Oligomerization Is Required for HIV-1 Nef-Induced Activation of the Src Family Protein-Tyrosine Kinase, Hck†

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ABSTRACT: Hck is a member of the Src protein-tyrosine kinase family and is expressed strongly in macrophages, an important HIV target cell. Previous studies have shown that Nef, an HIV-1 accessory protein essential for AIDS progression, binds and activates Hck through its SH3 domain. Structural analysis suggests that Nef forms oligomers in vivo, which may bring multiple Hck molecules into close proximity and promote autophosphorylation. Using bimolecular GFP fluorescence complementation, we show for the first time that Nef oligomerizes in living cells and that the oligomers localize to the plasma membrane. To test the role of Nef oligomerization in Hck activation, we fused Nef to the hormone-binding domain of the estrogen receptor (Nef-ER), allowing us to control its dimerization with 4-hydroxytamoxifen (4-HT). In Rat-2 fibroblasts co-expressing Nef-ER and Hck, 4-HT treatment induced Nef-ER dimer and tetramer formation, leading to Hck kinase activation and cellular transformation. The number of transformed foci observed with Nef-ER plus Hck in the presence of 4-HT was markedly greater than that observed with wild-type Nef plus Hck, suggesting that enforced oligomerization enhances activation of Hck by Nef in vivo. Enhanced transformation correlated with increased Hck/Nef complex formation at the plasma membrane. In complementary experiments, we observed that a Nef mutant defective for Hck SH3 domain binding (Nef-PA) suppressed Hck kinase activation and transformation by the wild-type Hck/Nef complex. This effect correlated with the formation of a ternary complex between wild-type Nef, Nef-PA, and Hck, suggesting that Nef-PA suppresses Hck activation by blocking wild-type Nef oligomerization. Finally, Nef-ER induced Hck activation in a 4-HT-dependent manner in the macrophage precursor cell line TF-1, suggesting that oligomerization is essential for signaling through Hck in a cell background relevant to HIV infection. Together, these data demonstrate that Nef oligomerization is critical to the activation of Hck in vivo, and suggest that inhibitors of oligomerization may suppress Nef signaling through Hck in HIV-infected macrophages, slowing disease progression.

The Nef protein of HIV-1[1] is an essential determinant of AIDS pathogenesis (reviewed in refs *1*−*3*). Nef is required for high-titer viral replication and is essential for the development of AIDS-like disease in SIV-infected monkeys (*4*). HIV strains with defective *nef* alleles have been isolated from patients with long-term, nonprogressive HIV infection, implicating Nef as a critical progression factor for AIDS (*5*, *6*). Furthermore, targeted expression of Nef to the T-cell and macrophage compartments of transgenic mice induces a severe AIDS-like syndrome, directly illustrating an essential role for this protein in disease pathogenesis (*7*).

Nef is a small myristylated protein with no known catalytic activity that binds to a diverse array of host cell signaling molecules (*1*−*3*). Nef promotes immune evasion of HIV-infected cells by inducing down-regulation of MHC class I proteins as well as the CD4 receptor through distinct molecular pathways (*8*−*10*). In addition to cell surface receptors, Nef also interacts with intracellular protein kinases, including several members of the Src tyrosine kinase family (*1*, *11*). One well-characterized Nef target kinase is Hck, a Src family member expressed primarily in myeloid leukocytes (*12*, *13*). Hck expression is particularly strong in macrophages, an important HIV target cell type (*14*, *15*). Biochemical and structural studies have established that Nef binds specifically to the Hck SH3 domain with unusually high affinity (*16*−*19*). This interaction is bipartite in nature, involving a highly conserved Nef proline-rich motif (PQVPxR) and the surface of SH3 as well as a very specific interaction of the Hck SH3 domain RT loop and a conserved hydrophobic pocket of Nef. Engagement of the Hck SH3 domain by Nef is sufficient to induce constitutive kinase activation both in vitro and in vivo (*20*−*22*). Recent genetic evidence suggests that this interaction contributes to Nef-induced AIDS-like disease in a transgenic mouse model (*23*). Whereas wild-type Nef expression is sufficient to produce AIDS-like disease in this model, mutations in the Nef

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[1] Abbreviations: HIV, human immunodeficiency virus; AIDS, acquired immunodeficiency syndrome; GFP, green fluorescent protein; ER, estrogen receptor hormone-binding domain; 4-HT, 4-hydroxytamoxifen; BiFC, bimolecular fluorescence complementation; YFP, yellow-shifted variant of GFP.

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proline-rich motif essential for Hck SH3 binding completely abolish this effect of Nef. Furthermore, Nef-induced disease progression is significantly delayed in mice in which both alleles of Hck have been disrupted by gene-targeting (*23*). Taken together, these data strongly suggest that Nef-induced activation of Hck contributes to HIV pathogenesis, and that selective inhibitors of this pathway may help to suppress the onset of AIDS.

The molecular basis of Nef-induced Hck activation has been clarified as a result of structural analyses of full-length, down-regulated Hck (*24*−*26*). These studies have shown that Hck, like Src, is held in an inactive conformation as a result of two intramolecular interactions. The first involves binding of the SH2 domain to the tyrosine-phosphorylated tail, a reaction that is catalyzed in vivo by a distinct kinase known as Csk (*27*). The second involves intramolecular engagement of SH3 with the linker joining the SH2 and kinase domains. Binding of Nef to the Hck SH3 domain displaces the linker and relieves its inhibitory effect on the kinase domain (*20*, *28*). Although linker displacement favors an active kinase conformation, full kinase activity also requires autophosphorylation of a conserved tyrosine residue in the activation loop. Structural studies show that the Nef protein can form dimers and higher order oligomers (*17*, *29*, *30*), which may contribute to Hck autophosphorylation by aligning two or more Hck kinase domains and promoting autophosphorylation by a trans mechanism. In this study, we provide direct evidence to support this hypothesis. First, we establish that Nef undergoes oligomerization in living cells using the technique of biomolecular GFP fluorescence complementation (*31*, *32*). We then show that regulated dimerization of Nef by fusion to the estrogen receptor ligand-binding domain allows for ligand-controlled Nef dimerization that correlates with sustained Hck activation in vivo. Conversely, we demonstrate that a Nef mutant defective for Hck SH3 binding but capable of dimerization suppresses Hck activation by wild-type Nef, forming a dead-end ternary complex with wild-type Nef and Hck. These data provide the first evidence that Nef forms oligomers in live cells, and show that oligomerization is essential for Hck activation. Our work identifies the Nef dimerization interface as a target for inhibitors of Nef-induced activation of Hck and possibly other kinases important for Nef signaling.

MATERIALS AND METHODS

Expression Vectors. To create the Nef-ER fusion construct, the coding sequence of HIV-1 Nef (SF2 strain) was amplified by PCR and subcloned into the mammalian expression vector, pCDNA3.1 (InVitrogen) to create the plasmid pCDNA3.1-Nef. The coding sequence of the murine ER ligand-binding domain (amino acids 281−599) was PCR-amplified from the plasmid pANMerCreMer (*33*). A point mutation in this ER sequence (G525R) abrogates estrogen-binding activity while retaining high affinity for the synthetic estrogen 4-hydroxytamoxifen (4-HT) (*34*). The ER segment was subcloned downstream and in-frame of the Nef C-terminus to generate pCDNA3.1-Nef-ER. Mutagenesis of Nef to create Nef-PA, a mutant in which prolines 72 and 75 are replaced with alanines, has been described elsewhere (*21*). Three sequential repeats of the coding sequence for the Flag epitope tag (DYKDDDDK) were added to the C-terminus of Nef-PA to distinguish it from wild-type Nef in co-expression experiments. Nef constructs were subcloned into the retroviral expression vector pSRαMSVtk*neo* (*35*), which carries a G418 resistance marker. The coding sequences of Hck (and EYFP as a negative control) were subcloned into the retroviral vector pMSCVpuro (Clontech), which carries a puromycin selection marker. Use of retroviral vectors carrying different selection markers permitted enforced expression of Hck and Nef proteins in co-infected cells by double antibiotic selection.

Bimolecular Fluorescence Complementation (BiFC) Analysis of Nef Oligomerization in Vivo. These experiments are based on recent observations by Hu et al. (*31*, *32*), which demonstrate that bringing two nonfluorescent fragments of green fluorescent protein (GFP) into close proximity as result of fusion to other interacting molecules leads to complementation of the GFP structure and a fluorescent signal. Experiments done here used an enhanced yellow-shifted variant of GFP, which we will refer to as YFP. To create BiFC vectors for HIV Nef, the coding sequence of N-terminal YFP amino acids Val 2 through Ala 154 were amplified from the plasmid vector pEYFP-C1 (Clontech), and ligated into the expression vector pCDNA3.1-Nef via a unique Acc III restriction site close to the Nef C-terminus. A similar approach was used to fuse Nef to the coding region for YFP C-terminal residues Ala 154−Lys 238. The resulting N- and C-terminal Nef-YFP fusions are referred to as Nef-YN and Nef-YC, respectively.

To test for fluorescence complementation, 293T cells were transfected with the Nef-YN and Nef-YC expression constructs either individually or in combination using standard calcium phosphate techniques (*21*, *22*, *36*, *37*). Parallel cultures were transfected with pCDNA3.1-Nef as a positive control for protein expression. Forty-eight hours later, cells were removed from the incubator and held at room temperature for 1−3 h prior to imaging to promote fluorophore maturation. Transfected cells were imaged using a Nikon TE300 inverted microscope with epifluorescence capability and a SPOT cooled CCD high-resolution digital camera and software (Diagnostic Instruments). Expression of each Nef protein was verified in cell lysates by immunoblotting with an anti-Nef antibody as described below.

Analysis of Nef Oligomerization by Co-Precipitation from Sf-9 Insect Cells. Construction of GST-Nef and GFP-Nef baculoviruses has been described elsewhere (*38*). Sf-9 insect cells were co-infected with either the GST or GST-Nef baculoviruses and the GFP-Nef baculovirus. Forty-eight hours later, cells were sonicated in Hck lysis buffer (50 mM Tris-HCl, pH 7.4, 50 mM NaCl, 1 mM EDTA, 10 mM $MgCl_2$, 1% Triton X-100), and GST proteins were precipitated with glutathione-agarose beads. Precipitates were collected by centrifugation, washed three times with Tris-buffered saline, and analyzed for associated GFP-Nef by immunoblotting with an anti-GFP antibody (Clontech). Expression of GST and GST-Nef was verified in the cell lysates by immunoblotting with an anti-GST antibody (Santa Cruz). Additional details of the GST-Nef precipitation assay can be found elsewhere (*38*).

Production of Recombinant Retroviruses and Transformation Assays. Retroviral expression vectors were used to generate high-titer retroviral stocks in 293T cells by co-transfection with an ecotropic packaging vector as described elsewhere (*21*, *22*, *36*, *37*). Rat-2 fibroblasts were obtained

from the ATCC and cultured in Dulbecco's modified Eagle's medium (DMEM) containing 5% FBS and 50 μg/mL gentamycin. Retroviral stocks were supplemented with Polybrene (hexadimethrine bromide, Sigma) to 4 μg/mL and added to Rat-2 fibroblasts in 6-well plates (2×10^5 cells/well). The plates were centrifuged at 1000*g* for 4 h at 18 °C to enhance infection efficiency (*39*). For co-infection experiments, cells were super-infected with the second retrovirus 24 h later. Two days after infection, cells were split into 60-mm culture dishes and cultured in the presence of both 800 μg/mL G418 and 2.5 μg/mL puromycin for two weeks. Nef-ER expressing cells were grown in the absence or presence of 4-HT (Sigma) at different concentrations ranging from 10 nM to 1 μM. Transformed foci were visualized by Wright-Giemsa staining and counted using a BioRad Imaging Densitometer and colony-counting software.

Retroviral Transduction of TF-1 Cells. TF-1 cells were obtained from the ATCC and cultured in RPMI medium containing 1 ng/mL GM-CSF, 10% FBS, and 50 μg/mL gentamycin. The pSRα-Nef-ER construct was used to generate amphotropic retroviruses by co-transfection of 293T cells as described above. Retroviral stocks were supplemented with Polybrene and added to TF-1 cells in 6-well plates (2.5×10^5 cells/well). Following incubation for 4 h at room temperature, retroviral supernatants were replaced with complete medium. Two days after infection, cells were selected with 800 μg/mL G418 for 10 days. TF-1/Nef-ER clonal cell lines were then established by limiting dilution, and Nef-ER expression was confirmed by immunoblotting. Nef-ER/TF-1 cell lines were then infected with an amphotropic Hck retrovirus carrying a puromycin selection marker as described above. The TF-1/Nef-ER/Hck cell population was selected with puromycin and Hck expression was confirmed by immunoblotting.

Immunoprecipitation and Immunoblotting. The antibodies used in this study include anti-Hck polyclonal (N-30; Santa Cruz Biotechnology), anti-Hck monoclonal (Transduction Laboratories), anti-Src phosphospecific (Src pY418; BioSource International), anti-phosphotyrosine (PY99; Santa Cruz), anti-Nef (monoclonal EH1; NIH AIDS Research & Reference Reagent Program), anti-Actin (MAB1501; Chemicon), and anti-FLAG (M2; Sigma). Rat-2 cells (90% confluent in 100-mm culture dishes) were lysed in either 1.0 mL of RIPA buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100, 0.1% SDS, 1 mM EDTA, 1% sodium deoxycholate) or in 1.0 mL of Hck lysis buffer. All buffers were supplemented with protease and phosphatase inhibitors (25 mM NaF, 2 mM Na_3VO_4, 2 mM PMSF, 25 μg/mL leupeptin, and 25 μg/mL aprotinin). To suppress nonspecific autophosphorylation of Hck during cell lysis and immunoprecipitation, buffers also contained the Src-selective kinase inhibitors PP2 (30 μM) and A-419259 (1 μM) (*40*). Cell lysates were clarified by centrifugation at 16000*g* for 10 min at 4 °C, and protein concentrations were determined using either the Bradford or BCA assay (Pierce). Aliquots of total protein were heated directly in SDS sample buffer and separated by SDS−PAGE. For Hck immunoprecipitation, protein concentrations were first normalized with lysis buffer, followed by addition of 1 μg of anti-Hck polyclonal antibody and 25 μL of protein G-Sepharose (50% slurry; Amersham Pharmacia Biotech). Following incubation for 2 h at 4 °C, immunoprecipitates were washed three times with 1.0 mL of RIPA buffer and heated in SDS sample buffer. Following SDS−PAGE, proteins were transferred to PVDF membranes for immunoblot analysis. Immunoreactive proteins were visualized with appropriate secondary antibody-alkaline phosphatase conjugates and NBT/BCIP colorimetric substrate (Sigma).

In Vivo Cross-Linking. The protein cross-linking reagent used in this study is the homobifunctional amine-reactive cross-linker disuccinimidyl suberate (DSS, Pierce). Fresh stock solutions of DSS (25 mM) were prepared in dry DMSO immediately prior to each experiment. Rat-2 cells expressing Nef-ER were washed with PBS, and DSS was added to a final concentration of 2 mM. Cells were incubated with DSS for 30 min at room temperature, and the reaction was stopped by adding Tris buffer (pH 7.4) to a final concentration of 50 mM. Cells were washed again with PBS and lysed in RIPA buffer. Lysates were clarified by centrifugation and protein concentrations were determined as described above. Protein aliquots were separated by SDS−PAGE and transferred to PVDF membranes, and Nef proteins were visualized by immunoblotting.

Subcellular Fractionation. Rat-2 cells were lysed by sonication in 500 μL of hypotonic buffer (10 mM Tris-HCl, pH 7.5, 5 mM EDTA) supplemented with protease and phosphatase inhibitors as described above. Lysates were centrifuged at 16000*g* for 10 min at 4 °C to pellet nuclei and unbroken cells. The supernatant was then recentrifuged at 100000*g* for 1 h at 4 °C, and the resulting supernatant was defined as the cytosolic fraction. The pellet was sonicated in RIPA buffer and centrifuged at 16000*g* for 10 min at 4 °C. The final supernatant was defined as the membrane fraction. Aliquots of each fraction were heated in SDS sample buffer prior to analysis by SDS−PAGE and immunoblotting.

RESULTS

Demonstration of Nef Oligomerization in Vivo by Co-Immunoprecipitation and BiFC. While crystallographic analysis has shown that HIV-1 Nef forms dimers, less evidence exists in support of Nef oligomerization in vivo. Before analyzing the potential role of Nef oligomerization in Hck activation, we first explored whether Nef formed stable oligomers in cells. In the first approach, we tagged Nef with either GFP or GST, and co-expressed the resulting Nef fusion proteins in Sf9 insect cells. GST-Nef was precipitated from infected cell lysates with glutathione-agarose, and analyzed for associated GFP-Nef by immunoblotting. As shown in Figure 1A, GFP-Nef readily precipitated with GST-Nef, while no complex was observed with GST alone, providing evidence for self-association of Nef in vivo.

As a second approach, we employed the technique of BiFC developed by Kerppola and co-workers to study the interaction and distribution of dimeric transcription factors in live cells (*31*, *32*). This technique takes advantage of the observation that bringing two nonfluorescent complementary fragments of YFP into close proximity by fusing them to interacting proteins leads to reassembly of a functional YFP structure. To apply this technique to HIV Nef, we created expression vectors for two Nef-YFP fusion proteins, the first

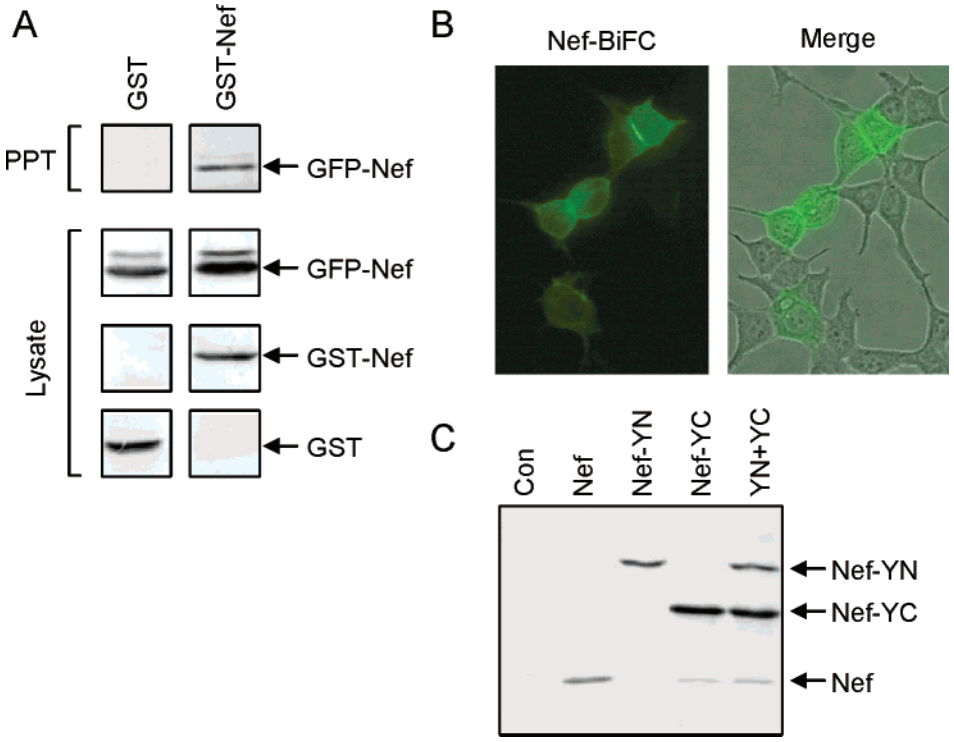

FIGURE 1: Oligomerization of HIV-1 Nef in vivo. (A) Sf9 insect cells were infected with GST or GST-Nef baculoviruses together with a GFP-Nef virus, and GST proteins were precipitated from clarified cell extracts 48 h later. Associated GFP-Nef was detected by immunoblotting (PPT; top). Cell lysates were immunoblotted with GFP or GST antibodies to control for expression of GFP-Nef, GST-Nef and GST. (B,C) Demonstration of Nef oligomerization in live cells by BiFC. 293T cells were transfected with expression vectors for Nef, Nef fused to an N-terminal YFP fragment (Nef-YN), a C-terminal YFP fragment (Nef-YC), or Nef-YN plus Nef-YC (YN+YC). Cells transfected with the parent vector served as a negative control (Con). Panel B shows YFP fluorescence from cells co-expressing both Nef-YN and -YC (Nef-BiFC; left), as well as a merged image of the same cells under fluorescence and bright field (merge; right). Note the enhanced fluorescence at the cell periphery, indicating that Nef oligomers localize to the cell membrane. No fluorescence was observed under any of the other transfection conditions (not shown). Panel C shows anti-Nef immunoblots of the transfected cell lysates to verify expression of each Nef protein. The positions of wild-type Nef and the Nef-YN and Nef-YC fusions are indicated by the arrows.

containing an N-terminal YFP fragment fused to the C-terminus of Nef (Nef-YN) and the second fused to a C-terminal YFP fragment (Nef-YC). The GFP fragments were fused to the C-terminal end of Nef to preserve the native N-terminal myristylation signal sequence. Nef-YN and Nef-YC were expressed individually or together in 293T cells, and the resulting fluorescent images are shown in Figure 1B. Cells expressing both Nef-YFP fusion proteins showed strong fluorescence, with remarkable localization to the cell periphery. In contrast, cells expressing either fragment alone showed no fluorescence (data not shown), despite strong expression as determined on immunoblots (Figure 1C). This result provides the first direct evidence for Nef oligomerization in live cells, and is consistent with the localization of Nef oligomers to the plasma membrane, a site essential for interaction with Hck (see below).

Enforced Dimerization of Nef by Fusion to the Estrogen Receptor Ligand Binding Domain (ER) Enhances Hck Activation in Vivo. Data shown in Figure 1 provide new evidence for Nef oligomerization in vivo, consistent with previous reports (*17*, *29*, *30*, *41*, *42*). This finding suggests that Nef may promote Src family kinase activation by juxtaposing two or more kinase monomers and promoting autophosphorylation through a trans mechanism. To test this idea, we created a regulated Nef dimer by fusing it to the hormone-binding domain of the estrogen receptor (Nef-ER), allowing us to control its oligomerization with the synthetic estrogen 4-HT. The ER domain was fused to the C-terminal end of Nef, keeping the native N-terminal myristylation signal sequence intact.

To study the role of oligomerization in Nef-induced Hck activation, we evaluated Hck activation by Nef-ER in Rat-2 fibroblast transformation assays. Previous studies from our laboratory have shown strong cell transformation upon co-expression of Hck and Nef in this model system, which correlates with stable Hck/Nef complex formation and constitutive Hck tyrosine kinase activation (*21*, *22*). Rat-2 cells were co-infected with recombinant Nef-ER and Hck retroviruses, and focus-forming activity was assessed as a function of 4-HT treatment. As shown in Figure 2, no transformed foci were observed in cells expressing Hck and Nef-ER in the absence of 4-HT. However, 4-HT treatment of cells co-expressing Nef-ER and Hck induced strong focus-forming activity. Interestingly, the number of transformed foci observed in the presence of 4-HT was 4−5-fold higher than cells expressing wild-type Nef and Hck, despite similar levels of Nef and Hck protein expression (Figure 3). Control plates show that expression of Hck, wild-type Nef, or Nef-ER individually did not induce transformation. Furthermore, cells expressing Nef-ER alone or co-expressing wild-type Nef and Hck were unaffected by 4-HT treatment.

We next correlated transformation with Hck kinase activity, which was evaluated in two ways. First, Hck autophosphorylation was assessed by immunoblotting with a phosphospecific antibody that recognizes the phosphorylated tyrosine residue in the activation loop of the kinase domain

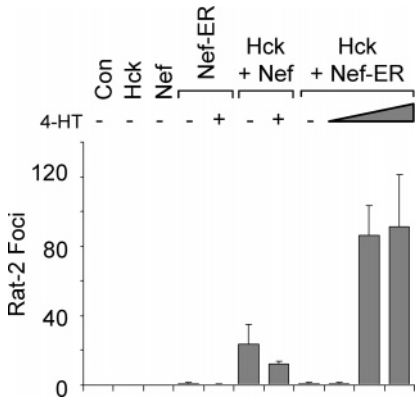

FIGURE 2: Enforced Nef dimerization enhances Hck transforming activity. Rat-2 fibroblasts were sequentially co-infected with either wild-type Nef or Nef-ER retroviruses in combination with either the Hck retrovirus or a GFP control virus. Infected cells were plated in triplicate under both G418 and puromycin selection to ensure co-expression of the Nef and Hck proteins, respectively. Cells expressing Nef-ER alone or wild-type Nef plus Hck were incubated in the presence or absence of 1 μM 4-HT. Cells co-expressing Nef-ER plus Hck were incubated with 0, 0.01, 0.1, or 1 μM 4-HT. Transformed foci were visualized after two weeks by Wright-Giemsa staining and counted using a scanning densitometer and colony-counting software. Data are presented as the mean number of foci $\pm$ standard deviation for a representative experiment. The entire experiment was repeated twice with comparable results.

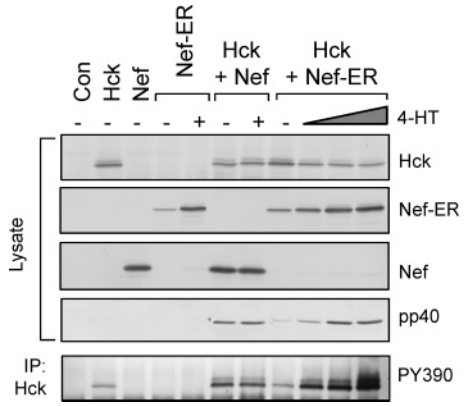

FIGURE 3: Nef-ER activates Hck in a 4-HT-dependent manner. Clarified cell lysates were prepared from each of the fibroblast cultures described in Figure 2 and analyzed by immunoblotting to verify Hck, Nef-ER, and wild-type Nef protein expression (top three panels). Phosphorylation of the endogenous Hck substrate protein p40 was detected on anti-phosphotyrosine immunoblots (pp40). Hck autophosphorylation was detected in anti-Hck immunoprecipitates with an antibody specific to the Hck activation loop tyrosine residue, Tyr 390 (PY390; bottom panel). Equivalent recovery of Hck in each immunoprecipitate was verified on an anti-Hck immunoblot (not shown). Control blots for actin verified equal protein loading for each of the lysate blots (not shown).

(PY390). Previous work from our lab has established the specificity of this antibody for the active form of Hck (*22*). Figure 3 shows that Hck autophosphorylation in the Nef-ER cells correlates closely with the transformation response. Very little PY390 immunoreactivity was observed on blots from untreated cells, while increasingly stronger signals were observed in transformed cells as a function of 4-HT concentration. To evaluate substrate phosphorylation by the Hck/Nef complex, we examined levels of the endogenous Hck substrate protein, pp40, on anti-phosphotyrosine immunoblots. Previous studies have shown that this protein is highly phosphorylated in Hck-transformed cells, and serves

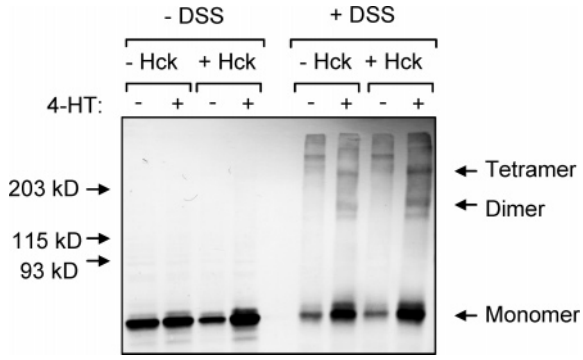

FIGURE 4: 4-HT-induces dimerization of Nef-ER in vivo. Rat-2 fibroblasts stably expressing Nef-ER either alone (−Hck) or together with Hck (+Hck) were cultured in the absence (−) or presence (+) of 1 μM 4-hydroxytamoxifen (4-HT). The cells were then incubated in the presence or absence of the lipid-soluble chemical cross-linker DSS at a final concentration of 2 mM in PBS for 30 min at room temperature. Cross-linking reactions were quenched by the addition of Tris buffer, and protein extracts were prepared and analyzed by immunoblotting using an anti-Nef antibody. The positions of the Nef-ER monomers, dimers, and tetramers are indicated by the arrows. Immunoblotting with an anti-ER antibody produced identical results (not shown). The positions of the molecular weight standards are indicated on the left.

as a useful marker for Hck activation in vivo (*21*, *22*). As shown in Figure 3, phosphorylated p40 was readily apparent in 4-HT-treated cells co-expressing Nef-ER but was virtually undetectable in the absence of 4-HT. Both Hck autophosphorylation and p40 tyrosine phosphorylation were also detected in control cells expressing wild-type Nef and Hck, but were very low or undetectable in control cell populations. As with the transformation data, cells expressing Nef-ER and Hck in the presence of 4-HT showed much higher levels Hck autophosphorylation compared to cells expressing Hck and wild-type Nef. Taken together, these data show that enforced dimerization of Nef through ER fusion leads to enhanced Hck activation and signaling in vivo, consistent with a requirement for Nef dimerization in the activation of this Src family member (see Discussion).

To verify that 4-HT actually affects the state of Nef-ER oligomerization in vivo, Rat-2 cells expressing Nef-ER either alone or together with Hck were incubated in the presence or absence of 4-HT. The cells were then treated with the bifunctional cross-linker, disuccinimidyl suberate (DSS), to stabilize the Nef-ER oligomers prior to analysis by SDS−PAGE and immunoblotting. As shown in Figure 4, two high molecular weight bands were readily detected in lysates from cells treated with 4-HT and DSS, which correspond very closely to the predicted molecular mass of Nef-ER dimers (130 kDa) and tetramers (260 kDa). Neither of these bands was observed in the absence of 4-HT treatment, although a single band of very high molecular weight was present which may correspond to a complex of Nef-ER with HSP90 and other molecular chaperones. 4-HT-induced Nef-ER oligomerization was not affected by co-expression of Hck, and no oligomerization was detected in the absence of 4-HT treatment. These experiments support the conclusion that 4-HT induces Nef-ER oligomerization, which in turn induces constitutive Hck activation and downstream signaling.

Enforced Dimerization Stabilizes Membrane Association of Nef-ER. Results presented in the previous section demonstrate that 4-HT-dependent dimerization of Nef-ER is not

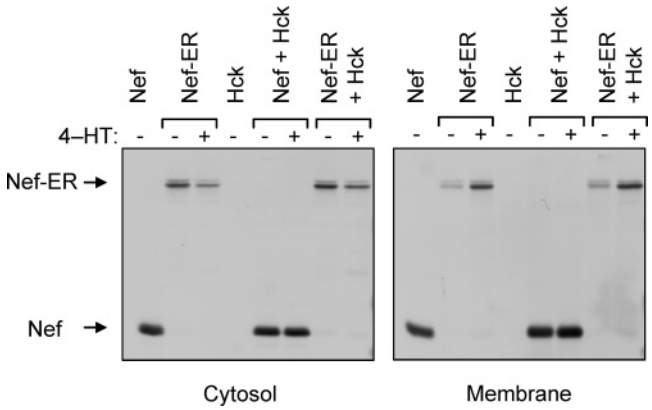

FIGURE 5: Dimerization enhances Nef-ER membrane localization. Rat-2 cells stably expressing wild-type Nef, Nef-ER, or Hck either alone or in the combinations shown were cultured in the presence (+) or absence (−) of 1 μM 4-HT and lysed in hypotonic buffer. Nuclei were removed by low-speed centrifugation, and the resulting supernatant was separated into cytosolic and membrane fractions by ultracentrifugation. The relative proportions of Nef and Nef-ER present in each fraction were analyzed by anti-Nef immunoblotting. The positions of Nef and Nef-ER are indicated by the arrows.

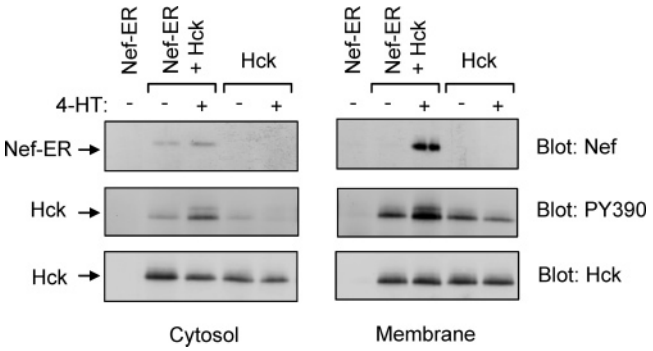

FIGURE 6: Dimerization stabilizes Nef-ER interaction with Hck in the membrane fraction. Rat-2 cells expressing Nef-ER or Hck either alone or in combination were cultured in the presence or absence of 1 μM 4-HT and separated into cytosolic and membrane fractions as described in Figure 5. Hck proteins were immunoprecipitated from each fraction and associated Nef-ER was visualized by immunoblotting (top panels). Hck autophosphorylation was assessed by immunoblotting with a phosphospecific antibody (PY390; middle panels). Hck immunoblots show equal recovery of Hck protein in the immunoprecipitates (bottom panels).

only required for Hck activation, but substantially enhances Hck signaling relative to the wild-type Hck/Nef complex. Because both Hck and Nef require membrane targeting for their biological activities (*1*, *43*−*45*), we investigated the effect of dimerization on the subcellular distribution of Nef-ER. Rat-2 cells expressing Nef-ER either alone or in combination with Hck were incubated in the presence or absence of 4-HT, and the relative proportion of Nef-ER present in the membrane and cytoplasmic fractions was determined by immunoblotting. As shown in Figure 5, Nef-ER was localized predominantly in the cytosolic fraction in the absence of 4-HT. Following 4-HT treatment, however, Nef-ER underwent a dramatic shift from the cytosolic to the membrane fraction, suggesting that dimerization stabilizes Nef interaction with the membrane. This effect was not influenced by the presence of Hck, suggesting that dimerization stabilizes a conformation of Nef that favors membrane association as suggested by Arold et al. (*3*, *29*). The effect of 4-HT was specific to cells expressing Nef-ER, and had no impact on the subcellular distribution of wild-type Nef.

We next investigated whether enhanced membrane targeting of Nef-ER affected its interaction with Hck. Rat-2 cells expressing Nef-ER, Hck, or a combination of the two were incubated in the presence or absence of 4-HT. Hck was then immunoprecipitated from the cytosolic and membrane compartments, and analyzed for associated Nef-ER by immunoblotting. As shown in Figure 6, low levels of Nef-ER associated with Hck in the cytosol in a 4-HT-independent manner. 4-HT treatment, however, induced a dramatic increase in Nef-ER/Hck complex formation in the membrane fraction. In addition, anti-pY390 immunoblots show that Hck is more strongly autophosphorylated in the 4-HT-treated membrane fraction. Control blots show that equal amounts of Hck are present in each immunoprecipitate. Taken together, these results suggest that oligomerization stabilizes Nef/Hck interaction at the cell membrane, promoting Hck autophosphorylation and kinase activation. These data are consistent with other studies showing that the active forms of these two signaling proteins localize to lipid rafts and other membrane compartments (*43*−*46*).

Suppression of Nef-Induced Hck Activation by Nef-PA, a Dimerizable but Hck Interaction-Deficient Mutant. In a complementary approach to investigate the role of Nef dimerization in Hck activation, we tested the effect of a Nef mutant lacking SH3-binding activity (Nef-PA) on signaling by the wild-type Nef/Hck complex. In this Nef mutant, the two highly conserved proline residues critical for Hck SH3 binding are substituted with alanines. Previous data from our laboratory have demonstrated that Nef-PA is unable to interact with or activate Hck either in the fibroblast system or a macrophage progenitor cell line (*21*, *47*). Because the Nef proline-rich motif responsible for SH3 binding is spatially separated from the dimerization interface in the crystal structures (*17*, *30*), we predicted that Nef-PA should retain the ability to dimerize both with itself and with wild-type Nef. If dimerization is required for Hck activation, then Nef-PA should act in a dominant-negative manner by forming nonproductive dimers with wild-type Nef. Such heterodimers are predicted to trap monomeric Hck in a nonproductive ternary complex.

To test Nef-PA for dominant-negative activity, Rat-2 fibroblasts were co-infected with a Hck retrovirus and either the wild-type Nef or Nef-PA retroviruses, or both. To enable us to distinguish Nef-PA from wild-type Nef on the same immunoblots, a triple Flag epitope tag was added to the C-terminus of Nef-PA. As shown in Figure 7A, co-expression of Hck with wild-type Nef resulted in strong focus-forming activity, while co-expression with Nef-PA was without effect, in agreement with our previous work (*21*). However, co-expression of Hck with wild-type Nef and Nef-PA reduced the number of transformed foci by more than half. Figure 7B shows that the suppressive effect of Nef-PA on transformation correlated with reduced levels of endogenous p40 tyrosine phosphorylation, consistent with the idea that Nef-PA interferes with Hck activation by wild-type Nef. Control blots show equivalent expression of Nef and Hck proteins across each of the cell populations.

Further support for the mechanism of Nef-PA suppression of Hck/Nef signaling comes from co-immunoprecipitation

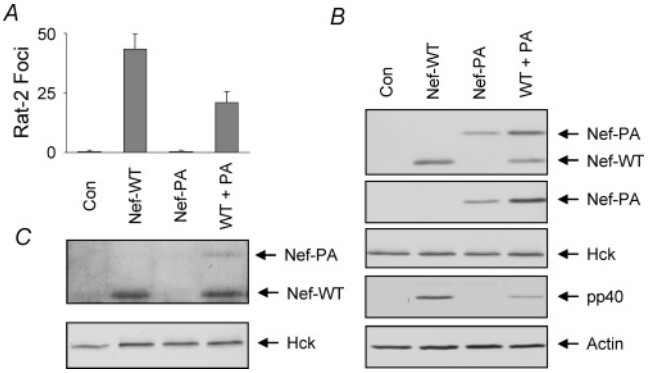

FIGURE 7: Suppression of Nef-induced Hck activation by the SH3 binding mutant, Nef-PA. Rat-2 fibroblasts were co-infected with a Hck retrovirus and either GFP, wild-type Nef (Nef-WT), Nef-PA, or both Nef viruses. (A) Focus assay. Transformed foci were visualized after two weeks by Wright-Giemsa staining and counted using a scanning densitometer and colony-counting software. Data are presented as the mean number of foci ± standard deviation for a representative experiment. The entire experiment was repeated twice with comparable results. (B) Analysis of protein expression and kinase activity. Clarified cell lysates were prepared from each of the cultures in part A, and analyzed by immunoblotting to verify expression of Nef-PA, Nef-WT, and Hck (top three panels). The top panel was blotted with the Nef antibody. The lower mobility of Nef-PA is due to the presence of a triple Flag-tag on its C-terminus. The second panel shows a replicate blot of Nef-PA with the anti-Flag antibody. The third panel shows equivalent expression of Hck on anti-Hck immunoblots. The fourth panel reveals phosphorylation of the endogenous Hck substrate p40 on anti-phosphotyrosine immunoblots (pp40). The bottom panel shows a control blot for actin to verify equal protein loading for each of the lysates. (C) Nef-PA forms a ternary complex with wild-type Nef and Hck. Clarified lysates were prepared from each of the cultures shown in part A, and Hck was immunoprecipitated and probed for associated Nef proteins by immunoblotting. The positions of wild-type Nef and Nef-PA are indicated by the arrows (top panel). A control immunoblot shows equal recovery of Hck protein in the immunoprecipitates (bottom panel).

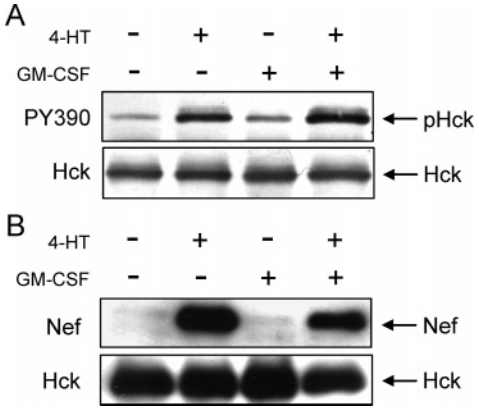

FIGURE 8: Nef-ER activates Hck in a 4-HT-dependent manner in the human macrophage precursor cell line, TF-1. TF-1 cells were co-infected with Nef-ER and Hck retroviruses and selected with G418 and puromycin as described under Experimental Procedures. (A) Cells were cultured in the presence or absence of 1 μM 4-HT for 24 h, washed, and then incubated in the presence or absence of GM-CSF and 4-HT for an additional 16 h as indicated. Hck was immunoprecipitated from clarified cell extracts and immunoblotted with a phosphospecific antibody against the Hck activation loop phosphotyrosine residue, P-Tyr 390 (PY390; top panel). Aliquots of the Hck immunoprecipitates were also blotted with the Hck antibody to verify equivalent protein recovery (lower panel). (B) Hck/Nef-ER complex formation. Cells were treated as described in part A. Hck was immunoprecipitated from each culture and associated Nef-ER was visualized by immunoblotting (top panel). Equivalent recovery of Hck in each immunoprecipitate was verified on an anti-Hck immunoblot (bottom panel).

experiments. Hck was immunoprecipiated from each of the Rat-2 cell populations described above, and analyzed for associated Nef by immunoblotting. As shown in Figure 7C, wild-type Nef was readily observed in the Hck immunoprecipitates from cells co-expressing these proteins, while Nef-PA failed to bind Hck on its own. However, Nef-PA was detected in Hck immunoprecipitates from cells co-expressing both wild-type Nef and Nef-PA, providing direct evidence for a Hck/Nef/Nef-PA ternary complex. Together these data strongly suggest that Nef-PA dimerizes with wild-type Nef, leading to sequestration of Hck as an inactive monomer.

Dimerization-Dependent Activation of Hck by Nef-ER in a Human Macrophage Precursor Cell Line. Previous studies from our laboratory established that Nef promotes survival signaling in the human myeloid leukemia cell line, TF-1 (*47*). Constitutive expression of Nef led to cytokine-independent proliferation in these cells, which correlated with constitutive activation of Hck. Here we used this model system to evaluate whether Nef dimerization is involved in Hck activation in a cell lineage relevant to HIV infection.

TF-1 cells were infected with the Nef-ER retrovirus described above, and selected in the presence of G418. Because TF-1 cells express low levels of endogenous Hck, we then created a population of TF-1/Nef-ER cells that express wild-type Hck at levels similar to those observed in mature macrophages. This was accomplished using the Hck retrovirus with puromycin as a selection marker (see Experimental Procedures). The resulting cell population was then examined for the activation of Hck in a 4-HT-dependent manner as described above for the fibroblast experiments. As shown in Figure 8A, treatment of these TF-1/Nef-ER cells with 4-HT strongly increased the extent of Hck autophosphorylation as judged by immunoblotting with the phosphospecific antibody. Note that Nef-ER-induced Hck autophosphorylation is independent of the presence of GM-CSF, which also induced a slight increase in Hck autophosphorylation on its own. Co-immunoprecipitation experiments show that Nef-ER/Hck complex formation is also enhanced by 4-HT (Figure 8B), consistent with the results in Rat-2 cells. These results suggest that Nef dimerization is important for both the recruitment and stimulation of Hck kinase activity in a cell lineage relevant to HIV infection.

DISCUSSION

Previous work from our laboratory and others has established that HIV-1 Nef binds strongly to the SH3 domain of Hck, leading to constitutive kinase activation both in vitro and in vivo (*20*−*22*, *37*, *38*, *47*). Hck is required for M-tropic HIV replication in cultured macrophages (*48*) and for Nef-driven AIDS-like disease in a transgenic mouse model (*7*, *23*), identifying the Hck/Nef complex as a possible target for anti-HIV therapy. Because the Nef protein forms dimers and higher-order oligomers in vitro and in vivo (*29*, *30*, *49*), activation of Hck by Nef may involve recruitment of multiple kinase monomers, followed by *trans*-autophosphorylation of the juxtaposed kinase domains. BiFC experiments presented in Figure 1 provide direct evidence that Nef oligomers localize to the plasma membrane, suggesting that Nef may substitute for natural upstream activators of Src family

kinases such as growth factor, cytokine, and antigen receptors, which also exist as oligomers in their active forms (*50*−*52*). Several lines of evidence presented here support this model.

In one set of experiments, we created a regulated dimer of Nef by fusing it to the hormone-binding domain of the estrogen receptor. Unlike wild-type Nef, the Nef-ER fusion protein failed to activate Hck in the absence of the dimerizer (4-HT), despite the ability of this protein to associate with Hck in the cytosol (Figure 6). This finding suggests that SH3 engagement per se may not be sufficient for Nef-induced activation, and that juxtaposition of at least two Hck monomers is essential for autophosphorylation and subsequent signaling. Addition of 4-HT and subsequent Nef dimerization led to Hck autophosphorylation and cellular transformation, consistent with this idea. Enforced dimerization of Nef-ER by 4-HT induced Hck autophosphorylation to a much greater extent than native Nef, and led to a stronger transformation response (Figures 2 and 3). One possible explanation for this observation is that enforced dimerization increases the lifetime of Nef at the plasma membrane, a subcellular site essential for both Nef and Hck signaling functions (*43*−*46*). As a result, Nef-ER may be able to recruit and activate more molecules of membrane-bound Hck relative to wild-type Nef. Data presented in Figures 5 and 6 support this idea, where the proportion of Nef-ER in the membrane fraction was found to dramatically increase in the presence of the dimerizer, as was the amount of Nef-ER bound to Hck. This observation is consistent with recent work by Walk et al., which shows that 4-HT induces accumulation of a similar Nef-ER fusion protein in lipid rafts from a Nef-ER-expressing T-cell line (*53*). The Hck fraction associated with Nef-ER at the membrane also showed enhanced phosphorylation at the activation loop, supporting the idea that association of Hck with Nef at the membrane is essential for Hck autophosphorylation. Activation of Hck by Nef at the membrane may also be essential for downstream signaling. Other work from our laboratory has shown that Nef-induced survival of human macrophage progenitor cells requires the Nef-membrane targeting signal (myristylation) and correlates with endogenous Hck and Stat3 activation (*47*). We extend this finding here by showing that Nef dimerization is also required for Hck recruitment and activation in a myeloid cell context (Figure 8).

Although we observed the association of cytoplasmic Nef-ER with Hck in the absence of 4-HT, Nef-ER complexes with Hck were not observed in the membrane fraction without 4-HT treatment (Figure 6). This observation suggests that Nef-ER may be sterically hindered from interaction with Hck due to chaperone binding, providing an alternative explanation for the lack of Hck activation by Nef-ER in the absence of 4-HT. However, this possibility does not diminish the observation that following enforced dimerization with 4-HT, Nef-ER undergoes dimerization and leads to very strong binding and activation of Hck. Comparison of the transforming activity of wild-type Nef plus Hck vs Nef-ER plus Hck in the presence of 4-HT underscores this view. Nef-ER induced a much stronger transforming signal from Hck in the presence of 4-HT than did wild-type Nef (Figure 2), despite the observations that Nef and Nef-ER are expressed to the same extent (Figure 3) and that both proteins localize to the membrane (Figure 5). This biological effect correlates with greatly enhanced autophosphorylation of Hck by Nef-ER in the presence of ligand (Figure 3), as well as strong Hck−Nef complex formation in the membrane fraction (Figure 6), supporting the hypothesis that Nef oligomers enhance Hck activation by promoting autophosphorylation at the plasma membrane.

The enhancement of Hck signaling observed upon 4-HT induced dimerization of Nef-ER at the membrane suggests that oligomerization may influence Nef interactions with other target proteins as well. Walk et al. (*53*) demonstrated 4-HT-inducible interaction of Nef with an endogenous 62 kDa Ser/Thr kinase activity in a T-cell line. This finding suggests that dimerization of Nef may be important for activation of kinases in addition to Hck, possibly through the *trans*-autophosphorylation mechanism proposed here. Induction of cell-surface CD4 and HLA downregulation by Nef-ER was also shown to be dependent upon 4-HT treatment, suggesting that other Nef functions also require dimerization. Interestingly, recent studies have shown that Nef-induced downregulation of MHC-I requires the Nef proline-rich motif (*8*), suggesting that recruitment and activation of Hck or other Src family members may help to couple Nef to the endocytic machinery.

In a complementary approach, we tested a Nef mutant that is defective for Hck binding for dominant-negative activity against the wild-type Hck/Nef complex. This mutant, Nef-PA, has alanine substitutions for the conserved prolines (Pro 72, Pro 75) essential for Hck SH3 engagement and kinase activation in vivo (*18*, *21*). This Nef motif is distinct from the dimerization interface in the Nef crystal structure, suggesting that Nef-PA should dimerize with wild-type Nef. Because the putative wild-type/mutant heterodimer has only one functional SH3-binding site, it was predicted to recruit only a single Hck molecule and therefore interfere with full kinase activation without a partner for trans-phosphorylation. Data presented in Figure 7 support this prediction. The Nef-PA mutant suppressed focus-forming activity by the wild-type Hck/Nef complex with a concomitant reduction in p40 tyrosine phosphorylation. Importantly, while co-immunoprecipitation experiments showed that Nef-PA was unable to interact directly with Hck, co-expression of Nef-PA with Hck and wild-type Nef led to the formation of a ternary complex. This experiment not only supports the idea that the Nef dimer is required for Hck kinase activation in vivo, but also provides an important proof-of-concept that inhibitors of Nef dimerization may suppress Nef-induced activation of Hck and other kinases important for AIDS pathogenesis.

In summary, data presented in this report support the conclusion that Nef dimerization is necessary for the Nef-induced activation of Hck. On the basis of these observations and previous data (*21*, *22*), we propose a two-step mechanism for Nef-induced Hck activation. First, each half of the Nef dimer recruits one molecule of Hck through its SH3 domain. Nef binding also displaces the Hck SH3 domain from its inhibitory intramolecular interaction with the SH2-kinase linker. Previous studies have shown that Nef-SH3 engagement pushes Hck toward a more active conformation by relieving the inhibitory strain of linker interaction with the αC helix of the kinase domain (*20*, *28*). The resulting reorganization of the catalytic cleft exposes the activation loop tyrosine, positioning it for autophosphorylation. Because Nef can oligomerize, SH3-based recruitment also serves to

juxtapose two or more Hck monomers. In the second step, the activation loop tyrosine of one Nef-bound Hck monomer is *trans*-phosphorylated by the other, and vice versa. This step requires a close proximity of two Hck molecules so that the activation loop of one kinase domain can reach the substrate binding site of the other. We have observed that wild-type Hck can *trans*-phosphorylate kinase-defective Hck on its activation loop, consistent with this proposed mechanism for Nef-induced autophosphorylation (H. Ye and T. Smithgall, unpublished observations). Autophosphorylation locks the kinase domain in the active conformation, with the catalytic cleft accessible for substrate binding and phosphorylation (*54*). Thus, the active Hck/Nef complex displays multiple surfaces for selective inhibitor discovery that are unique to HIV-infected cells, including the Nef dimer interface, the Nef-SH3 domain binding site, as well as the active kinase domain of Nef-associated Hck.

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