Virology **264,** 5–15 (1999)
Article ID viro.1999.9937, available online at http://www.idealibrary.com on IDEAL®

The Conserved Core of Human Immunodeficiency Virus Type 1 Nef Is Essential for Association with Lck and for Enhanced Viral Replication in T-Lymphocytes

Hua Cheng,* James Hoxie, and Wade P. Parks*,[1]

*Department of Microbiology and Pediatrics, New York University School of Medicine, New York, New York 10016; and †Hematology-Oncology Division, Department of Medicine, University of Pennsylvania Medical Center, Philadelphia, Pennsylvania 19104

Received May 21, 1999; returned to author for revision June 28, 1999; accepted July 27, 1999

The Nef protein of the primate lentiviruses, including human immunodeficiency virus type 1 (HIV-1) and simian immunodeficiency virus (SIV), is a myristylated protein associated with increased viral replication and enhanced pathogenicity. Both the potentiation of T-lymphocyte activation and the enhanced serine-phosphorylation of HIV-1 capsid by Nef correlate with increased viral replication. We report the functional interactions of the Nef proteins with Src kinases. The Nef proteins from HIV-1 and SIV bind to Lck as well as Hck, Lyn, and Fyn. The SH3 and SH2 domains of Lck are sufficient for coprecipitation with non-tyrosine-phosphorylated Nef proteins. The conserved core region of HIV-1 Nef is essential for the interaction with Lck and is also important for enhanced HIV-1 replication in T-lymphocytes. In addition, we show that SIV and HIV-1 Nef proteins are differentially tyrosine-phosphorylated. The kinase-active Lck tyrosine-phosphorylates SIVmac239 Nef but does not phosphorylate HIV-1 Nef. These data suggest that the association of Nef and Lck is central to the enhanced viral replication of HIV-1 and SIV in T-lymphocytes.

INTRODUCTION

Primate lentiviruses possess two characteristics central to the understanding of the role of accessory genes like nef. One characteristic is that this retroviral subfamily can infect and replicate in nondividing cells; second, the family is both T-lymphocyte and macrophage tropic. Thus a model of functions of these accessory genes should account for these characteristics. *nef* RNA is the major species of human immunodeficiency virus type 1 (HIV-1) viral mRNA expressed immediately postintegration of the provirus (Cullen, 1991; Robert-Guroff *et al.,* 1990). Despite several intriguing biological characteristics of the Nef proteins, their functions are incompletely understood as they affect both viral replication and pathogenicity (reviewed in Rater *et al.,* 1995; Trono, 1995). Deletion of the *nef* gene results in a decreased rate of HIV-1 replication *in vivo* and, in the case of simian immunodeficiency virus (SIV), decreases virulence (Chakrabarti *et al.,* 1996; Kestler *et al.,* 1991; Novembre *et al.,* 1996). Furthermore, an intact *nef* gene is necessary for HIV-1 replication in quiescent T-lymphocytes or monocytes (Miller *et al.,* 1994; Spina *et al.,* 1994). Nef interacts with cellular factors of the signal transduction pathways and alters T-lymphocyte activation, as well as affecting viral replication in quiescent T-lymphocytes or monocytes (Miller *et al.,* 1994; Ratner *et al.,* 1995; Spina *et al.,* 1994). Nef has been shown to down-regulate the surface expression of CD4 (Ratner *et al.,* 1995; Trono, 1995) and MHC I (Collins *et al.,* 1998).

Several observations suggest that the enhanced viral replication by Nef could be related to its ability to promote T-lymphocyte activation and proliferation (Baur *et al.,* 1994; Du *et al.,* 1996; Hanna *et al.,* 1998; Skowronski *et al.,* 1993). It has been shown that both HIV-1 Nef and SIVmac239 Nef enhanced SIV viral replication in an interleukin (IL)-2-dependent rhesus monkey T-lymphocyte line in the absence of exogenous IL-2 and, also, increased IL-2 production (Alexander *et al.,* 1997). Alexander *et al.* (1997)also reported that v-Ras but not c-Ras replaced the Nef function (Alexander *et al.,* 1997), supporting the hypothesis that Nef increases viral replication through potentiation of T-lymphocyte signaling pathways. Other potential mechanisms of Nef-enhanced replication include enhancement of virus infectivity, facilitation of reverse transcription of viral RNA genome, and stimulation of proviral DNA synthesis (Aiken *et al.,* 1995; Chowers *et al.,* 1994; Miller *et al.,* 1994; Spina *et al.,* 1994). These effects may be caused by increased serine-phosphorylation of the HIV-1 matrix protein induced by Nef (Swingler *et al.,* 1997), because the phosphorylation of HIV-1 matrix at serine residues is required for the virus infectivity (Bukrinskaya *et al.,* 1996; Gallay *et al.,* 1995). Nef-associated cellular serine kinases have been suggested to mediate this effect (Baur *et al.,* 1997).

Although the mechanism of potentiating cellular func-

[1] To whom reprint requests should be addressed at Department of Pediatrics, 550 First Avenue, RM TH-501A. Fax: (212) 263-8172. E-mail: parksw01@ mcrcr.med.nyu.edu.

0042-6822/99 $30.00

tions by Nef remains to be elucidated, this function may be associated with Nef's ability to interact with Src family kinases in T-lymphocytes and macrophages. At least three Src family tyrosine kinases, including Hck, Lyn, and Lck, have been documented to associate with Nef (Baur *et al.,* 1997; Collette *et al.,* 1996; Greenway *et al.,* 1996; Saksela *et al.,* 1995). HIV-1 Nef binds to Hck through its conserved proline-rich motif (PXXP repeat) and increases its kinase activity (Lee *et al.,* 1995, 1996; Moarefi *et al.,* 1997; Saksela *et al.,* 1995). The interaction of Nef and Lck appears to be controversial. Using the same conditions that are optimal for the interaction of Nef and Hck, the association of Nef with Lck was not detected (Moarefi *et al.,* 1997; Saksela *et al.,* 1995). Greenway *et al.* (1996) and Collette *et al.* (1996) found the PXXP motif of Nef directly interacts with Lck; HIV-1 Nef inhibited the kinase activity of Lck. In contrast, Baur *et al.* (1997) observed that the Nef–Lck association is bridged by a cellular serine kinase and that Nef's N-terminal α-helix domain is sufficient for formation of the complex.

In an attempt to clarify the potential functional linkages of Nef with Src kinases, particularly Lck, we reassessed structural and functional motifs of the Nef proteins derived from both HIV-1 and SIV necessary for the association with Src kinases. We found that both Nef proteins physically interact with Lck independent of the PXXP motif in addition to the binding to Hck, Lyn, and Fyn *in vivo*. The conserved core of Nef is associated with the SH3 and SH2 domains of Lck; only SIV Nef is tyrosine-phosphorylated by Lck. We also showed that the core region of HIV-1 Nef is essential for enhanced HIV-1 replication in T-lymphocytes.

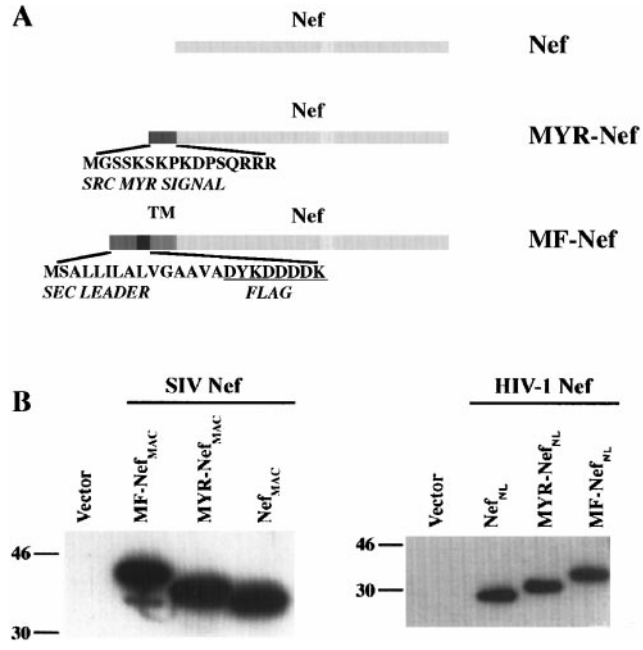

FIG. 1. Expression of the native and membrane-targeted Nef proteins. (A) Schematic representation of Nef expression constructs. The native Nef was constructed by fusion of human CD4 5′-UT with the *nef* gene derived from SIVmac239 or HIV-1 pNL4–3. MYR-Nef contains a v-Src myristylation signal peptide followed by the intact *nef* gene. MF-Nef expresses a membrane-targeted, nonmyristylated Nef. The *nef* gene derived from SIV mac239 encodes 263 aa, and HIV-1 Nef encodes 206 aa. MYR, myristylation signal peptide derived from v-Src; SEC, mouse preprotrypsin secretory leader; TM, transmembrane domain from IGFR. (B) Expression of the Nef proteins in transiently transfected 293T cells using LipofectAMINE reagent. The cell lysates were directly immunoblotted with anti-SIV Nef (left panel) or anti-HIV-1 Nef AG11 (right panel). MAC indicates that the *nef* gene is from SIVmac239, and NL represents the *nef* protein derived from HIV-1 pNL4–3.

RESULTS

The Nef proteins were expressed in potentially biological active forms

The Nef protein contains an N-terminal myristylation peptide that is required for plasma membrane attachment. Nef is also localized in cellular compartments, including cytoplasm and nucleus, and is present in nonmembrane fraction (Kienzle *et al.,* 1992; Murti *et al.,* 1993; Ranki *et al.,* 1994). To determine the relative importance of the myristylation or the membrane association for the biological activities of Nef, we used several approaches to express native Nef protein, as well as to express membrane-targeted Nef, using different artificial membrane-targeting signal peptides (depicted in Fig. 1A). First, the native *nef* genes were constructed to express a 34-kDa (SIVmac239 Nef) or a 27-kDa (HIV-1 Nef) protein in transiently transfected 293T cells (Fig. 1B). Second, a well-characterized Src myristylation signal peptide (MYR) was added to the N-terminus of Nef (MYR-Nef) to facilitate Nef's membrane attachment (Aronheim *et al.,* 1994; Resh, 1994). The MYR-Nef protein is presumably processed in the same intracellular compartment as the native Nef protein. Third, we constructed a myristylation-deficient membrane-targeted Nef (MF-Nef; depicted in Fig. 1A). MF-Nef was created by fusion of the *nef* gene with fragments sequentially encoding a preprotrypsin secretory leader, an extracellular Flag epitope tag, and an artificial transmembrane domain derived from insulin-like growth factor I receptor. MF-Nef was designed to translate from polyribosomes in the ER, transported to the cell surface, and anchored to the cytoplasmic leaflet through the transmembrane domain. We assessed the ability of three Nef expression forms to down-modulate cell surface expression of CD4 receptor because this function is conserved for both HIV-1 and SIV Nef proteins (Ratner *et al.,* 1995; Trono, 1995). All expression forms of Nef reduced the CD4 expression as measured by FACS analysis (data not shown), suggesting that they are potentially biological active forms.

Nef interacts with Src family kinases

To assess the physical interactions of the Nef proteins from HIV-1 and SIV with the lymphocyte-specific Src kinase Lck, a transient cotransfection was performed using Nef expression vector and a wild-type Lck in 293T cells. The SIV Nef proteins, including MF-Nef$_{MAC}$, MYR-Nef$_{MAC}$, and the native Nef$_{MAC}$, were coprecipitated with Lck, using anti-Lck for immunoprecipitation (Fig. 2A, top panel, lanes 4–6, respectively). The SIV Nef proteins were not coprecipitated when a nonspecific control anti-CD3 was used for immunoprecipitation (Fig. 2A, top panel, lanes 1–3). Similarly, all three forms of HIV-1 Nef were also found to associate with Lck as detected by the *in vivo* association assay (Fig. 2A, bottom panel, lanes 4–6). In Fig. 2A (bottom panel, lane 4), the native 27-kDa Nef band overlapped with IgG light chain. The interaction of this native Nef protein with Lck is also shown in Fig. 3.

We next investigated the *in vivo* association of the Nef proteins derived from both HIV-1 and SIV with these three other Src kinases: Hck, Lyn, and Fyn. Both kinases are tagged with an influenza hemagglutinin (HA) epitope. Hsp70-HA (heat shock protein 70) was used as a control. As shown in Fig. 2B, HIV-1 Nef was coprecipitated with Hck, Lyn, or Fyn but not with Hsp70. Similarly, SIV Nef was also coprecipitated with Hck and Fyn but not precipitated with Hsp-70. A weak interaction of SIV Nef and Lyn was also detected (Fig. 2B, top panel). The amount of Nef protein precipitated by Hck was at least threefold greater than that precipitated by Lyn (Fig. 2B), indicating that the Nef proteins have higher affinity for Hck than for Lyn.

The *in vivo* interaction of Nef with Src kinases requires membrane association

Because the Nef proteins are also present in cytoplasmic fraction in addition to the membrane fraction, we tested the association of the non-membrane-bound Nef with Lck and Hck. By attaching a Flag epitope at the N-terminus of the native Nef, a nonmyristylated non-membrane-targeted Nef (Flag-Nef) was generated (depicted in Fig. 3A). MF-Nef$_{NL}$ or Flag-Nef$_{NL}$ was cotransfected with Lck or Hck into 293T cells followed by the *in vivo* association assay. As demonstrated in Fig. 3B, only the membrane-targeted Nef protein (MF-Nef) was coprecipitated with either Lck or Hck. The Flag-Nef was not precipitated by Lck and also not by Hck *in vivo*. Similar results were obtained when SIV Nef was used (data not shown).

The conserved core region of HIV-1 Nef is required for association with the SH3 and SH2 domains of Lck

To further assess which specific domains of Lck mediate the association with Nef, recombinant GST-fusion proteins, including GST-LckSH2, GST-LckSH3, and GST, were used for *in vitro* interaction assay. In Fig. 4A, the

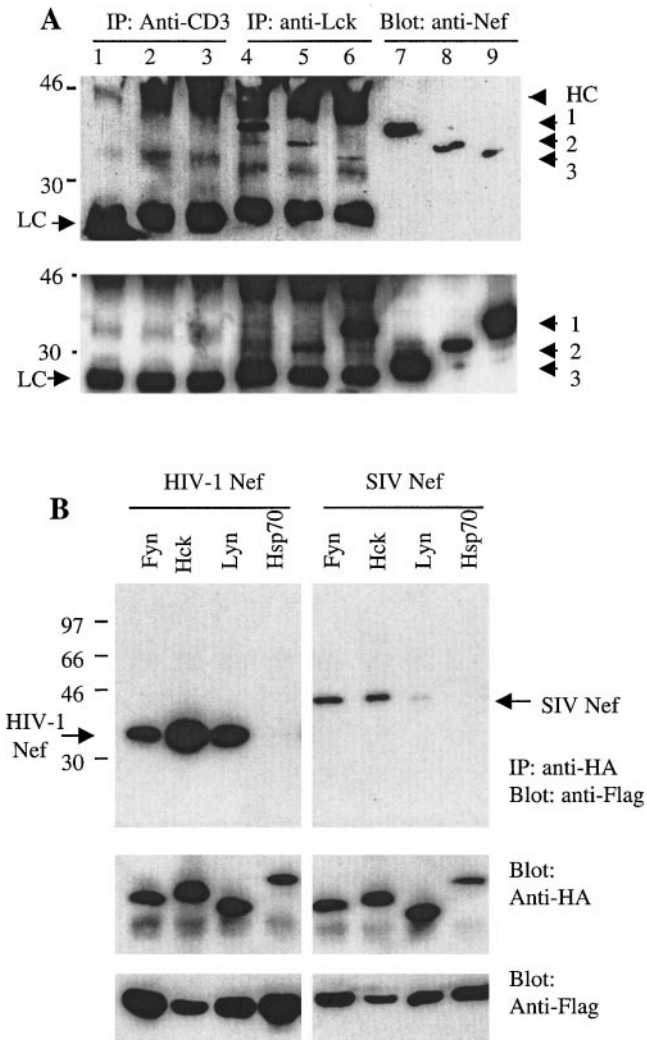

FIG. 2. SIV and HIV-1 Nef proteins physically interact with Src family kinases *in vivo*. (A) The *in vivo* association assay was performed by a transient cotransfection of native Nef$_{MAC}$, MYR-Nef$_{MAC}$, or MF-Nef$_{MAC}$ with Lck into 293T cells. The cellular extracts were precleared with normal mouse serum and protein G agarose and immunoprecipitated with mouse anti-CD3 (lanes 1–3) as a negative control or with mouse anti-Lck (lanes 4–6). The immunoprecipitates were analyzed by immunoblotting using anti-SIV Nef. Protein extracts from the transfected cells were directly immunoblotted with anti-SIV Nef (lanes 7–9). Nef$_{MAC}$, lanes 3, 6, and 9; MYR-Nef$_{MAC}$, lanes 2, 5, and 8; and MF-Nef$_{MAC}$, lanes 1, 4, and 7 (top panel). The transient cotransfection of HIV-1 Nef and Lck expression plasmids was performed using the procedure similar to the above. The cellular extracts were immunoprecipitated with anti-CD3 (lanes 1–3) or anti-Lck (lanes 4–6), followed by immunoblotting with anti-HIV-1 Nef (AG11). An equal amount of cellular proteins was directly immunoblotted with AG11 (lanes 7–9). Nef$_{NL}$, lanes 1, 4, and 7; MYR-Nef$_{NL}$, lanes 2, 5, and 8; and MF-Nef$_{NL}$, lanes 3, 6, and 9 (bottom panel). LC indicates IgG light chain; HC, for IgG heavy chain. Arrows 1–3 indicate MF-Nef, MYR-Nef, and the native Nef, respectively. (B) MF-Nef$_{NL}$ or MF-Nef$_{MAC}$ was cotransfected with Fyn-HA, Hck-HA, Lyn-HA, or Hsp70-HA. The protein extracts were precleared with normal rabbit serum and protein A beads, followed by immunoprecipitation using rabbit anti-HA. The precipitates were transferred to a nitrocellulose membrane and analyzed by immunoblot with anti-Flag M2 (top panel). The membrane was stripped with 0.2 M glycine, pH 2.0, and reblotted with mouse anti-HA (middle panel) to detect the expression of Fyn, Hck, Lyn, and Hsp-70. The equal protein extracts were directly immunoblotted with anti-Flag M2 for detecting Nef expression levels (bottom panel). IP, immunoprecipitation.

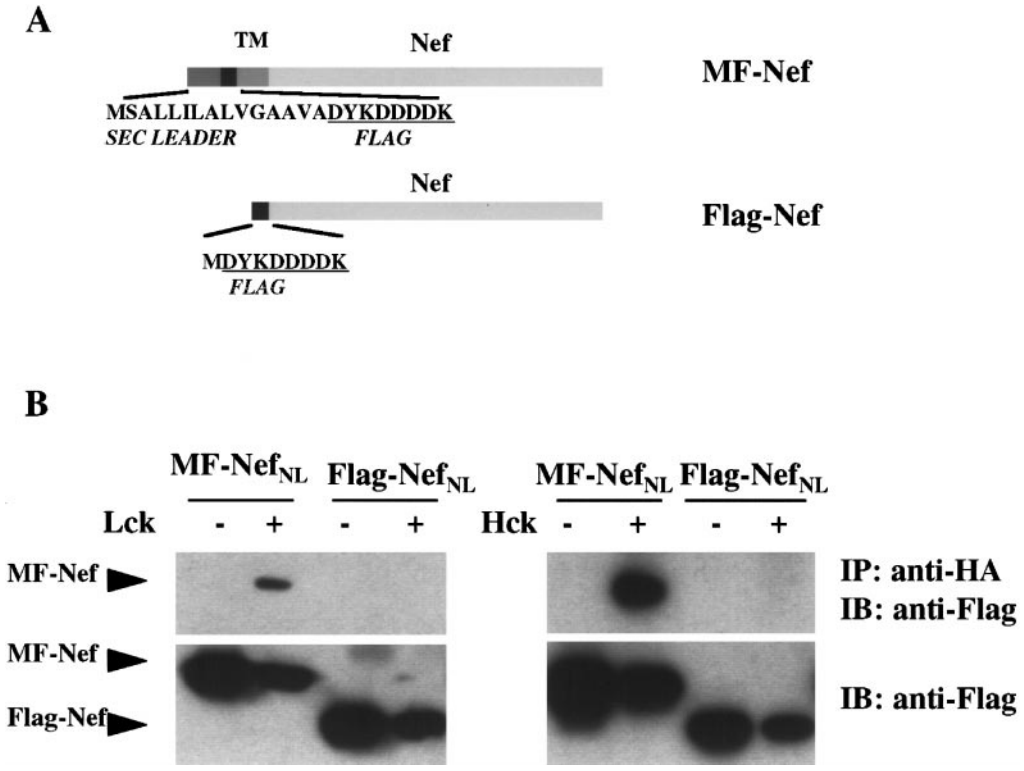

FIG. 3. Membrane association of Nef is required for the interaction with Lck and Hck. (A) Schematic structures of membrane-targeted Nef (MF-Nef) and non-membrane-targeted nonmyristylated Nef (Flag-Nef). (B) MF-Nef$_{NL}$ or Flag-Nef$_{NL}$ was cotransfected with either Lck or Hck-HA in 293T cells. The *in vivo* association assay was performed as described in legend to Fig. 2.

Nef proteins prepared from transiently expressed cellular extracts were coprecipitated using GST-Lck SH3 or GST-LckSH2 but not by GST. These data indicate that either the SH3 domain or the SH2 domain of Lck is sufficient for the association with either HIV-1 Nef or SIV Nef in non-tyrosine-phosphorylated forms, because both Nef proteins transiently expressed in 293T cells were not tyrosine-phosphorylated (data not shown).

Mutagenesis was performed to generate HIV-1 Nef variants with internal deletions in the central core to test whether these peptide sequences mediate Nef–Lck interaction (Table 1). Selected peptide sequences in the central region that are conserved among HIV-1 Nef alleles and SIV Nef include NefΔVPLR (amino acids [aa] 74–77), NefΔILD (aa 109–111), NefΔGPG (aa 130–132), and NefΔKLVPV (aa 144–148). The wild-type Nef and NefΔVPLR, which contains a deletion mutation of the major PXXP motif within the core region, were coprecipitated by GST-LckSH3 recombinant protein, but NefΔILD, NefΔGPG, and NefΔKLVPV were not (Fig. 4B, top left). We further assessed several additional conserved aa in the core of HIV-1 Nef. The deletion of Y81K82, H89F90, D123W124, or F139G140 abolished the association with Lck, whereas deletion of G95G96 retained the association of Nef with Lck (Fig. 4B, top right).

The associations of the native Nef and various Nef mutants of HIV-1 with Hck were also assessed. In consistency with previous reports, the PXXP motif of HIV-1 Nef is essential for interaction with Hck, because NefΔVPLR with mutations in the major PXXP motif abolished the association with Hck (Fig. 4B, bottom left). The conserved core of HIV-1 Nef appeared not to be essential for the interaction with Hck, because mutations of the conserved peptides of Nef did not abolish the association with Hck (Fig. 4, bottom right). Thus the interaction patterns of Nef and Lck or Nef and Hck are apparently different, although some peptide sequences of Nef overlap.

SIV Nef, but not HIV-1 Nef, is tyrosine-phosphorylated by Lck

To examine the tyrosine-phosphorylation of Nef by Lck, the *in vivo* tyrosine-phosphorylation assay was performed by a transient cotransfection using a kinase active form of Lck (Lck$_{CA}$) with MF-Nef$_{MAC}$ or with MYR-Nef$_{NL}$. As shown in Fig. 5A (top panel), SIV Nef was phosphorylated by Lck. In contrast, under identical conditions, HIV-1 Nef was not phosphorylated by Lck. The tyrosine-phosphorylation of SIV Nef requires the kinase activity of Lck, because a kinase-deficient variant of Lck (Lck$_{KM}$) (Wange *et al.,* 1996) did not phosphorylate the SIV

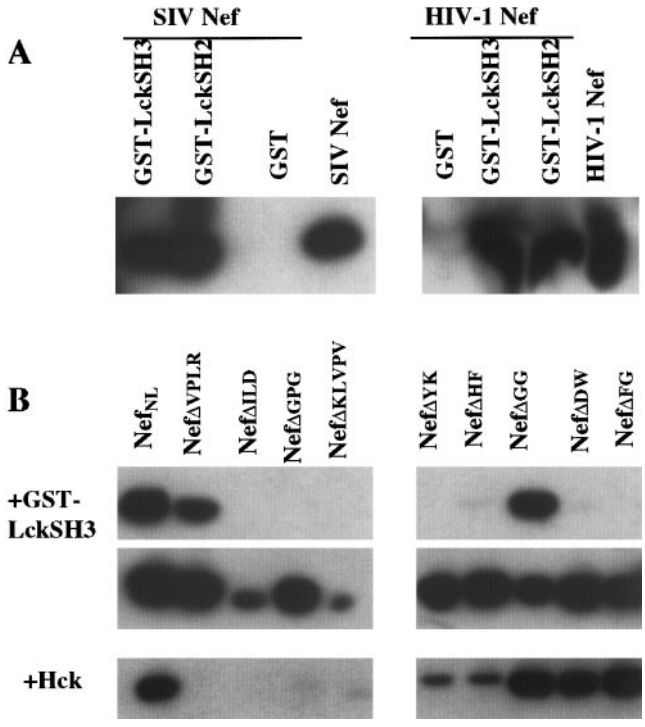

FIG. 4. The conserved core region of Nef is essential for interaction with the SH3 and SH2 domains of Lck. (A) The SH3 or SH2 domain of Lck is sufficient for the association with Nef. Equal amounts of cellular extracts were prepared from the transfected cells with either the native SIV Nef protein (left panel) or the native HIV-1 Nef (right panel) expressed in 293T cells. The lysates were precleared with GST-coated beads followed by incubating with 4 μg of GST-fusion protein-coated beads (GST-LckSH3, GST-Lck SH2, or GST alone) at 4°C for 2 h. The precipitates were analyzed by immunoblotting with anti-SIV Nef (left panel) or with anti-HIV-1 Nef AG11 (right panel). (B) The Nef proteins expressed in 293T cells, including the native HIV-1 Nef and the Nef mutants NefΔ74VPLR77, NefΔ109ILD111, NefΔ130GPG133, NefΔ144KLVPV148, NefΔY81K82, NefΔH89F90, NefΔG95G96, NefΔD123W124, and NefΔF139G140, were incubated with GST-LckSH3-coated beads. The precipitates were immunoblotted with anti-Nef AG11 (top panel). The Nef protein levels were shown at the middle panel. In addition, the native Nef and the Nef mutants as shown above were transiently cotransfected with Hck-HA. The protein extracts were precleared and immunoprecipitated with anti-HA, followed by immunoblotting using anti-HIV-1 Nef (bottom panel).

Nef protein (Fig. 5A, right panel). Lck also phosphorylated the native SIV Nef and the MYR-Nef$_{MAC}$ in a transient cotransfection of 293T cells (Fig. 5B, top panel). In addition, we observed that Flag-Nef$_{MAC}$ was not phosphorylated by Lck *in vivo* (data not shown), indicating that the tyrosine-phosphorylation of SIV Nef requires its membrane association.

The association of Nef and Lck is important for enhanced HIV-1 replication in T-lymphocytes

Finally, we examined the functional relevance of the Nef-Lck association with Nef's enhancement of viral replication. We selected several representative HIV-1 Nef mutants, including NefΔVPLR, NefΔILD, NefΔGPG, and NefΔKLVPV, as well as the wild-type Nef$_{NL}$, and inserted these Nef fragments into the infectious DNA backbone of HIV-1 (pNL4-3) to replace the native *nef* locus. Infectious viruses with various Nef mutants were generated by transient transfection of the proviral DNAs into 293T cells, and these viruses were used to subsequently infect the human T-lymphocyte line CEM X174. All four mutant viruses showed decreased levels of viral replication compared with the wild-type virus as quantified with p24 titer (Fig. 6). As shown earlier, NefΔILD, NefΔGPG, and NefΔKLVPV did not associate with Lck, indicating that the Nef–Lck complex is likely to be necessary for the enhanced viral replication in T cells. Intriguingly, NefΔVPLR, which contains mutations in the major PXXP motif, also showed a decreased ability to enhance viral replication. These data suggest that the Nef–Lck association is necessary but not sufficient for this enhanced replication. Nef may require one domain for Lck association and the PXXP motif for an optimal biological activity in promoting viral replication.

DISCUSSION

HIV-1 Nef was coprecipitated with several cellular proteins in T cells; Lck and CD4 were identified among Nef-associated cellular factors previously (Greenway *et al.,* 1995). However, a direct interaction of Nef and Lck was not observed in subsequent reports (Moarefi *et al.,* 1997; Saksela *et al.,* 1995; Sawai *et al.,* 1994). Instead, two groups demonstrated that Hck, a Src family tyrosine kinase closely related to Lck, binds directly to HIV-1 Nef with high affinity (Moarefi *et al.,* 1997; Saksela *et al.,* 1995). Mutational studies and crystal structure analysis revealed that HIV-1 Nef associates with Hck via the direct interaction of the proline-rich motif and the SH3 domain (Lee *et al.,* 1995, 1996; Saksela *et al.,* 1995). Because Hck is present in monocytes and B-lymphocytes and is not present in T cells, the biological significance of the direct Nef–Hck interaction remains unclear. To further evaluate the association of Nef and Lck, Col-

TABLE 1

Internal Deletions of the Core Region of HIV-1 Nef

Plasmids	Mutations
Nef	Wild type
NefΔVPLR	Deletion VPLR (aa 74–77)
NefΔILD	Deletion ILD (aa 109–111)
NefΔGPG	Deletion GPG (aa 130–132)
NefΔKLVPV	Deletion KLVPV (aa 144–148)
NefΔYK	Deletion YK (aa 81–82)
NefΔHF	Deletion HF (aa 89–90)
NefΔGG	Deletion GG (aa 95–96)
NefΔDW	Deletion DW (aa 123–124)
NefΔFG	Deletion FG (aa 139–140)

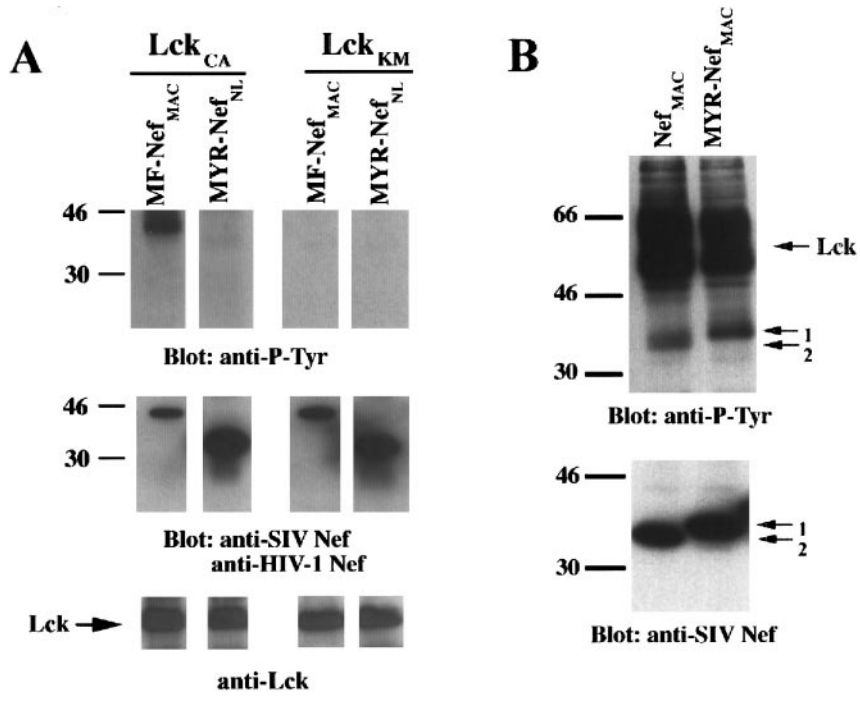

FIG. 5. SIVmac239 Nef, but not HIV-1 Nef, is tyrosine-phosphorylated by Lck. (A) The *in vivo* kinase assay was performed by cotransfection of MYR-Nef$_{NL}$ (1 μg) or MF-Nef$_{MAC}$ (1 μg) with Lck$_{CA}$ (kinase active Lck, 0.6 μg) or Lck$_{KM}$ (kinase deficient Lck, 0.6 μg) into 293T cells. The cellular extracts from the transfected cells were immunoprecipitated with rabbit anti-Flag for MF-Nef or rabbit antiserum specific for HIV-1 Nef followed by immunoblotting with combined anti-phosphotyrosine antibodies PY20 and 4G10 (top panel). The membrane was stripped with 0.2 M glycine (pH 2.0) and reblotted with anti-SIV Nef or AG11 (middle panel). The protein levels of Lck are indicated in the bottom panel. (B) Tyrosine-phosphorylation of the native Nef$_{MAC}$ and MYR-Nef$_{MAC}$ by Lck$_{CA}$ were detected directly by immunoblotting using anti-phosphotyrosine antibodies in transiently cotransfected 293T cells. The protein level of SIV Nef is indicated in the bottom panel. Arrow 1, MYR-Nef$_{MAC}$; arrow 2, native SIV Nef. The autophosphorylated Lck is indicated with the upper arrow.

lette *et al.* (1996) and Greenway *et al.* (1996) reported a direct interaction of Nef with Lck. They showed that HIV-1 Nef is tyrosine-phosphorylated in Jurkat T cells and was specifically coprecipitated by the SH3 and SH2 domains of Lck, and the PXXP motif of HIV-1 Nef was responsible for interaction with Lck. In subsequent studies, Baur *et al.* (1997) observed that a conserved amphipathic α-helix in the N-terminus of Nef of HIV-1 and SIV is sufficient to form a complex with Lck and a cellular serine kinase. This serine kinase can be distinguished from serine kinases p62/p72 identified by Sawai *et al.* (1994) and presumably bridges Nef to Lck (Baur *et al.,* 1997). In the present study, the PXXP motif of Nef is not sufficient for association with Lck. Mutagenesis analysis indicates that the conserved core of Nef is important for formation of the Nef–Lck complex. In addition, the Nef variants with mutations in the core region but with an intact N-terminus abrogated the interaction with Lck. The N-terminal α-helix domain of Nef contains several basic aa such as arginines similar to Src N-terminal domain. These basic aa are essential for membrane attachment because mutations in this region abolish membrane association of Src kinase (Resh, 1994). It appears that the N-terminal α-helix domain of Nef probably serves as an additional signal for membrane targeting instead of serving as the direct binding site for Lck. Our study shows that the membrane attachment of Nef is essential for *in vivo* interaction with Lck.

Although overall amino acid sequence homology shared by both Nef proteins from HIV-1 and SIV is about 38%, the two Nef proteins exhibit similar biological and biochemical features (Ratner *et al.,* 1995; Trono, 1995), suggesting that they share a common peptide structure. In addition to the conserved myristylation signal peptide, the core region of Nef is conserved with peptide sequence homology of about 50–80% (Shugars *et al.,* 1993). Several peptide sequences distributed in four highly conserved blocks within the core region of Nef are crucial for interacting with Lck and/or for maintaining protein stability as demonstrated in the present study. The amino acid sequence 130GPG133, Y81K82, H89F90, D123W124, and F139G140 of Nef is critical for the Nef–Lck association. In addition, the HIV-1 Nef mutants with internal deletions at 109ILD111 and 144KLVPV148 are expressed at lower levels compared with that of wild-type HIV-1 Nef, suggesting that these regions may be important for maintaining Nef protein stability. It is not clear whether these peptide sequences serve as the binding sites for

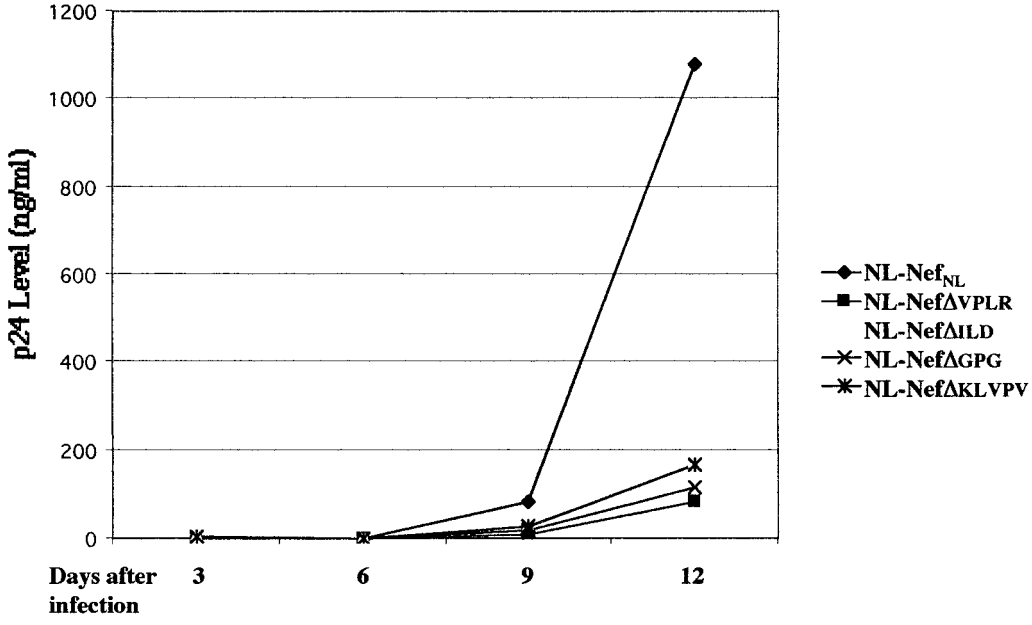

FIG. 6. The association of Nef and Lck is important for enhanced HIV-1 replication in human T cells. CEMX174 cells (1×10^6) were infected with 1 ng (p24) of the wild-type virus NL-Nef$_{NL}$ and the mutant viruses NL-NefΔ74VPLR77, NL-NefΔ109ILD111, NL-NefΔ130GPG133, and NL-NefΔ144KLVPV148 according to the procedure described in Materials and Methods. The culture media were collected for p24 titer analysis. The p24 levels (ng/ml) from each virus-infected culture at several time points were measured using ELISA.

Lck. It appears that Nef is associated with Lck via an interaction that is distinct from the Nef–Hck interaction. In addition, the SH2 domain of Lck binds to HIV-1 Nef via an atypical interaction, an observation consistent with Dutartre *et al.* (1998). We also found that this SH2 domain is mediating the interaction with SIV Nef that is not spontaneously tyrosine-phosphorylated when expressed in 293T cells. The mechanism of this type of interaction is not currently understood. There probably is an intermediate protein involved in the interaction between Nef and Lck. This putative connector might contain SH3- and SH2-binding domains mediating direct interaction with Lck; this possibility remains to be addressed. Nevertheless, the conserved regions of Nef are necessary for maintaining the integrity of the core structure that is required for formation of the Nef–Lck complex.

The tyrosine-phosphorylation of the SIV Nef protein has been implicated to correlate with the pathogenesis of simian AIDS reported by Du *et al.* (1995, 1996). SIV YEnef containing the putative SH2 binding domain resulted in marked phosphorylation by v-Src and was associated with a rapid disease progression compared with wild-type SIVmac 239. We have demonstrated that a kinase-active Lck tyrosine-phosphorylates wild-type SIVmac239 Nef in the absence of the YE motif. Thus the following scenario may occur. Ligation of TCR and CD4–Lck complex in quiescent T cells leads to an immediate activation of Lck upstream of the TCR-directed signaling pathway (Glaichenhaus *et al.,* 1992; Wange *et al.,* 1996; Weil *et al.,* 1996). Activated Lck can subsequently phosphorylate SIV Nef; therefore, SIV Nef could serve as a substrate of Lck and acquire a full activity through phosphorylation for enhanced virus infectivity *in vivo*. A second scenario may also occur. Regardless of tyrosine-phosphorylation of Nef and alteration of the Lck kinase activity, Nef may serve as an adapter that links Lck to other signaling molecules for modulation of cellular signaling pathways. Nef has been previously found to associate with PKC-θ, MAP kinase, c-Raf, serine kinases p62/p72, and p21-activated kinase (PAK), in addition to Src kinases (Baur *et al.,* 1994; Greenway *et al.,* 1996; Hodge *et al.,* 1998; Nunn *et al.,* 1996; Sawai *et al.,* 1994; Smith *et al.,* 1996). This evidence supports the possibility that Nef behaves as an adapter.

The finding that Nef increased IL-2 production and enhanced virus replication in rhesus macaque T cells further supports that Nef modulates signaling cascades (Alexander *et al.,* 1997). Because HIV-1 long terminal repeat contains an IL-2-like enhancer, transcriptional factors involved in T cell activation can positively regulate viral replication (Cheng *et al.,* 1998; Cullen, 1991; Kawakami *et al.,* 1988; Nabel *et al.,* 1990; Siekevitz *et al.,* 1987). This regulatory mechanism could be essential for initiating a productive viral replication at the early stage after viral entry. Finally, the differential tyrosine-phosphorylation and the distinct association patterns of Nef with Src kinases suggest that although HIV-1 Nef and SIV Nef share several biological functions, the precise mech-

anisms to exert these functions may differ. Future studies of the detailed structural basis of the Nef–Lck complex and identification of the putative connectors could provide more information concerning the molecular mechanism of Nef-enhanced viral replication.

MATERIALS AND METHODS

Cell lines and antibodies

The 293T cells were maintained in DMEM with 10% FBS and 50 μg/ml gentamicin (GIBCO BRL, Grand Island, NY). CEMX174 cells were cultured in RPMI 1640 medium supplemented with 10% FBS and 50 μg/ml gentamicin.

Polyclonal antibodies against HIV-1 Nef were obtained from AIDS Research and Reference Reagent Program (Bethesda, MD). Anti-Flag, anti-HA, and anti-Lck antibodies and GST-LckSH3 and GST-LckSH2 fusion proteins were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-Flag M2 was from IBI (New Haven, CT). Anti-phosphotyrosine antibodies PY20 and 4G10 were purchased from Transduction Laboratories (Lexington, KY) and Upstate Biotechnology, Inc. (Lake Placid, NY) respectively. FITC-labeled anti-CD4 was from Becton Dickinson (San Jose, CA).

To prepare mouse monoclonal anti-SIV Nef antibody, the full length of SIVmac239 *nef* gene was amplified using PCR and cloned in pGEX4T-1 (Pharmacia, Uppsala, Sweden) to generate GST-SIV Nef fusion protein. The fusion protein was purified by absorption to glutathione–agarose beads followed by 10% SDS–PAGE electrophoresis. The fusion protein was eluted from the SDS–PAGE gel with PBS containing 0.1% SDS. The proteins were dialyzed with PBS. Using purified GST-SIV Nef fusion protein, several mouse hybridoma cell clones producing antibodies for SIV Nef were generated. The pooled hybridoma culture supernatants were used for immunoblotting analysis.

Plasmid DNA constructions and site-directed mutagenesis

The native Nef genes were derived from pNL4–3 (HIV-1) and from pCMV/CD8-SIVmac239 Nef (Sawai *et al.,* 1994) using PCR with *pfu* DNA polymerase (Stratagene, La Jolla, CA). The PCR fragments were cloned into phEFneo vector, a modified version of pEF-BOS (Mizushima *et al.,* 1990). MYR-Nef was generated by fusion of a fragment containing the 5′-UT region and myristylation sequence of the *v-src* gene with the *nef* gene fragment. To generate a myristylation-deficient membrane-targeted Nef, a transmembrane domain derived from human insulin-like growth factor I receptor was initially ligated with the *nef* gene fragment. The fused fragment was inserted into phEF/MFneo vector (a modified version of phEFneo) to generate phEF/MF-Nef_{NL}. A nonmyristylated and non-membrane-targeted Nef (Flag-Nef) was constructed by inserting the *nef* gene into phEF/Flag vector. The internal deletion mutants of *nef*, NefΔ74VPLR77, NefΔ109ILD111, NefΔ130GPG133, NefΔ144KLVPV148, NefΔY81K82, NefΔH89F90, NefΔG95G96, NefΔD123W124, and NefΔF139G140 were created by a PCR-based mutagenesis. SIVmac239 Nef expression vectors were constructed using the same approaches as HIV-1 Nef to create the expression plasmids for native SIV Nef (Nef_{MAC}), MYR-Nef_{MAC}, or MF-Nef_{MAC}.

The cDNAs that express Fyn, Hck, Lyn, and Hsp-70 were obtained from a human lymph node cDNA library (Edge BioSystems, Gaithersburg, MD) using PCR. All of these cDNAs were cloned into the expression vector pCEF with a C-terminal influenza HA epitope tagging. Murine Lck cDNAs, including wild-type, kinase-deficient, and kinase-active forms of Lck, were from Dan Littman (New York University School of Medicine, New York, NY) and were subcloned into phEFneo vector.

DNA transfection, immunoprecipitation, and immunoblotting

Transfection of 293T cells was performed with 6-well plates using LipofectAMINE reagent (GIBCO BRL) according to the manufacturer's recommendation. Transiently transfected 293T cells were washed and resuspended in 4°C PBS. Cells were lysed in 0.4 ml of RIPA buffer (50 mM Tris–Cl, pH 7.6, 100 mM NaCl, 1% Nonidet P-40) containing 5 μg/ml leupeptin, 5 μg/ml aprotinin, and 1 mM PMSF on ice for 30 min. The cellular extracts were clarified by centrifugation at 14,000 rpm for 10 min at 4°C. Primary antibodies were added to the supernatants, followed by incubation for 2 h at 4°C. After incubation, protein A– or G–agarose beads were added to the antibody–antigen mixture for incubation for 1 h on a rotator at 4°C. The beads were washed four times with the RIPA buffer, and 20 μl of 2× SDS loading buffer was then added to the beads. The immunoprecipitates were boiled for 4 min, resolved on 12% SDS–PAGE, and transferred to a nitrocellulose membrane. The membrane was blocked with 5% nonfat milk in T-TBS buffer (50 mM Tris–Cl pH 7.6, 100 mM NaCl, and 0.1% Tween 20) and subsequently immunoblotted with primary antibody diluted in T-TBS buffer containing 5% nonfat milk at 37°C for 1 h. After being washed three times with T-TBS buffer, the membrane was immunoblotted with secondary antibody conjugated with horseradish peroxidase. The antigen–antibody complex was detected using the ECL system (Amersham, Arlington Heights, IL). The expressed pro-

teins were also analyzed by direct immunoblotting without the immunoprecipitation step.

Detection of *in vivo* association of Nef and Src kinases

The 293T cells were transiently cotransfected with Nef and the expression plasmid for Hck-HA, Lyn-HA, or Hsp70-HA. Similarly, the three different expression forms of Nef from both HIV-1 and SIV were cotransfected with the murine Lck expression plasmid. The transfected cells were collected 24 h posttransfection and were lysed in the RIPA-containing protease inhibitors at 4°C for 30 min. After clarification by centrifugation, the cellular extracts were precleared with normal rabbit serum and protein A beads, when rabbit antibody was used for immunoprecipitation or normal mouse serum and protein G beads if mouse antibody is used, and followed by immunoprecipitation using rabbit anti-HA or anti-Lck. The immunoprecipitates were analyzed by immunoblotting using anti-Flag (M2) or anti-Nef.

Assessment of *in vitro* association of Nef and Src kinases

Cellular extracts containing native Nef proteins expressed in 293T cells were prepared in the RIPA buffer containing protease inhibitors. The cellular extracts were precleared with GST-bound beads at 4°C for 2 h. GST-LckSH3-, GST-LckSH2-, or GST-coated beads (4 μg each) were then added to the cellular extracts. Incubation was performed at 4°C for 2 h with rotation. After incubation, the beads were washed four times with RIPA buffer, and the precipitates were resolved on 12% SDS–PAGE. Immunoblotting was performed using anti-SIV Nef or anti-HIV-1 Nef.

In vivo tyrosine-phosphorylation assay

Nef expression plasmids and the kinase-active Lck (Lck_{CA}) or the kinase-mutant Lck (Lck_{KM}) were cotransfected into 293T cells. The cellular extracts were prepared in RIPA buffer containing cellular protease inhibitors, including PMSF (1 mM), leupeptin (5 μg/ml), and aprotinin (5 μg/ml), and the phosphatase inhibitor sodium vanadate (1 mM). After clarification by centrifugation, the cellular supernatants were immunoprecipitated with rabbit anti-Flag for immunoprecipitation of Flag-tagged SIV Nef or rabbit anti-HIV-1 Nef, followed by immunoblotting using combined anti-phosphotyrosine PY20 and 4G10.

HIV-1 infection assay

The Nef mutants of HIV-1, including the native Nef_{NL}, NefΔ74VPLR77, NefΔ109ILD111, NefΔ130GPG133, and NefΔ144KLVPV148, were inserted back to the infectious DNA (pNL4–3) at the *Bam*HI and *Xho*I sites to generate pNL-Nef_{NL}, pNL-NefΔ74VPLR77, pNL-NefΔ109ILD111, pNL-NefΔ130GPG133, and pNL- NefΔ144KLVPV148. The infectious proviral DNAs were used for transient transfection of 293T cells, and the viruses in the cell-free medium were collected. The CEMX 174 cells (1×10^6) were infected with 1 ng of viruses (quantified as p24 titer by ELISA) for 8 h, and the cells were washed three times and cultured in complete medium for 12 days. The culture medium was collected and applied for measurement of p24 titer by ELISA.

ACKNOWLEDGMENTS

We thank John Hurst for FACS analysis, Dan Littman for murine Lck cDNA plasmids and pEF-BOS vector, Lu-hai Wang for pNM1 (IGFR) and pMV-Src plasmids, and Cecilia Cheng-Mayer and Yaoxing Huang for pCMV/CD8-SIV Nef plasmid and for assistance with the HIV-1 infection assay, respectively. This work is supported by a Carter-Wallace AIDS fellowship and in part by CFAR Pilot Project Program.

REFERENCES

Aiken, C., and Trono, D. (1995). Nef stimulates human immunodeficiency virus type 1 proviral DNA synthesis. *J. Virol.* **69,** 5048–5056.

Alexander, L., Du, Z., Rosenzweig, M., Jung, J. U., and Desrosiers, R. C. (1997). A role for natural simian immunodeficiency virus and human immunodeficiency virus type 1 nef alleles in lymphocyte activation. *J. Virol.* **71,** 6094–6099.

Aronheim, A., Engelberg, D., Li, N., Al-Alawi, N., Schlessinger, J., and Karin, M. (1994). Membrane targeting of the nucleotide exchange factor Sos is sufficient for activating the Ras signaling pathway. *Cell.* **78,** 949–961.

Baur, A. S., Sass, G., Laffert, B., Willbold, D., Cheng-Mayer, C., and Peterlin, B. M. (1997). The N-terminus of Nef from HIV-1/SIV associates with a protein complex containing Lck and a serine kinase. *Immunity* **6,** 283–291.

Baur, A. S., Sawai, E. T., Dazin, P., Fantl, W. J., Cheng-Mayer, C., and Peterlin, B. M. (1994). HIV-1 Nef leads to inhibition or activation of T cells depending on its intracellular localization. *Immunity* **1,** 373–384.

Briggs, S. D., Sharkey, M., Stevenson, M., and Smithgall, T. E. (1997). SH3-mediated Hck tyrosine kinase activation and fibroblast transformation by the Nef protein of HIV-1. *J Biol Chem.* **272,** 17899–17902.

Bukrinskaya, A. G., Ghorpade, A., Heinzinger, N. K., Smithgall, T. E., Lewis, R. E., and Stevenson, M. (1996). Phosphorylation-dependent human immunodeficiency virus type 1 infection and nuclear targeting of viral DNA. *Proc. Natl. Acad. Sci. USA* **93,** 367–371.

Chakrabarti, B. K., Maitra, R. K., Ma, X. Z., and Kestler, H. W. (1996). A candidate live inactivatable attenuated vaccine for AIDS. *Proc. Natl. Acad. Sci. USA* **93,** 9810–9815.

Cheng, H., Tarnok, J., and Parks, W. P. (1998). Human immunodeficiency virus type 1 genome activation induced by human T-cell leukemia virus type 1 Tax protein is through cooperation of NF-kappaB and Tat. *J. Virol.* **72,** 6911–6916.

Chowers, M. Y, Spina, C. A., Kwoh, T. J., Fitch, N. J., Richman, D. D., and Guatelli, J. C. (1994). Optimal infectivity in vitro of human immunodeficiency virus type 1 requires an intact nef gene. *J. Virol.* **68,** 2906–2914.

Collette, Y., Dutartre, H., Benziane, A., Ramos-Morales, Benarous, R., Harris, M., and Olive, D. (1996). Physical and functional interaction of Nef with Lck. HIV-1 Nef-induced T-cell signaling defects. *J. Biol. Chem.* **271,** 6333–6341.

Collins, K. L., Chen, B. K., Kalams, S. A., Walker, B. D., and Baltimore, D.

(1998). HIV-1 Nef protein protects infected primary cells against killing by cytotoxic T lymphocytes. *Nature* **391,** 397–401.

Cullen, B. R. (1991). Regulation of human immunodeficiency virus replication. *Annu. Rev. Microbiol.* **45,** 219–250.

Du, Z., Ilyinskii, P. O., Sasseville, V. G., Newstein, M., Lackner, A. A., and Desrosiers, R. C. (1996). Requirements for lymphocyte activation by unusual strains of simian immunodeficiency virus. *J. Virol.* **70,** 4157–4161.

Du, Z., Lang, S. M., Sasseville, V. G., Lackner, A. A., Ilyinskii, P. O., Daniel, M. D., Jung, J. U., and Desrosiers, R. C. (1995). Identification of a nef allele that causes lymphocyte activation and acute disease in macaque monkeys. *Cell* **82,** 665–674.

Dutartre, H., Harris, M., Olive, D., and Collette, Y. (1998). The human immunodeficiency virus type 1 Nef protein binds the Src-related tyrosine kinase Lck SH2 domain through a novel phosphotyrosine independent mechanism. *Virology* **247,** 200–211.

Gallay, P., Swingler, S., Song, J., Bushman, F., and Trono, D. (1995). HIV nuclear import is governed by the phosphotyrosine-mediated binding of matrix to the core domain of integrase. *Cell* **83,** 569–576.

Glaichenhaus, N., Shastri, N., Littman, D. R., and Turner, J. M. (1992). Requirement for association of p56lck with CD4 in antigen-specific signal transduction in T cells. *Cell* **64,** 511–520.

Greenway, A., Azad, A., and McPhee, D. (1995). Human immunodeficiency virus type 1 Nef protein inhibits activation pathways in peripheral blood mononuclear cells and T-cell lines. *J. Virol.* **69,** 1842–1850.

Greenway, A., Azad, A., Mills, J., and McPhee, D. (1996). Human immunodeficiency virus type 1 Nef binds directly to Lck and mitogen-activated protein kinase, inhibiting kinase activity. *J. Virol.* **70,** 6701–6708.

Hanna, Z., Kay, D. G., Rebai, N., Guimond, A., Jothy, S., and Jolicoeur, P. (1998). Nef harbors a major determinant of pathogenicity for an AIDS-like disease induced by HIV-1 in transgenic mice. *Cell* **95,** 163–175.

Hodge, D. R., Dunn, K. J., Pei, G. K., Chakrabarty, M. K., Heidecker, G., Lautenberger, J. A., and Samuel, K. P. (1998). Binding of c-Raf1 kinase to a conserved acidic sequence within the carboxyl-terminal region of the HIV-1 Nef protein. *J. Biol. Chem.* **273,** 15727–15733.

Kawakami, K., Scheidereit, C., and Roeder, R. G. (1988). Identification and purification of a human immunoglobulin-enhancer-binding protein (NF-kappaB) that activates transcription from a human immunodeficiency virus type 1 promoter in vitro. *Proc. Natl. Acad. Sci. USA* **85,** 4700–4704.

Kestler, H. W., 3d, Ringler, D. J., Mori, K., Panicali, D. L., Sehgal, P. K., Daniel, M. D., and Desrosiers, R. C. (1991). Importance of the nef gene for maintenance of high virus loads and for development of AIDS. *Cell* **65,** 651–662.

Kienzle, N., Bachmann, M., Muller, W. E., and Muller-Lantzsch, N. (1992). Expression and cellular localization of the Nef protein from human immunodeficiency virus-1 in stably transfected B-cells. *Arch. Virol.* **124,** 123–132.

Lee, CH, Leung, B., Lemmon, M. A., Zheng, J., Cowburn, D., Kuriyan, J., and Saksela, K. (1995). A single amino acid in the SH3 domain of Hck determines its high affinity and specificity in binding to HIV-1 Nef protein. *EMBO J.* **14,** 5006–5015.

Lee, C. H., Saksela, K., Mirza, U. A., Chait, B. T., and Kuriyan, J. (1996). Crystal structure of the conserved core of HIV-1 Nef complexed with a Src family SH3 domain. *Cell* **85,** 931–942.

Luo, W., and Peterlin, B. M. (1997). Activation of the T-cell receptor signaling pathway by Nef from an aggressive strain of simian immunodeficiency virus. *J. Virol.* **71,** 9531–9537.

Markovitz, D. M., Hannibal, M., Perez, V. L., Gauntt, C., Folks, T. M., and Nabel, G. J. (1990). Differential regulation of human immunodeficiency viruses (HIVs): A specific regulatory element in HIV-2 responds to stimulation of the T-cell antigen receptor. *Proc. Natl. Acad. Sci. USA* **87,** 9098–9102.

Miller, M. D., Warmerdam, M. T., Gaston, I., Greene, W. C., and Feinberg, M. B. (1994). The human immunodeficiency virus-1 nef gene product: A positive factor for viral infection and replication in primary lymphocytes and macrophages. *J. Exp. Med.* **179,** 101–113.

Mizushima, S., and Nagata, S. (1990). pEF-BOS, a powerful mammalian expression vector. *Nucleic Acids Res.* **18,** 5322.

Moarefi, I., LaFevre-Bernt, M., Sicheri, F., Huse, M., Lee, C H., Kuriyan, J., and Miller, W. T. (1997). Activation of the Src-family tyrosine kinase Hck by SH3 domain displacement. *Nature* **385,** 650–653.

Murti, K. G., Brown, P. S., Ratner, L., and Garcia, J. V. (1993). Highly localized tracks of human immunodeficiency virus type 1 Nef in the nucleus of cells of a human $CD4^+$ T-cell line. *Proc. Natl. Acad. Sci. USA.* **90,** 11895–11899.

Nabel, G., and Baltimore, D. (1990). An inducible transcription factor activates expression of human immunodeficiency virus in T cells *Nature* **326,** 711–713.

Novembre, F. J., Lewis, M. G., Saucier, M. M., Yalley-Ogunro, J., Brennan, T., McKinnon, K., Bellah, S., and McClure, H. M. (1996). Deletion of the nef gene abrogates the ability of SIV smmPBj to induce acutely lethal disease in pigtail macaques. *AIDS Res. Hum. Retroviruses* **12,** 727–736.

Nunn, M. F., and Marsh, J. W. (1996). Human immunodeficiency virus type 1 Nef associates with a member of the p21-activated kinase family. *J. Virol.* **70,** 6157–6161.

Ranki, A., Lagerstedt, A., Ovod, V., Aavik, E., and Krohn, K. J. (1994). Expression kinetics and subcellular localization of HIV-1 regulatory proteins Nef, Tat and Rev in acutely and chronically infected lymphoid cell lines. *Arch. Virol.* **139,** 365–378.

Ratner, L., and Niederman, T. M. (1995). Nef. *Curr. Top. Microbiol. Immunol.* **193,** 169–208.

Resh, M. D. (1994). Myristylation and palmitylation of Src family members: the fats of the matters. *Cell* **76,** 411–413.

Robert-Guroff, M., Popovic, M., Gartner, S., Markham, P., Gallo, R. C., and Reitz M. S. (1990). Structure and expression of tat-, rev-, and nef-specific transcripts of human immunodeficiency virus type 1 in infected lymphocytes and macrophages. *J. Virol.* **64,** 3391–3398.

Saksela, K., Cheng, G., and Baltimore, D. (1995). Proline-rich (PxxP) motifs in HIV-1 Nef bind to SH3 domains of a subset of Src kinases and are required for the enhanced growth of Nef+ viruses but not for down-regulation of CD4. *EMBO J.* **14,** 484–491.

Sawai, E. T., Baur, A., Struble, H., Peterlin, B. M., Levy, J. A., and Cheng-Mayer, C. (1994). Human immunodeficiency virus type 1 Nef associates with a cellular serine kinase in T lymphocytes. *Proc. Natl. Acad. Sci. USA* **91,** 1539–1543.

Sawai, E. T., Baur, A. S., Peterlin, B. M., Levy, J. A., and Cheng-Mayer, C. (1995). A conserved domain and membrane targeting of Nef from HIV and SIV are required for association with a cellular serine kinase activity. *J. Biol. Chem.* **270,** 15307–15314.

Schwartz, O., Marechal, V., Danos, O., and Heard, J. M. (1995). Human immunodeficiency virus type 1 Nef increases the efficiency of reverse transcription in the infected cell. *J. Virol.* **69,** 4053–4059.

Shugars, D. C., Smith, M. S., Glueck, D. H., Nantermet, P. V., Seillier-Moiseiwitsch, F., and Swanstrom, R. (1993). Analysis of human immunodeficiency virus type 1 nef gene sequences present in vivo. *J. Virol.* **67,** 4639–4650.

Siekevitz, M., Josephs, S. F., Dukovich, M., Peffer, N., Wong-Staal, F., and Greene, W. C. (1987). Activation of the HIV-1 LTR by T cell mitogens and the transactivator protein of HTLV-I. *Science* **238,** 1575–1578.

Skowronski, J., Parks, D., and Mariani, R. (1993). Altered T cell activation and development in transgenic mice expressing the HIV-1 nef gene. *EMBO J.* **12,** 703–713.

Smith, BL, Krushelnycky, BW, Mochly-Rosen, D, Berg, P. (1996). The HIV nef protein associates with protein kinase C theta. *J. Biol. Chem.* **271,** 16753–16757.

Spina, C. A., Kwoh, T. J., Chowers, M. Y., Guatelli, J. C., and Richman, D.D. (1994). The importance of nef in the induction of human immunodeficiency virus type 1 replication from primary quiescent CD4 lymphocytes. *J. Exp. Med.* **179,** 115–123.

Swingler, S., Gallay, P., Camaur, D., Song, J., Abo, A., and Trono, D. (1997). The Nef protein of human immunodeficiency virus type 1 enhances serine phosphorylation of the viral matrix. *J. Virol.* **71,** 4372–4377.

Trono, D. (1995). HIV accessory proteins: Leading roles for the supporting cast. *Cell* **82,** 189–192.

Wange, R. L., and Samelson, L. E. (1996). Complex complexes: Signaling at the TCR. *Immunity* **5,** 197–205.

Weil, R., and Veillette, A. (1996). Signal transduction by the lymphocyte-specific tyrosine protein kinase p56lck. *Curr. Top. Microbiol. Immunol.* **205,** 63–87.