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Affinity of Src Family Kinase SH3 Domains for HIV Nef in Vitro Does Not Predict Kinase Activation by Nef in Vivo†

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ABSTRACT: Nef is an HIV accessory protein required for high-titer viral replication and AIDS progression. Previous studies have shown that the SH3 domains of Hck and Lyn bind to Nef via proline-rich sequences in vitro, identifying these Src-related kinases as potential targets for Nef in vivo. Association of Nef with Hck causes displacement of the intramolecular interaction between the SH3 domain and the SH2-kinase linker, leading to kinase activation both in vitro and in vivo. In this study, we investigated whether interaction with Nef induces activation of other Src family kinases (Lyn, Fyn, Src, and Lck) following coexpression with Nef in Rat-2 fibroblasts. Coexpression with Nef induced Hck kinase activation and fibroblast transformation, consistent with previous results. In contrast, coexpression of Nef with Lyn was without effect, despite equivalent binding of Nef to full-length Lyn and Hck. Furthermore, Nef was found to suppress the kinase and transforming activities of Fyn, the SH3 domain of which exhibits low affinity for Nef. Coexpression with Nef did not alter c-Src or Lck tyrosine kinase or transforming activity in this system. Differential modulation of Src family members by Nef may produce unique downstream signals depending on the profile of Src kinases expressed in a given cell type.

Nef is a highly conserved gene found in human and simian immunodeficiency viruses (HIV-1, HIV-2, SIV) that has an important role in AIDS progression (*1*−*3*). Rhesus monkeys infected with strains of SIV lacking Nef do not develop high viral loads or progress to AIDS-like disease (*4*). Similarly, individuals infected with *nef*-defective strains of HIV experience long-term nonprogressive HIV infection and low viral loads (*5*, *6*). Targeted expression of Nef in the T-cells and macrophages of transgenic mice is sufficient to produce an AIDS-like syndrome, providing further evidence that Nef is an important determinant of AIDS pathogenesis (*7*). Although these studies implicate Nef in HIV replication and disease progression, the mechanism of Nef action at the molecular level is still unclear.

Nef is a 25−30 kDa myristylated protein with no known catalytic activity that appears to interact with cellular proteins involved in signal transduction pathways. Protein kinases represent one important group of Nef-associated proteins,

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and both serine/threonine and tyrosine kinases have been shown to associate with Nef (*2*). Tyrosine kinases of the Src family, including Hck, Lyn, Fyn, and Lck, are of particular interest because of their multiple roles in lymphocyte and macrophage signaling pathways (*8*). Nef−Hck interaction requires the Hck SH3 domain, which binds to a Nef proline-rich sequence with high affinity (*9*). This Nef proline-rich motif is highly conserved among known HIV isolates (*10*). Nef interacts with the Lyn and Hck SH3 domains to a similar extent in filter binding assays (*11*), suggesting that Lyn may also be a target for Nef in vivo. In contrast to Hck and Lyn, the SH3 domains of Fyn, Lck, and Src bind to Nef with 10-fold lower affinity than Hck as determined by isothermal titration calorimetry (*12*). In the case of Lck, however, the SH2 domain and possibly other regions may contribute to Nef interaction (*13*, *14*).

The X-ray crystal structures of Src and Hck demonstrate that the SH3 and SH2 domains are involved in intramolecular interactions that negatively regulate tyrosine kinase activity (*15*−*17*). The SH2 domain associates with the C-terminal regulatory tyrosine as a result of phosphorylation by Csk, while the SH3 domain interacts with a polyproline type II helix located between the SH2 and kinase domains (SH2-kinase linker). Mutations of the conserved tyrosine residue in the C-terminal tail, the SH2-kinase linker, and also the SH2 or SH3 domains all release Src family kinase activity and induce oncogenic transformation of fibroblasts, presumably by disturbing these intramolecular regulatory contacts (*18*−*23*).

Previous work in our laboratory has established that coexpression of Hck and Nef in fibroblasts induces Hck activation and leads to cellular transformation (*19*, *24*). Hck activation by Nef requires the Nef proline-rich motif, implicating displacement of the Hck SH3 domain from the SH2-kinase linker in the activation mechanism. Here we extended this study to other members of the Src kinase family to determine if the affinity of Nef−SH3 domain interaction is generally predictive of Src kinase activation using the same model system. Unlike Hck, no increase in transformation, kinase activation, or endogenous protein phosphorylation was observed following coexpression of Nef with Fyn, Lyn, Src, or Lck in Rat-2 fibroblasts. This result was particularly surprising for Lyn because of the strong interaction of its SH3 domain with Nef in vitro (*11*). In addition, Nef was found to inhibit the transforming and kinase activities of Fyn. These findings suggest that interaction of Nef with kinases other than Hck may involve contacts in addition to or outside of the SH3 domain, and have implications for the overall impact of Nef on Src family kinase signaling in different cell types.

MATERIALS AND METHODS

Retroviral Expression Constructs. Construction of the human Hck cDNA clone as well as retroviral expression vectors for Hck and Nef has been described elsewhere (*24*, *25*). Partial cDNA clones of Fyn and Lyn were obtained from the ATCC. Full-length clones were constructed by RT-PCR amplification of the required 5′ sequences and splicing the resulting products together with the partial clones via unique internal restriction sites. The nucleotide sequences of all PCR-derived cDNA fragments were verified by automated DNA sequence analysis. cDNA clones of human c-Src and Lck were graciously provided by Dr. Owen Witte, Howard Hughes Medical Institute, UCLA, and Dr. Mario Stevenson, University of Massachusetts Medical School, respectively. All Src family cDNAs were subcloned into the retroviral vector pSRαMSVtkneo (*26*). Retroviral stocks were generated by cotransfection of 293T cells with the retroviral vectors and an ecotropic packaging vector as described elsewhere (*24*). Negative control retroviruses were prepared using the empty pSRαMSVtkneo parent vector.

Transformation Assays. Rat-2 fibroblasts were obtained from the ATCC and grown in Dulbecco's modified Eagle's medium (DMEM) containing 5% fetal bovine serum and 50 μg/mL gentamycin. For transformation assays, Rat-2 fibroblasts (2×10^4) were plated in 6-well tissue culture plates 1 day prior to infection. The following day, cells were infected with Nef or negative control retroviruses. Polybrene (4 μg/mL) was added, and plates were centrifuged at 1000*g* for 4 h at 18 °C to enhance infection efficiency (*27*). After infection, virus-containing medium was aspirated and replaced with 5 mL of fresh medium. The next day, the cells were re-infected with the Hck, Src, Lyn, Fyn, or Lck retroviruses using the same procedure. Two days later, cells were trypsinized and equally divided into four 60 mm dishes, and 5 mL of medium containing G-418 (800 μg/mL) was added. G-418-containing medium was replaced every 3 days for 14 days. At day 14, transformed foci were visualized by Wright−Giemsa staining. Duplicate plates from the same experiment were used to verify Nef and Src family kinase expression and to assess kinase activity.

In Vitro Kinase Assays. Kinase assays were performed as described previously (*19*, *24*). Briefly, Rat-2 fibroblasts (confluent 60 mm culture dish) were flash-frozen on liquid nitrogen and lysed by thawing in 1.0 mL of modified RIPA buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100, 0.1% SDS, 1 mM EDTA, and 1% sodium deoxycholate) supplemented with 20 mM NaF, 1 mM Na_3VO_4, and 50 μM $NaMoO_4$. The lysates were clarified by centrifugation at 100000*g* for 15 min at 4 °C, and protein concentrations were determined using the Bradford assay (Pierce). Clarified lysates (1.0 mg total protein) were incubated with 1 μg of anti-Hck, anti-Lyn, anti-Src, or anti-Fyn polyclonal antibodies (Santa Cruz Biotechnology) or anti-Lck monoclonal antibody (Santa Cruz Biotechnology) and 20 μL of protein G−Sepharose (50% slurry; Pharmacia) for 2 h at 4 °C. Immunoprecipitates were washed twice with 1.0 mL of RIPA buffer followed by two washes with 1.0 mL of kinase buffer (50 mM HEPES, pH 7.4, 10 mM $MgCl_2$). Kinase buffer (20 μL) containing 1 μg of the tyrosine kinase substrate p50 [50 kDa GST fusion protein containing residues 331−443 of the Src substrate protein Sam 68 (*28*, *29*); Santa Cruz Biotechnology] and 5 μCi of [γ-^{32}P]ATP (3000 Ci/mmol; Dupont/New England Nuclear) was added, and the reactions were incubated for 15 min at 30 °C. Reactions were stopped by adding SDS−PAGE sample buffer and heating to 95 °C for 5 min. Src kinase proteins were resolved by SDS−PAGE, transferred to PVDF membranes, and probed with anti-Hck, anti-Lyn, or anti-Fyn monoclonal antibodies (Transduction Laboratories), anti-Src polyclonal antibody (Santa Cruz Biotechnology), or anti-Lck monoclonal antibody (Santa Cruz Biotechnology). Immunoprecipitated Src kinase protein levels were quantitated by densitometry

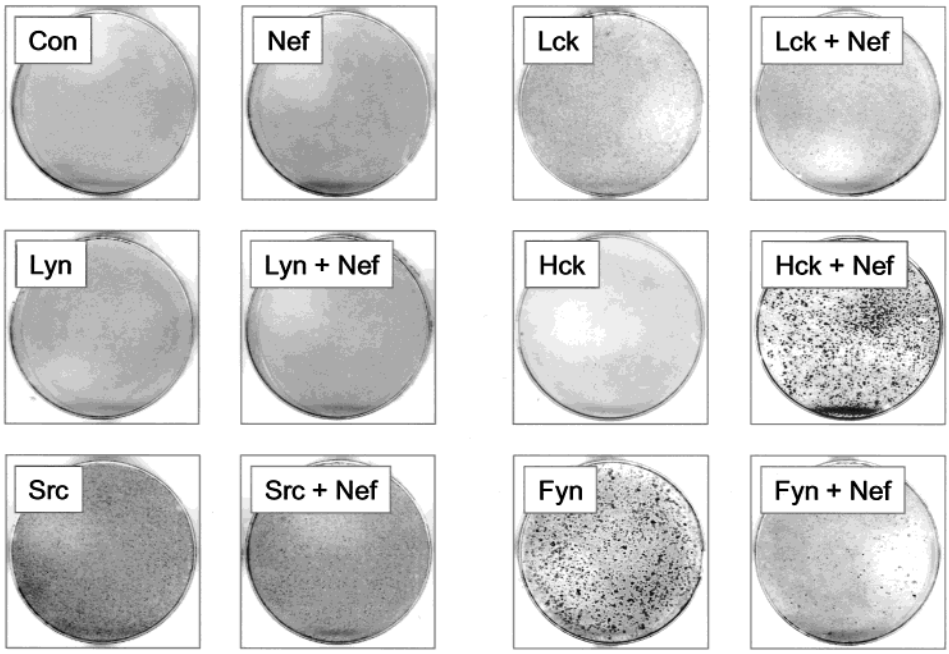

FIGURE 1: Focus-forming assay of Rat-2 fibroblasts coexpressing Src family kinases and Nef. Rat-2 fibroblasts were sequentially coinfected with Nef and Src kinase retroviruses (Lyn + Nef, Src + Nef, Lck + Nef, Hck + Nef, Fyn + Nef) as described under Materials and Methods. Parallel cultures were coinfected with a negative control retrovirus carrying only the neo selection marker and the Src retroviruses (Lyn, Src, Lck, Hck, Fyn). Additional controls included infection with the control virus alone (Con) or in combination with Nef (Nef). Infected cells were selected with G-418 for 14 days, and transformed foci were visualized by Wright−Giemsa staining. Three independent experiments produced comparable results; a representative plate is shown.

(Molecular Dynamics Densitometer). Radiolabeled Src kinases and p50 were visualized and quantitated by storage phosphor imaging (Molecular Dynamics PhosphorImager), and relative changes in kinase activity were normalized to protein levels.

Analysis of Tyrosine Phosphorylation of Endogenous Proteins. Rat-2 fibroblasts expressing various Src kinases (Hck, Src, Lyn, Fyn, or Lck) in the presence or absence of Nef were flash-frozen on liquid nitrogen and lysed by thawing in 1.0 mL of RIPA buffer as described above. The lysates were clarified by centrifugation at 100000*g* for 15 min at 4 °C, and protein concentrations were determined using the Bradford Assay (Pierce). SDS sample buffer was added directly to the clarified lysates. Lysate proteins (30 μg) were resolved by SDS−PAGE, transferred to PVDF membranes, and probed with anti-phosphotyrosine antibodies (PY20; Transduction Laboratories). Crude lysates were also probed with anti-Hck, anti-Lyn, or anti-Fyn monoclonal antibodies (Transduction Laboratories), the anti-Src polyclonal antibody (Santa Cruz Biotechnology), the anti-Lck monoclonal antibody (Santa Cruz Biotechnology), or the anti-Nef antibody to verify expression of each respective protein.

Expression of Nef and Src Kinases in Sf-9 Insect Cells and GST−Nef Binding Assay. The coding region of Nef from the SF2 strain of HIV-1 was amplified by PCR and subcloned into the baculovirus transfer vector pVL-GST (*30*), downstream and in-frame with the coding sequence of glutathione *S*-transferase. The Fyn, Lyn, and Lck cDNAs were subcloned into the baculovirus transfer vector pVL1392. The resulting transfer vectors were used to produce recombinant baculoviruses in Sf-9 insect cells using Baculogold baculovirus DNA as described elsewhere (*30*). Production of recombinant Hck and c-Src baculoviruses has been described previously (*25*, *31*).

To investigate binding of GST-Nef to Src kinases, Sf-9 cells were coinfected with the Src family kinase baculoviruses and either the GST-Nef or the GST control baculovirus. Forty-eight hours after infection, cells were lysed 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) containing 2 mM PMSF, 25 μg/mL leupeptin, 10 μg/mL aprotinin, 25 mM NaF, and 2 mM Na_3VO_4. Glutathione−agarose beads were added to the clarified cell lysates, and the reactions were incubated for 2 h at 4 °C. Following incubation, the beads were washed with RIPA buffer, and bound proteins were eluted by heating in SDS−PAGE sample buffer. Nef-associated Src family kinases were detected by immunoblotting using the antibodies described above. As a positive control for kinase expression, aliquots of the cell lysates were analyzed on the same immunoblot. Expression of GST and GST-Nef were confirmed on duplicate immunoblots with an anti-GST antibody (Santa Cruz Biotechnology).

RESULTS

Modulation of Fibroblast Transformation by Coexpression of Different Src Family Kinase Members with Nef. Previous work from our laboratory has shown that SH3-mediated interaction with Nef is sufficient to activate Hck and induce cellular transformation of Rat-2 fibroblasts (*19*, *24*). To determine if Nef affects other Src family kinase members in a similar manner, Rat-2 fibroblasts were coinfected with Nef and Src, Lyn, Fyn, or Lck retroviruses. Parallel cultures were infected with the Src retroviruses and a control virus carrying only the neo selection marker, while fibroblasts coexpressing Nef and Hck served as a positive control. The coinfected cells were assayed for transforming activity using a focus-forming assay, and the results are shown in Figure 1. In the absence of Nef, no evidence of transformation was

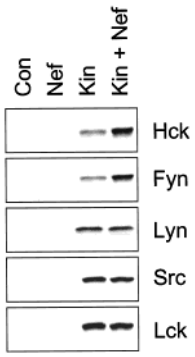

FIGURE 2: Western blot analysis of Rat-2 cells following coinfection with Nef and Src kinase retroviruses. Cultures of Rat-2 fibroblasts expressing Src family kinases alone (Kin) or together with Nef (*Kin + Nef*) were lysed in RIPA buffer. Extracts from fibroblasts infected with the control retrovirus alone (Con) or a combination of the control and Nef viruses (Nef) were used as negative controls. The clarified whole-cell lysates were analyzed for expression of each of the Src family members by immunoblotting. The primary antibody used for each immunoblot is indicated on the right.

observed in cells expressing Hck or Lyn. In contrast, multiple small foci were observed with c-Src and Lck, while Fyn induced the formation of many large foci. These results demonstrate the different degrees of Src family kinase regulation in Rat-2 fibroblasts, and are in agreement with previous experiments showing that overexpression of wild-type c-Src, Lck, and Fyn in fibroblast cell lines results in cellular transformation (*32*−*35*).

Coinfection of fibroblasts with the different Src kinase family members and Nef produced several unanticipated results. First, no transformation resulted from coexpression of Lyn and Nef. This result is surprising since previous studies have shown that the SH3 domain of Lyn binds to Nef with the same relative affinity as the Hck SH3 domain (*11*). Second, coexpression of Fyn and Nef led to an inhibition of cellular transformation. This result was unexpected because the low affinity of the Fyn SH3 domain for Nef suggested that the two proteins may not interact (*11*, *12*). Finally, coexpression of Src and Lck with Nef did not affect transforming activity compared to expression of the kinase alone. Coexpression of Hck with Nef resulted in strong focus-forming activity, consistent with previous results (*19*, *24*). Expression of all five Src family members in the presence and absence of Nef was verified by immunoblotting, and the results are shown in Figure 2. The results of the transformation assays suggest that activation of Hck may be a unique outcome of the interaction of Src family kinases with HIV Nef.

Differential Regulation of Src Family Kinase Activity by Nef. To determine whether the Nef-induced changes in Src family kinase transforming activity correlate with changes in kinase activity, the Src kinases were immunoprecipitated from the infected Rat-2 fibroblast populations shown in Figure 1. Src kinases were incubated in vitro with [γ-^{32}P]-ATP and a 50 kDa GST−Sam 68 fusion protein (p50) as substrate. As shown in Figure 3, Lyn, Src, and Lck showed no change in kinase activity following coexpression with Nef. However, the kinase activity of Fyn was significantly inhibited following coexpression with Nef, which agrees with the observed suppressive effect of Nef on Fyn focus-forming activity. As previously reported, the Hck tyrosine kinase was activated upon coexpression with Nef, both in terms of

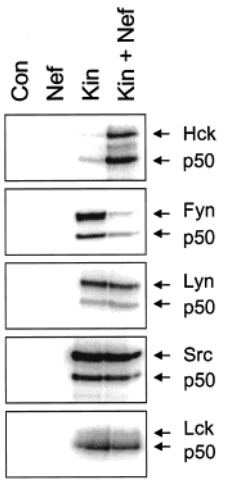

FIGURE 3: Coexpression with Nef modulates the kinase activity of Hck and Fyn but not Src, Lyn, or Lck. Cultures of Rat-2 fibroblasts expressing Src family kinases alone (Kin) or together with Nef (Kin + Nef) were lysed in RIPA buffer. Extracts from fibroblasts infected with the control retrovirus alone (Con) or a combination of the control and Nef viruses (Nef) were used as negative controls. Clarified lysates were incubated with the corresponding anti-Src kinase antibodies and protein G−Sepharose. The resulting immunoprecipitates were washed and incubated with a 50 kDa GST−Sam 68 fusion protein (p50) as a substrate and [γ-^{32}P]ATP. Samples were resolved by SDS−PAGE and transferred to PVDF membranes, and phosphorylated proteins were visualized by storage phosphor imaging. The positions of the autophosphorylated kinases and the labeled p50 substrate proteins are indicated by the arrows. The blots were probed with the corresponding anti-Src kinase antibodies to ensure equivalent recovery of each kinase in the presence and absence of Nef (data not shown).

autophosphorylation and p50 substrate phosphorylation (*19*, *24*). These data suggest that Nef directly modulates Hck and Fyn kinase activity in vivo.

Endogenous Substrate Phosphorylation in Rat-2 Fibroblasts Coexpressing Src Kinase Family Members and Nef. Previous work from our laboratory has demonstrated tyrosine phosphorylation of endogenous proteins following transformation by coexpression of Hck and Nef as well as constitutively activated Hck mutants (*19*, *24*). The most prominent phosphoprotein observed in these experiments was a 40 kDa protein (p40) which represents a convenient endogenous transformation-associated tyrosyl phosphoprotein marker. To assess the tyrosine phosphorylation of p40 and other endogenous proteins by Hck, Fyn, Lyn, Src, and Lck in the presence and absence of Nef, immunoblots of whole-cell protein extracts were probed with anti-phosphotyrosine antibodies. As shown in Figure 4, fibroblasts transformed by the combination of Hck and Nef exhibited prominent phosphorylation of p40 as well as autophosphorylated Hck. Transformation by Fyn is also associated with strong tyrosine phosphorylation of p40 and several other endogenous proteins, as well as a 59 kDa protein that is likely to represent autophosphorylated Fyn itself. Coexpression of Fyn with Nef resulted in the complete inhibition of p40 phosphorylation as well as a significant reduction in Fyn autophosphorylation. Rat-2 fibroblasts expressing Lyn either in the presence or in the absence of Nef showed little evidence of endogenous tyrosine phosphoproteins, consistent with the lack of transformation. Finally, fibroblasts expressing Lck and Src showed evidence of kinase autophosphorylation as well as p40 phosphorylation, but the intensity of these phosphory-

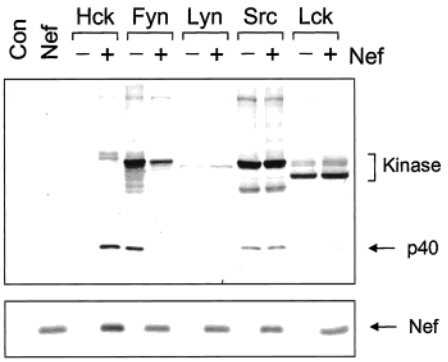

FIGURE 4: Tyrosine phosphorylation of endogenous proteins by activated Src kinase family members. Cultures of Rat-2 fibroblasts expressing the indicated Src family kinases alone (−) or together with Nef (+) were lysed in RIPA buffer. Extracts from fibroblasts infected with the control retrovirus alone (Con) or in combination with the Nef virus (Nef) were used as negative controls. Top: Clarified lysate proteins (30 μg) were immunoblotted with the anti-phosphotyrosine monoclonal antibody PY20. The positions of the major immunoreactive band p40 as well as the autophosphorylated kinases themselves are indicated. Bottom: Nef expression was verified by immunoblotting a duplicate membrane with anti-Nef monoclonal antibodies (*24*).

lated bands was not changed in the presence of Nef. No detectable endogenous protein−tyrosine phosphorylation was observed in fibroblasts expressing Nef alone or in control fibroblasts infected with an empty retrovirus. Expression of Nef was verified by immunoblotting in each case (Figure 4). These results agree with the transformation data presented in Figure 1 as well as the in vitro kinase activity data shown in Figure 3.

Analysis of Nef−Src Family Kinase Interaction in a Baculovirus/Sf-9 Cell System. Data presented above show that coexpression of Lyn and Nef does not induce Lyn activation. This result is surprising, given that the Lyn SH3 domain has been shown to interact with Nef almost as strongly as the Hck SH3 domain (*11*). To investigate whether Nef can form a stable complex with full-length Lyn as well as the other Src family members used in this study, a baculovirus-based coexpression system was employed. For these experiments, Nef was expressed as a GST fusion protein in Sf-9 insect cells together with each of the Src family kinase members. The GST−Nef fusion protein was then precipitated from cell lysates using glutathione−agarose beads, and the presence of associated Src kinases was analyzed by immunoblotting. As shown in Figure 5, GST−Nef bound strongly to Hck and Lyn, consistent with previous reports of Nef interaction with their SH3 domains (*11*). GST−Nef was also observed to bind to c-Src, which is somewhat surprising given the low affinity of the Src SH3 domain for Nef (*12*). On the other hand, GST−Nef did not coprecipitate Fyn or Lck, which agrees with published reports suggesting low affinity of Nef for the SH3 domains of these Src family members (*11*, *12*). Lack of interaction with Fyn suggests that the inhibition of Fyn kinase and transforming activities by Nef in Rat-2 fibroblasts may require additional cellular factors (see Discussion).

DISCUSSION

Previous studies have shown that various members of the Src tyrosine kinase family may represent targets for HIV-1

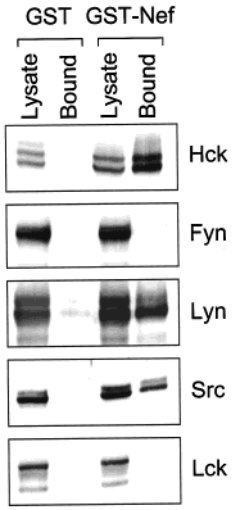

FIGURE 5: Binding of Src family kinases to HIV Nef in Sf-9 cells. Sf-9 cells were coinfected with the Src family kinase baculoviruses indicated (right-hand column) and either a GST−Nef or a GST baculovirus. Forty-eight hours after infection, GST and GST−Nef were precipitated from clarified cell lysates with glutathione−agarose beads. The beads were washed extensively with RIPA buffer, and the presence of associated Src kinases was assessed by immunoblotting with Src-kinase-specific antibodies (Bound). As a positive control for kinase expression, aliquots of the cell lysates were analyzed on the same immunoblot (Lysate). Additional control blots verified equivalent levels of GST and GST−Nef expression (data not shown). All experiments were repeated at least twice with comparable results.

Nef (*2*). In particular, SH3-dependent interaction with Nef activates Hck both in vivo and in vitro (*24*, *36*). These results suggest that the affinity of Src kinase SH3 domains for Nef may determine the responsiveness to Nef-mediated activation in vivo. We tested this hypothesis by coexpressing five different Src kinases with Nef in Rat-2 fibroblasts, and found that Nef does not enhance the kinase activity of Fyn, Lyn, Src, or Lck in this system. Based on a recent study showing the relatively low affinities of Nef for the SH3 domains of Fyn, Src, and Lck for Nef relative to Hck (*12*), it is not surprising that Nef was unable to induce activation of these Src family members. However, the inability of Nef to activate Lyn was unexpected, given that previous filter binding assays suggest that the SH3 domains of Lyn and Hck interact with Nef with similar affinities (*11*). In addition, the binding assays shown here demonstrate strong interactions of Nef with full-length Hck and Lyn (Figure 5). Furthermore, structural studies have revealed that the SH3 domains of Hck and Lyn share an RT loop Ile residue critical for interaction with Nef; this Ile is not found in the SH3 domains of other members of Src tyrosine kinase family (*9*, *37*, *38*). Despite these similarities, we have recently observed that alanine substitutions of the conserved prolines in the SH2-kinase linker region do not release Lyn tyrosine kinase or transforming activity in fibroblasts (E. Lerner and T. Smithgall, unpublished data). This is in marked contrast to Hck, where substitution of the homologous proline residues converts Hck into a potent transforming oncogene (*19*). This observation suggests that the SH3 domain may not have a dominant role in the regulation of Lyn kinase activity and may explain how Lyn can interact with Nef in an SH3-dependent manner without an effect on kinase activity. Alternatively, Nef may make additional contacts within Lyn that serve to stabilize the closed, inactive conformation of the kinase domain.

Our transformation assays revealed that overexpression of wild-type Fyn results in oncogenic transformation of Rat-2 fibroblasts, consistent with previous work in NIH 3T3 cells (*35*). These results suggest that Fyn is not as tightly regulated as other Src family members in fibroblasts. However, when Nef was coexpressed with Fyn, cellular transformation was almost completely blocked. Suppression of Fyn-induced transformation correlated with an inhibition of Fyn tyrosine kinase activity as well as reduced tyrosine phosphorylation of endogenous proteins, suggesting a direct interaction between the proteins in vivo. Nef-mediated suppression of transformation and kinase activity was unique to Fyn, and did not occur with Src or Lck which also exhibited constitutive tyrosine kinase activity. Despite the clear inhibition of Fyn transforming and kinase activities in fibroblasts, we were unable to detect a stable complex of Nef with Fyn in the Sf-9 cell overexpression system. Consistent with this result, Nef did not affect Fyn autophosphorylation in Sf-9 cells either (data not shown). However, the Sf-9 cell expression system differs in several important ways from fibroblasts, where inhibition is observed. First, tail phosphorylation of Fyn by Csk in fibroblasts, although unable to completely repress Fyn kinase activity, may induce a conformational change that promotes interaction with Nef. Second, both Fyn and Nef are modified by N-terminal myristylation, and are expected to localize to the same membrane compartment in fibroblasts. Local concentration effects resulting from colocalization may promote direct interaction and kinase inhibition despite the low apparent affinity for the interaction in vitro. Finally, it should be noted that a crystal structure of the Fyn SH3 domain complexed with Nef has recently been reported, providing further evidence that these proteins have the potential to interact (*39*).

Several studies have investigated the interaction of Nef with Lck, a Src family member expressed specifically in T lymphocytes. In one study, Nef was shown to bind to both the SH3 and SH2 domains of Lck in coprecipitation experiments with GST−SH domain fusion proteins. Although the extent of Nef interaction with the individual SH2 and SH3 domains was modest, stronger interaction was observed with a joint SH3−SH2 construct. Interaction of the full-length Nef and Lck proteins was also observed, leading to an inhibition of Lck tyrosine kinase activity both in vitro and in vivo (*13*). A second study produced similar results in terms of Lck kinase inhibition by Nef, although a role for the Lck SH2 domain in Nef binding was not observed (*40*). In contrast, our data show that coexpression of Lck with Nef in fibroblasts does not affect Lck kinase activity or influence cellular transformation. Consistent with this finding, we were not able to detect Nef−Lck complexes in Sf-9 cells. A possible explanation for these differences may relate to the allelic variants of Nef used. All of our studies to date have used Nef from the SF2 strain of HIV-1 (*19*, *24*), while the studies described above employed Nef from either HIV-1 Bru/Laï (*13*) or NL4-3 (*40*). Sequence variations in Nef may affect its affinity for the Lck SH2 and SH3 domains. Indeed, a recent report has shown that Nef derived from SIVmac239 stimulates rather than inhibits Lck (*41*). In this case, the activation mechanism appears to involve the SH2 rather than the SH3 domain.

The precise role of Src family kinases in HIV replication and AIDS pathogenesis remains to be determined. However, work presented here shows that Nef differentially influences the kinase activities of Hck and Fyn, which are primarily expressed in macrophages and T-cells, respectively. Constitutive activation of Hck by Nef may extend the lifespan of HIV-infected macrophages, contributing to the high viral loads that can persist in end-stage disease even after T-cell depletion. Recent work in our laboratory has shown that Nef can generate a signal for survival in a monocytic cell line, and that this signal correlates with activation of endogenous Hck and requires the Stat3 transcription factor downstream (*42*). On the other hand, work presented here demonstrates that Nef can inhibit Fyn kinase activity. Together with the inhibition of Lck by some forms of Nef (*13*, *40*), inhibition of Fyn may suppress T-cell development. Consistent with this hypothesis is the finding that T-cell maturation is completely compromised in mice in which both Fyn and Lck have been disrupted by gene targeting (*43*). In addition, HIV Nef has recently been shown to sensitize CD4+ T-lymphocytes to apoptosis via up-regulation of both Fas and Fas ligand (*44*). Whether or not this effect is also related to the ability of Nef to interfere with Lck or Fyn signaling will require further investigation.

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