length Itk-VC construct in 293T cells. Cells were fixed and immunostained for Nef and kinase protein expression (anti-V5 antibody) 30 h later. Cultures were analyzed by confocal microscopy for Nef/Itk interaction by BiFC (*green*), Nef expression (*blue*), and Itk expression (*red*). Representative images are shown along with a merged image in the *bottom panel*.

of each of the Nef alleles yielded a strong BiFC signal that localized to the cell membrane. Expression of both interacting partners was confirmed in individual cells by immunostaining with anti-Nef and anti-V5 (epitope tag for Itk) antibodies, and the merged images show co-localization. This result demonstrates that interaction of Nef with Itk is a highly conserved property of a diverse set of HIV-1 subtypes.

Selective Itk Inhibitor Blocks HIV Infectivity and Replication in a Nef-dependent Manner—Previous work has shown that HIV infectivity and replication are sensitive to the selective Itk inhibitor, BMS-509744 (26). This compound is remarkably selective for Itk and does not inhibit other Tec family members or Src family kinases (44). However, the molecular mechanism linking HIV-1 infection to activation of Itk is not known. To explore the role of Nef in the activation of Itk in the context of HIV-1 infection, we first evaluated Nef-dependent HIV-1 infectivity in the presence or absence of this compound in the TZM-bl reporter cell line (45). In this system, infectivity is measured by stimulation of a luciferase reporter gene that is driven by the HIV-1 LTR following infection with wild-type or Nef-deleted (ΔNef) HIV-1. As shown in Fig. 8*A*, infectivity was reduced by almost 50% in the absence of Nef, consistent with prior studies (21). Incubation of cells with BMS-509744 at a concentration of 3 μM suppressed wild-type HIV-1 infectivity to the same level as Nef-defective HIV (Fig. 8*B*). By contrast, this Itk inhibitor had no effect on infectivity of Nef-defective HIV-1, supporting a requirement for Nef expression in its mechanism of action.

To assess the role of Nef-dependent Itk activity in the context of HIV-1 replication, we turned to CEM-T4 lymphoblasts, which support viral replication in a Nef-dependent manner (21, 22). As shown in Fig. 9*A*, wild-type HIV-1 replicated more efficiently than the Nef-deleted virus over a wide range of viral inputs in CEM-T4 cells. Addition of BMS-509744 blocked wild-type virus replication in a concentration-dependent manner, with a reduction of almost 80% at the highest concentration tested (3 μM; Fig. 9*B*). Remarkably, this Itk inhibitor had no effect on the replication of Nef-defective HIV-1 over the same concentration range, suggesting that Nef couples HIV-1 to Itk signaling in T cell hosts. Note that Nef-deleted HIV-1 input was 10-fold higher than wild-type for these experiments (500 *versus*

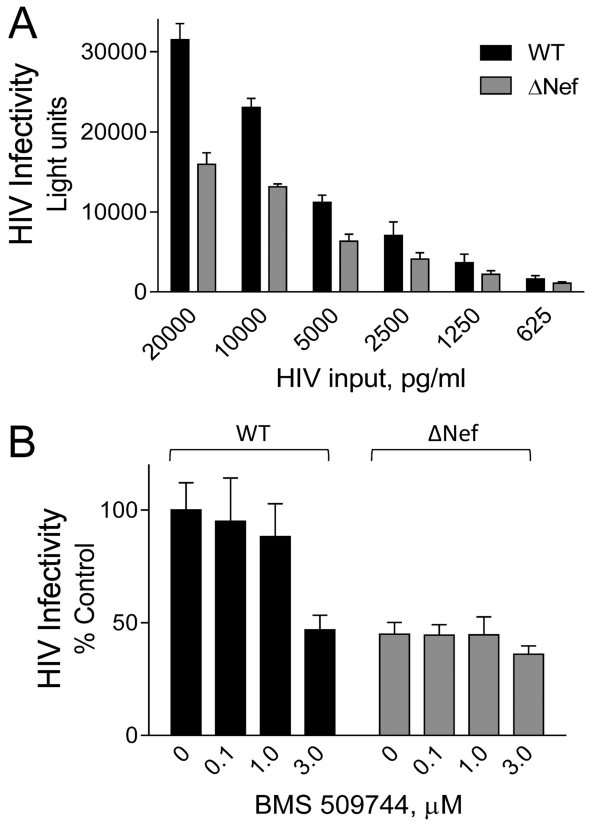


FIGURE 8. **Itk inhibitor BMS-509744 blocks HIV infectivity in a Nef-dependent manner.** *A,* HIV-1 infectivity is Nef-dependent. TZM-bl cells were infected with wild-type and Nef-deleted (ΔNef) HIV NL4-3, and viral infectivity was assessed 2 days later as luciferase activity. Results are plotted as relative light units ± S.E. ($n = 3$). *B,* inhibition of HIV infectivity by BMS-509744 is Nef-dependent. TZM-bl cells were preincubated overnight with BMS-509744 over the range of concentrations shown, followed by infection with wild-type and ΔNef HIV NL4-3. Infectivity was assessed 2 days later as luciferase activity. Results are presented as mean percent of HIV-1 infectivity observed in the presence of the DMSO carrier solvent alone ± S.E. ($n = 3$).

50 pg/ml p24 equivalents) to compensate for the reduced infectivity and replication of the mutant. In addition to CEM-T4 cells, we also tested the effect of BMS-508744 on HIV-1 replication in Jurkat T cells. Unlike CEM-T4 cells, HIV-1 replication is not affected by Nef in this cell line (Fig. 9*C*) (23). As shown in Fig. 9*D*, treatment of Jurkat cells with BMS-509744 had no

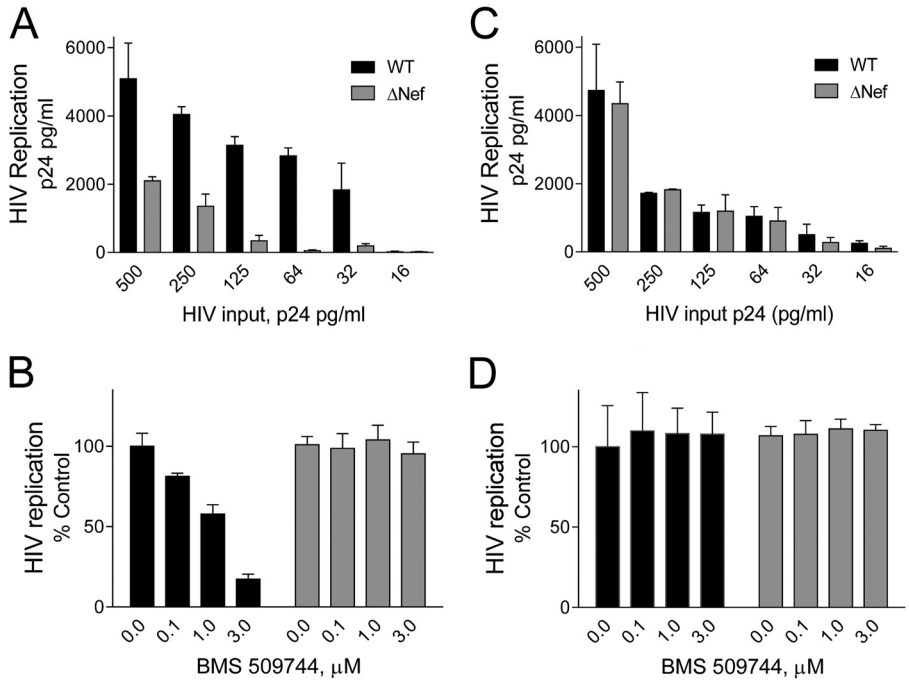


FIGURE 9. **Inhibition of HIV replication by the Itk inhibitor BMS-509744 requires Nef.** *A,* HIV-1 replication in CEM-T4 cells is enhanced by Nef. CEM-T4 cells were infected with wild-type and Nef-deleted (ΔNef) HIV NL4-3 and viral replication was assessed 10 days later by p24 Gag ELISA. Results are plotted as mean p24 levels ± S.E. ($n = 3$). *B,* inhibition of HIV-1 replication by BMS-509744 requires Nef in CEM-T4 cells. Following overnight preincubation with BMS-509744, cells were infected with the wild-type and ΔNef viruses. Input of ΔNef HIV was increased by 10-fold relative to the wild-type virus to compensate for the reduced replication of the mutant virus. HIV replication was determined by p24 ELISA 10 days later and is expressed as mean percent of replication observed with the DMSO-treated controls ± S.E. ($n = 3$). *C,* HIV-1 replication is independent of Nef in Jurkat T cells. Jurkat cells were infected with wild-type and ΔNef HIV NL4-3, and viral replication was assessed 10 days later by p24 Gag ELISA. Results are plotted as mean p24 levels ± S.E. ($n = 3$). *D,* BMS-509744 has no effect on HIV-1 replication in Jurkat T cells. Jurkat cells were preincubated overnight with BMS-509744 before infection with the wild-type or ΔNef virus. HIV replication was determined by p24 ELISA 10 days later and is expressed as mean percent of replication observed with the DMSO-treated controls ± S.E. ($n = 3$).

effect on either wild-type or Nef-defective HIV-1 replication, supporting a requirement for Nef in the mechanism of action of this compound on viral replication. Independent experiments verified that BMS-509744 did not exhibit cytotoxicity in Jurkat, CEM-T4, or TZM-bl cells over the range of concentrations tested in these experiments (data not shown).

Nef Stimulates Itk Kinase Activity—Results presented so far demonstrate that HIV-1 Nef interacts with Itk at the plasma membrane and that the enhancement of HIV-1 infectivity and replication by Nef are sensitive to a selective Itk kinase inhibitor. These observations imply that interaction with Nef stimulates Itk kinase activity. To test this possibility directly, we transfected 293T cells with full-length Itk and Nef either alone or in combination. Itk was then immunoprecipitated from the transfected cell lysates and probed with anti-phosphotyrosine antibodies. As shown in Fig. 10*A*, Itk was not detectably autophosphorylated when expressed alone in 293T cells, consistent with low intrinsic kinase activity as reported elsewhere (46). However, co-expression of Itk with Nef resulted in a dramatic increase in Itk phosphotyrosine content, consistent with Nef-dependent enhancement of kinase activity. We then repeated this experiment in the presence of the Itk inhibitor, BMS-509744, and observed a concentration-dependent decrease in Itk phosphotyrosine content. Nef-dependent Itk tyrosine phosphorylation was almost completely blocked at an inhibitor concentration of 1.0 μM (Fig. 10*A*), which agrees with the concentration of this compound required to inhibit Nef-dependent HIV replication and infectivity. Very similar results were obtained following immunoblot analyses of the transfected cell lysates (Fig. 10*B*). In this case, a strong tyrosine-phosphorylated band was observed in cells co-expressing Nef and Itk but not in cells expressing either protein alone. Tyrosine phosphorylation of this band, which migrates at ~72 kDa, is also potently inhibited by BMS-509744, and therefore it is likely to represent autophosphorylated Itk.

The observation that Nef induces activation of Itk and possibly other Tec family members raises interesting questions about the mechanism of kinase activation, especially in light of previous findings that Nef also binds and activates Hck and other Src family kinases (see Introduction). Previous work from our group and others has shown that Nef forms dimers in cells and that dimerization is important for Src family kinase activation by Nef as well as other Nef functions (21, 27, 47, 48). These observations suggest that a single Nef dimer may recruit two kinase molecules, resulting in juxtaposition of their kinase domains and subsequent activation by trans-phosphorylation of their kinase domain activation loops. In theory, a Nef dimer could recruit two heterologous kinases, *i.e.* a Src family kinase and a Tec family member. Nef-dependent formation of such a heterodimer could lead to stimulation of both kinases through a similar mechanism. Indeed, physiological activation of Itk requires, in part, trans-phosphorylation by a Src family kinase in the context of T cell receptor activation (25).

Summary and Conclusions—Here, we describe the use of BiFC to probe the interaction of Nef with Tec family kinases through their SH3 domains in a cellular context. We first vali-

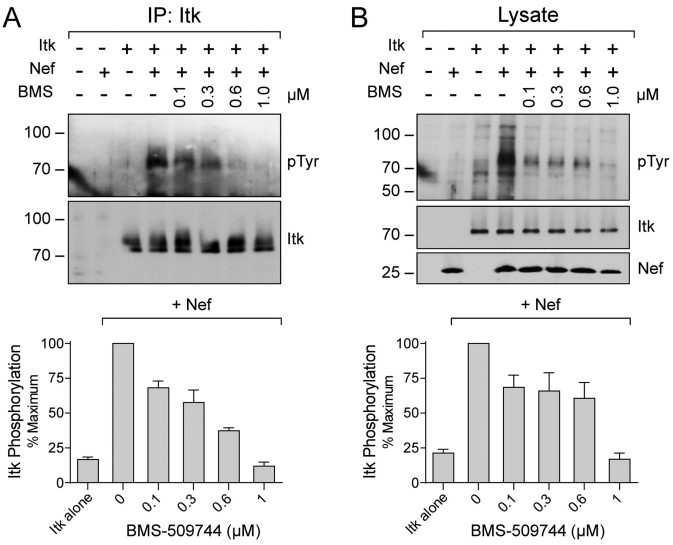


FIGURE 10. **Co-expression with Nef induces Itk activation that is sensitive to the selective Itk inhibitor BMS-509744.** Full-length Itk kinase and Nef were expressed in 293T cells either alone or in combination in the absence or presence of increasing concentrations of the Itk inhibitor BMS-509744. *A,* cells were lysed 30 h later, and Itk immunoprecipitates (*IP*) were analyzed by immunoblotting for phosphotyrosine content (*pTyr*) as well as Itk protein expression. Representative blots are shown at the *top*. This experiment was repeated in triplicate, and the relative phospho-Itk and Itk protein levels were quantitated using the LiCor Odyssey infrared imaging system. Relative signal intensities were corrected for background and used to calculate ratios of the phospho-Itk to Itk levels. The resulting ratios were then normalized to the highest value (phospho-Itk in presence of Nef without inhibitor) and are presented in the *bar graph* as the mean normalized ratio ± S.E. ($n = 3$). *B,* lysates from the same cell cultures in *A* were immunoblotted directly with antibodies to phosphotyrosine (*pTyr*) as well as Itk and Nef. A representative blot is shown at the *top*, and the data were quantitated and processed as per *A*.

dated the assay using the Src family kinase Hck, one of the best characterized SH3 binding partners for Nef. The Hck SH3 domain and full-length Hck both interacted strongly with Nef by BiFC, providing assay validation as well as new evidence for this interaction at the cellular level. Using the BiFC approach, we went on to show that Nef also interacts with the Tec family kinases Bmx, Btk, and Itk but not Tec or Txk. Experiments with truncated forms of Bmx, Btk, and Itk lacking the SH2 and kinase domains also produced bright, membrane-localized BiFC signals in the presence of Nef, supporting interaction through the SH3 domain. Sequence and structural alignment revealed that Tec and Txk have alanine and arginine, respectively, in place of the SH3 domain RT loop isoleucine critical for interaction with the Nef hydrophobic pocket, suggesting a possible explanation for their failure to interact. Replacement of this alanine in the Tec SH3 domain with isoleucine restored Nef binding, supporting a role for the RT loop in Nef recognition and suggesting that Src and Tec family kinases interact with Nef through very similar mechanisms. Nef allelic variants representing all M group HIV-1 clades interacted strongly with Itk in this assay, demonstrating that the Nef-Itk interaction is highly conserved in all major HIV-1 subtypes.

We also found that a highly selective small molecule inhibitor of Itk activity, BMS-509744, inhibited both HIV-1 infectivity and replication in a Nef-dependent manner, supporting a functional link between Nef and Itk in the HIV-1 life cycle. We also show for the first time that co-expression with Nef induces Itk autophosphorylation, which is sensitive to inhibition by BMS-509744. Consistent with these observations, this inhibitor had no effect on HIV replication in Jurkat T cells, which support HIV replication in a Nef-independent manner. These results suggest that Nef directly bridges HIV-1 infection with Itk signaling in T cells. Our results complement the recent observations of Schiralli Lester *et al.* (43), which show co-localization of Itk and HIV-1 Gag proteins to the plasma membrane at sites of F-actin accumulation and lipid rafts in HIV-1-infected T cells. They also reported that Itk inhibitors disrupt Itk co-localization with viral Gag as well as virus-like particle release. Taken together, these results suggest that Nef may play a role in the recruitment of Itk to this subcellular compartment and its activation, which in turn contributes to viral egress.

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