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The N-Terminus of Nef from HIV-1/SIV Associates with a Protein Complex Containing Lck and a Serine Kinase

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Summary

The Nef protein of human and primate lentiviruses is a key factor in HIV/SIV pathogenesis. Here we report that Nef associates with two different kinases, forming a multiprotein complex at the far N-terminus of the viral protein. One of the kinases was identified as Lck, whereas the second protein was found to be a serine kinase that phosphorylated Nef and Lck in vitro and could be discriminated from the serine kinase identified previously. The Nef-associated kinase complex (NAKC) was demonstrated in COS cells, in HIV-infected cells, and in vitro using recombinant Lck and Nef proteins. Deletion of a short amphipathic α-helix in the N-terminus, which was found to be conserved in all Nef proteins, inhibited association of the NAKC and significantly reduced virion infectivity.

Introduction

The genomes of the human and simian immunodeficiency viruses (HIV and SIV) encode an unusually high number of regulatory genes (*tat*, *rev*, *vif*, *vpu*, *nef*, *vpr*, and *vpx* in HIV-2 and SIV) and thus are considered complex retroviruses (for review see Subbramanian and Cohen, 1994; Cullen, 1995). Four of these genes, *vif*, *vpu*, *vpr*, and *nef*, are dispensable for virus replication in a number of in vitro systems and were therefore termed "nonessential" (for review see Trono, 1995). In the infected host, however, the *nef* gene appears to be a key factor in the pathogenesis of SIV/HIV infection. This is based on studies in Rhesus monkeys, which upon infection with *nef*-deleted strains of SIV did not develop high virus loads or progress to AIDS (Kestler et al., 1991). The importance of Nef in the virus pathogenesis was further supported by the discovery of deleted *nef* genes in HIV-infected, long-term nonprogressing individuals (Deacon et al., 1995; Kirchhoff et al., 1995).

Although the importance of Nef for the viral life cycle has not been questioned, its function at the molecular level remains to be determined. To date, at least three functions for Nef have been described. First, Nef leads to the rapid endocytosis of CD4 from the surface of infected or transfected cells. The implication of this effect of Nef is not clear. The virus could, for example, block superinfection of cells by reducing the number of receptors on the surface or, alternatively, modulate T cell activation pathways through increased levels of free Lck in the cytoplasm (Rhee and Marsh, 1994; Aiken et al., 1994). Second, Nef was reported to increase viral infectivity in vitro in a single cycle infection assay (Miller et al., 1994). This report confirmed other studies showing higher levels of viral replication in T cells with *nef*-competent HIV as compared with their *nef*-deleted counterparts (Spina et al., 1994; Chowers et al., 1994; Zazopoulos and Haseltine, 1993). Based on mutational analysis, down-regulation of CD4 and increase of viral infectivity were mapped to different regions of the molecule (Saksela et al., 1995; Goldsmith et al., 1995). Third, Nef modulates T cell activation pathways. Stable expression of Nef in T cell lines perturbed T cell receptor (TCR)-mediated activation of numerous cellular factors, including NF-κB and interleukin-2 (Greenway et al., 1995; Niederman et al., 1992; Luria et al., 1991). The inhibition of T cell signaling seems to depend on the subcellular localization of Nef as shown with a CD8–Nef chimeric fusion protein stably expressed in Jurkat cells. In these cell lines, CD8–Nef was almost exclusively found in the cytoplasm, which correlated with inhibition of TCR-mediated induction of tyrosine phosphorylation and Ca^{2+} mobilization. Cells with higher levels of CD8–Nef at the plasma membrane, however, not only had restored signaling pathways, but were found to be activated T cells (Baur et al., 1994). In line with these results, a SIV Nef allele was recently identified exhibiting profound effects on T cell activation and cell transformation (Du et al., 1995).

To understand the multiple effects of Nef, identification of proteins that associate with Nef is critical. Until now, numerous cellular factors have been implicated in binding to Nef, among them two different kinase families. In studies with transfected and infected cell lines, a serine kinase was reported to coprecipitate with Nef of HIV and SIV (Sawai et al., 1994, 1995). In other reports, using recombinant proteins, the tyrosine kinases Lck, Hck, and Lyn associated with Nef (Collette et al., 1996; Greenway et al., 1995; Saksela et al., 1995). Both kinase families were found to bind the more conserved central part of Nef. The role of either kinase family in the above described functions of Nef, however, is unclear. In this study, we report that the N-terminus of Nef associates with a protein complex (referred to as NAKC, for Nef-associated kinase complex) containing Lck and a serine kinase that is distinct from the PAK-related kinase described recently (Sawai et al., 1994; Lu et al., 1996). Our results suggest that the NAKC is required for Nef-mediated increase of viral infectivity and therefore the function of Nef.

Results

A Kinase Activity Associates with the N-Terminus of Nef

In previous studies on the effects of Nef on T cell signaling (Baur et al., 1994; Sawai et al., 1994, 1995), we expressed a CD8–Nef chimera (SF2 strain) in T and COS cells. In these studies we found that Nef associated specifically with a serine kinase (Sawai et al., 1994). Characteristically, this kinase did not bind to N-terminal fragments of Nef (Sawai et al., 1995). An example of these results is shown in Figure 1A, lanes 1–3. Full-length CD8–Nef (CN), as well as a truncated form of Nef encoding the first 94 amino acids (CN.94) and truncated CD8 (CT), consisting of the extracellular and transmembrane part of CD8, were transfected into COS cells. After 2 days, the chimeras were immunoprecipitated with anti-CD8 and in vitro kinase assays were performed. As shown in lane 2 of Figure 1A, a single band of molecular mass 62 kDa (p62) was detected with the full-length CN chimera, representing the kinase itself or a substrate (Sawai et al., 1994). No activity was detected with the truncated Nef construct (Figure 1A, lane 3). We repeated this experiment using Mn^{2+} instead of Mg^{2+} in the kinase buffer. As shown in Figure 1A, lanes 4–6, in addition to p62 a second band of about 53 kDa was detected using the full-length CN chimera (lane 5, open arrow), and a single prominent band was observed with CN.94 (lane 6, closed arrow). Other bands visible in Figure 1A (lanes 5 and 6) are probably nonspecific. To confirm that the same amounts of CN chimeras were expressed, we blotted the immunoprecipitates from Figure 1A with an anti-Nef rabbit serum. In Figure 1B, the expected Nef bands at around 53 kDa (CN, lanes 2 and 5) and 42 kDa (CN.94, lanes 3 and 6) are shown. The additional bands in the Mn^{2+}-based kinase assay (Figure 1A, lanes 4–6) comigrated with the Nef bands in Figure 1B (compare open and closed arrows in Figures 1A and 1B). These results implied that the CN chimeras were phosphorylated by a second kinase, which associated with the N-terminus of Nef.

In T Cells Lck and a Second Kinase Associate with the N-Terminus of Nef

Following immunoprecipitation and in vitro kinase assays, N-terminal subfragments of Nef derived from stable Jurkat cell lines were also found to be phosphorylated. As shown in Figure 1C, kinase reactions with CN.49 and CN.94, coding for the N-terminal 49 and 94 amino acids of Nef, revealed the presence of phosphorylated bands corresponding to the molecular mass of the chimeras (lanes 3 and 4). In addition, a protein with a molecular mass comparable with Lck (56–60 kDa) was coprecipitated (Figure 1C, lanes 3 and 4). For comparison, Lck was immunoprecipitated from Jurkat control (J.CT) cells and subjected to the same in vitro kinase assay (Figure 1C, lane 1). The 60 kDa protein was not observed in immunoprecipitates of truncated CD8 (Figure 1C, lane 2, CT) or a control CN construct coding for the C-terminal 47–211 amino acids of Nef (lane 5, CN.47/211). To ascertain the identity of the 60 kDa band, the immunoprecipitates shown in Figure 1C were reimmunoprecipitated with a Lck-specific antibody. As shown in Figure 1D, Lck was found in immunoprecipitates of

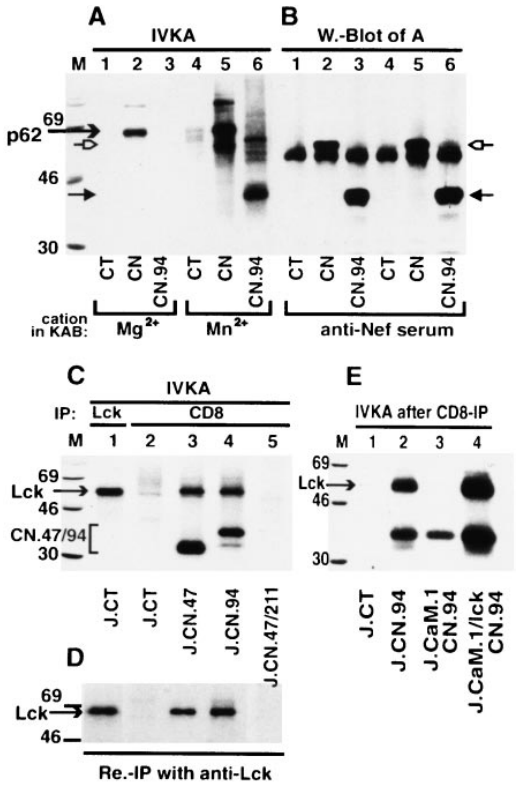

Figure 1. N-Terminal Fragments of Nef Associate with Two Different Kinase Activities in COS and T Cells

(A) In vitro kinase assay and Western blot of CD8–Nef (CN) chimeras transfected into COS cells. Two different CN chimeras and a CD8 control were transfected into COS cells, immunoprecipitated with an anti-CD8 MAb, and subjected to an in vitro kinase assay (IVKA) using Mg^{2+} or Mn^{2+} cations in the kinase buffer. After separation by 12.5% SDS–PAGE, the immunoprecipitates were transferred onto nitrocellulose. CT (CD8 truncated) was used as control; CN encodes the full-length Nef protein, and CN.94 encodes the N-terminal 94 amino acids of Nef.

(B) Western blot analysis of (A). The immunoprecipitates described in (A) were blotted with an anti-Nef serum. Note that bands in lane 5 of (A) and (B) migrate with the same mobility (open arrows), as well as bands in lane 6 of (A) and (B) (closed arrows).

(C) IVKA with Lck (lane 1) and different CN chimeras (CN.47 and CN.94, lanes 3 and 4) after immunoprecipitation (IP) from Jurkat (J.) cell lines. For control, CT and CN.47/211, coding for the C-terminal 47–211 amino acids of Nef, were used (J.CT, lane 2; J.CN.47/211, lane 5). CN.47 and CN.94 associate with a protein of same molecular mass as Lck, indicated by an arrow. The lower bands in lanes 3 and 4 represent phosphorylated CN.47 and CN.94, respectively.

(D) Reimmunoprecipitation of Lck with a specific antibody from in vitro kinase reactions shown in (C). Each lane in (C) corresponds to the respective lower lane in the panel. Lck could be reimmunoprecipitated from in vitro kinase reactions with Lck (serving as positive control), CN.47, and CN.94 (lanes 1, 3, and 4).

(E) In vitro kinase reaction with a short CN chimera (CN.94) immunoprecipitated from Jurkat (J.CN.94, lane 2), J.CaM.1 (J.CaM.1.CN.94, lane 3), and J.CaM.1 cells reconstituted with *lck* (J.CaM.1/lck.CN.94, lane 4). The lower bands in lanes 2–4 represent phosphorylated CN.94.

Lck itself (lane 1), serving as positive control, as well as in CN.49 and CN.94 (lanes 3 and 4). Thus, the 60 kDa band was identified as Lck. To determine whether the short CN chimeras were phosphorylated by Lck, CN.94 was expressed in J.CaM.1 cells, which lack expression of Lck (Straus and Weiss, 1992) and in J.CaM.1 cells reconstituted with *lck* (J.CaM.1/lck; Straus and Weiss, 1992). After obtaining stable cell lines, CN.94 was immunoprecipitated and subjected to an in vitro kinase assay. As shown in Figure 1E, Lck association was seen in Jurkat (lane 2) and J.CaM.1/lck cells (lane 4), but not in J.CaM.1 cells (lane 3). Since CN.94 was phosphorylated in the absence of Lck in T cells (lane 3) and in COS cells (Figure 1), we have concluded that a second kinase associates with the N-terminus of Nef independent of the presence of Lck.

Lck and a Serine Kinase Colocalize at the N-Terminus of Nef

To map the binding site of both kinases, stable Jurkat cell lines were established with several deletion mutants of CN, coding for the N-terminal 94, 49, 35, 26, 22, 20, and 15 amino acids of Nef. Truncated CD8 (CT) and a CN chimera encoding amino acids 47–105 were used as controls. The subfragments of Nef were immunoprecipitated and subjected to in vitro kinase assays as described above. These experiments revealed that a chimera as short as CN.22 still bound both kinases (Figure 2A, lane 6), whereas CN.20 or CN.15 (Figure 2, lanes 7 and 8) or any of the controls (Figure 2, lanes 1 and 9) did not. To confirm that all constructs were expressed at comparable levels, the respective stable cell lines (J.CN.94 to J.CN.45/105) were metabolically labeled with ^{35}S and the CN chimeras were immunoprecipitated with anti-CD8 (Figure 2B).

Next, the phosphorylated amino acid residues in Lck and phosphorylated CN.47 (shown in Figure 2A, lane 3) were identified. For this purpose, the in kinase reactions were transferred to nitrocellulose, from which both bands were excised (indicated by arrows) and subjected to phosphoamino acid analyses. As shown in Figure 2C, we found Lck to be phosphorylated on a tyrosine as well as on a serine residue, whereas CN.47 was only phosphorylated on a serine residue. The tyrosine phosphorylation was likely to be the result of the autophosphorylation activity of Lck, as described elsewhere (Straus and Weiss, 1992). The serine phosphorylation was obviously due to the associating serine kinase, which phosphorylated both Lck and CN.47. Taken together, these results indicated that both kinase activities colocalized at the N-terminus of Nef.

Binding of Nef to the N-Terminal Kinase Complex and p62

To ascertain whether phosphorylation of full-length Nef as well as binding to Lck and p62 occur simultaneously, a C-terminal-tagged (AU-1) Nef allele (nef.4-AU) derived from uncultured peripheral blood mononuclear cells (PBMC) of an HIV-infected individual, was investigated in COS cells and in vitro kinase assays. As shown in Figure 3A, cotransfection of nef.4-AU with mock DNA,

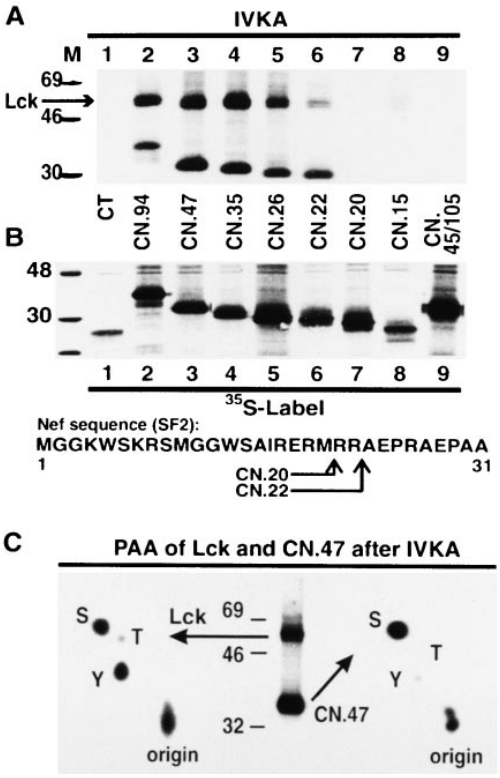

Figure 2. Binding and Characterization of Kinase Activities Associating with the N-Terminus of Nef

(A) Increasingly shorter forms of CD8–Nef were immunoprecipitated from Jurkat cell lines, and in vitro kinase assays were performed as described in Figure 1. The constructs ranged from amino acid 94 (CN.94, lane 2) to amino acid 15 (CN.15, lane 8). For control, CT (CD8 truncated) was used (lane 1) and CN.49/105, expressing amino acids 49–105 of Nef, was fused to truncated CD8 (lane 9).

(B) To demonstrate comparable levels of expression of all constructs described in (A), cell lines were metabolically labeled with ^{35}S and immunoprecipitated with anti-CD8. The sequence at the bottom of the figure shows the N-terminal 31 amino acids of Nef (SF2), indicating by arrows where Nef was truncated in CN.20 and CN.22.

(C) Phosphoamino acid analysis (PAA) of the upper (Lck) and lower band (CN.47) also shown in lane 3 of (A). Lck is phosphorylated on a tyrosine and serine residue, whereas CN.47 was only phosphorylated on serine. The positions of unlabeled phospho-serine (S), phospho-threonine (T), and phospho-tyrosine (Y) are indicated.

Lck, or Fyn resulted in Nef phosphorylation and binding of p62 and Lck, but not Fyn (lanes 2–4, see arrows). Interestingly, the band representing Nef shifted upon cotransfection with Lck but not Fyn (Figure 3A, lanes 3 and 4), suggesting that nef.4-AU, unlike CN.47 (see Figure 2), was phosphorylated by Lck. These results indicated that association of Nef with the N-terminal kinase complex (NAKC) can occur simultaneously to the binding of p62. For further confirmation, SIV_{mac239}-Nef was cotransfected with mock DNA or Lck (Figure 3A, lanes 5 and 6) into COS cells and immunoprecipitated with an anti–SIV Nef antibody. Phosphorylation of SIV Nef, binding of Lck, and p62 was observed as in nef.4-AU. (Figure 3A, lane 6). As in HIV-1 Nef, the N-terminus of SIV Nef was phosphorylated and bound to Lck, as could

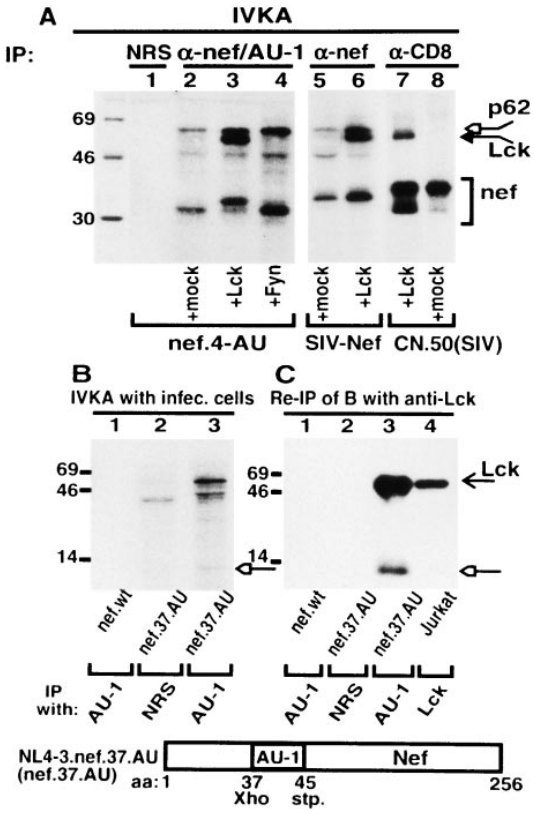

Figure 3. Phosphorylation of Nef and Association with Lck in COS and HIV-Infected Cells

(A) In vitro kinase assay (IVKA) with a C-terminal-tagged (AU-1), primary Nef protein (Nef.4-AU), nonfused SIV_{mac239}-Nef, and a CN chimera coding for the first 50 amino acids of SIV_{mac239}-Nef, cotransfected with mock DNA (lanes 2, 5, and 8), Lck (lanes 3, 6, and 7), or Fyn (lane 4) into COS cells. Prior to IVKAs, Nef was immunoprecipitated with a nonspecific rabbit serum (NRS, lane 1), anti-AU-1 (lanes 2–4), anti–SIV Nef serum (lanes 5 and 6), or anti-CD8 (lanes 7 and 8). The position of p62 (Sawai et al., 1994), Lck, and phosphorylated Nef are indicated by arrows and bracket.

(B) IVKA after immunoprecipitation of N-terminal Nef fragments from HIV-infected cells. As depicted at the bottom of (B) and (C), an oligonucleotide encoding an AU-1 tag and a stop codon was introduced into the XhoI site of the *nef* reading frame from the NL4-3 provirus (NL4-3.nef.37. AU). Cells infected with NL4-3.nef.37.AU (nef.37.AU) or wild-type NL4-3 (nef.wt) were lysed and immunoprecipitated with anti-AU-1 (lanes 1 and 3) or a nonspecific rabbit serum (NRS, lane 2). Kinase reactions were analyzed by 15% SDS–PAGE. The position of the putative phosphorylated N-terminal Nef fragment (46 amino acids) is indicated by an open arrow in (B) and (C).

(C) Reimmunoprecipitation of Lck with a specific MAb from kinase reactions described in (B). For comparison, Lck was immunoprecipitated from Jurkat cells and subsequently reimmunoprecipitated in an analogous way (lane 4). The Lck signal of the control (lane 4) is less intense since we used lysates from only 5×10^6 uninfected as compared with 40×10^6 infected cells (lanes 1–3) for immunoprecipitation.

be shown in cotransfections with a CN chimera, encoding for the first 50 amino acids of SIV_{mac239}-Nef (CN.50, Figure 3A, lanes 7 and 8).

Association of Lck with the N-Terminus of Nef in Infected Cells

To demonstrate the association of Lck with the N-terminus of Nef in infected cells, we introduced an oligonucleotide coding for an AU-1 tag plus a stop codon into the Xho site of the *nef* allele of an HIV-1 proviral clone (NL4-3; see the bottom of Figure 3), creating a 37 amino acid N-terminal fragment of Nef. We used this strategy for two reasons: first, we wanted to dissect N-terminal binding of Lck to Nef from a potential binding to the conserved central part as reported recently (Collette et al., 1996), and, second, N-terminal fragments of Nef, but not the full-length Nef protein, are readily expressed at the plasma membrane of Jurkat cells where Lck is located (Baur et al., 1994). Jurkat cells were permanently infected with the recombinant (NL4-3.nef.37.AU) and the wild-type NL4-3 virus without AU-1 tag, serving as control (NL4-3.nef.wild-type). Cytoplasmic lysates equivalent to 4×10^6 cells from each cell line were immunoprecipitated with anti-AU-1 or a nonspecific rabbit serum and subjected to an in vitro kinase reaction. An aliquot of the reaction (one tenth) was separated by 15% SDS–polyacrylamide gel electrophoresis (SDS–PAGE) (Figure 3B), whereas the remainder of the reaction was reimmunoprecipitated with a specific antibody against Lck. As shown in Figure 3C, Lck could be reimmunoprecipitated from the in vitro reactions at significant levels (lane 3, see upper arrow). Along with Lck, a second band was observed (see open arrow in Figure 3C), probably representing the phosphorylated N-terminal fragment of Nef. This short fragment was also seen as a faint signal in the initial immunoprecipitation (see arrow in Figure 3B). For comparison, Lck was immunoprecipitated from Jurkat cells and subsequently reimmunoprecipitated in the same manner (Figure 3C, lane 4).

Assembly of the Nef-Associated Kinase Complex In Vitro with Recombinant Proteins and Cytoplasmic Lysates of Jurkat Cells

Next, we asked whether the Nef-associated kinase complex could be assembled in vitro, using baculovirus-derived glutathione S-transferase (GST)–Lck and prokaryotically expressed Nef. Along with an unrelated protein (up), serving as control, the *nef.4* allele was expressed and purified using the pET system (Novagen). In Figure 4A, aliquots of the purified proteins (13 μg) are shown in a Coomassie-stained 15% SDS–polyacrylamide gel. To assemble the complex, 1 μg of GST–Lck and 5 μg of recombinant Nef or up were incubated in the presence of bovine serum albumin (BSA) or cytoplasmic lysates from Jurkat or Sf9 cells as indicated in Figure 4B. After the incubation, GST–Lck was immunoprecipitated and an in vitro kinase assay was performed. As seen in Figure 4B, GST–Lck coimmunoprecipitated with a phosphorylated Nef protein, and probably with N-terminal fragments of Nef (indicated by an asterisk; see also Figure 4A), in the presence of Jurkat lysates (lane 4), but not in the presence of BSA (lane 2) or Sf9 lysates (lane 6). No association or a nonspecific phosphorylation activity was seen with the unrelated protein (Figure 4B, lanes 3 and 5). Phosphoamino acid analysis with the phosphorylated Nef protein revealed the same result as in Figure 2, namely that Nef was phosphorylated mainly on a serine residue(s) (data not shown). Using this experimental approach, we did not see an association with p62. Likewise, incubation of baculovirus-derived Nef

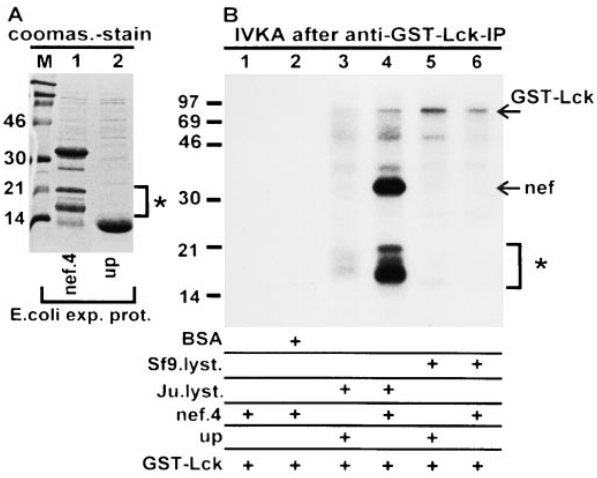

Figure 4. Association of Recombinant Nef and Lck Proteins In Vitro

(A) Coomassie staining of prokaryotically expressed and purified Nef.4 and an unrelated protein (up). The asterisk indicates probable degradation products of Nef, which could not be removed by the purification process.

(B) In vitro kinase assay after immunoprecipitation of GST–Lck, which was incubated with Nef (nef.4) in the presence of lysis buffer (lane 1), BSA (lane 2), Jurkat lysate (Ju.lyst., lane 4), or lysate from Sf9 cells (Sf9.lyst., lane 6). Similarly, GST–Lck was incubated with a control protein in the presence of Jurkat (lane 3) or Sf9 cellular lysate (lane 5) prior to the kinase reaction. Owing to the short exposure, autophosphorylation of GST–Lck (see arrow) was low. Association and phosphorylation of Nef was only seen in the presence of Jurkat lysates (lane 4). Phosphorylated bands indicated by the asterisk probably represent N-terminal fragments of Nef as indicated in (A) (see the same asterisk in [A]).

proteins with Jurkat lysates did not reveal binding of p62 (data not shown). In conclusion, these results demonstrated that the binding of Lck to Nef as well as the phosphorylation of Nef required at least a third factor, which was present in cytoplasmic lysates of T but not Sf9 cells.

The NAKC Is Required for Increased Viral Infectivity

Next, we evaluated the functional relevance of the NAKC for viral replication. The N-terminus of HIV-1 Nef shows considerable heterogeneity and has almost no homology to Nef of SIV_{mac239}. Moreover, it was found to be a separate domain (residues 1–57 in HIV-1 NL4-3; Freund et al., 1994a, 1994b), distinguishable from the more conserved C-terminal part of Nef. However, the amino acids 16–22 (SF2:IRERMRR) are well preserved in HIV-1 strains. Among HIV-2/SIV strains the corresponding region is similarly preserved and resembles the HIV-1 motif ($SIV/HIV\text{-}2_{cons}$:LRERLLRAR). We therefore analyzed the N-termini (amino acids 1–57) of 42 randomly selected HIV and SIV Nef sequences for possible secondary structure similarities, applying the PhD network (Rost and Sander, 1993). In all cases, an overall basic charge and an amphipathic α-helical structure was predicted for residues 14–21 (Figure 5). In view of this finding, the IRERMRR motif was deleted, and the resulting clone (CN.Δ16–22 or untagged Δ16–22) was subsequently tested for Nef phosphorylation, p62/Lck binding, and CD4 down-regulation. In a second clone, we introduced a larger deletion in the N-terminus (amino acids 11–40 and Δ11–40) to determine whether additional domains upstream and downstream of the IRERMRR motif would affect the function of Nef. As shown in Figure 6A, both mutants (CN fusion proteins CN.Δ16–22; CN.Δ11–40) still bound p62 but were not phosphorylated like the wild-type CN (compare lanes 1 with 2 and 3; for the identity of the phosphorylated band, indicated by P-CN and an arrow in lane 1, see also Figures 1A and 1B). Consistent with the idea of a protein complex binding to the N-terminus of Nef, several additional phosphorylated bands, which are usually observed in kinase assays with Nef, were absent with both mutants (compare lanes 1 with 2 and 3 in Figure 6A and lanes 3–5 in Figure 6B).

Upon cotransfection with Lck, both mutants had a reduced capacity to bind the tyrosine kinase (Figure 6B, compare lanes 3–5); however, they were not completely unable to do so. The latter result prompted us to reevaluate whether, in COS cells, parts of Nef other than the N-terminus would contribute to the binding of Lck. As demonstrated in Figure 6C, in COS cells, but not in T cells (see Figure 1C), CN.47–211 still associated with Lck, although to a much lesser degree than the N-terminus of Nef represented by CN.47 (Figure 6C). A possible mechanism for this interaction will be discussed below. Like the wild-type SF2 Nef protein, both mutants (without CD8 tag) down-regulated CD4 after cotransfection into COS cells (Figure 7A). For comparison and control,

AA:	1	16 20	24
SIV-S4	MGGAISKKQYKRG	GN**L**RER**L**L	QAR
SIV-MA	MGGTISMRRSRST	GD**L**RQR**L**L	RAR
SIV-AG	MGLGNSKPQHKKQ	LS**L**WHA**L**H	KTR
HV2-RO	MGASGSKKHSRPP	RG**L**QER**L**L	RAR
HV2-NZ	MGASGSKKRSKPL	QG**L**QER**L**L	QAR
HV2-D1	MGASGSKKRSEHS	QG**L**RER**L**L	RAR
HV1-SC	MGGKWSKRSVVGW	PT**V**RER**M**R	KTE
HV1-PV	MGGKWSKSSVIGW	PA**V**RER**M**R	RAE
HV1-MA	MGGKWSKSSIVGW	PK**I**RER**I**R	RTP
SIV_{mac239}	MGGAISMRRSRPS	GD**L**RQR**L**L	RAR
Nef.4	MGGKWSKSSVIGW	PT**V**RER**M**R	RAE

Figure 5. Clustal V Multiple Sequence Alignment of HIV and SIV Nef Proteins

Sequences were obtained from the SwissProt data bank. Secondary structure prediction using the PhD network (Rost and Sander, 1993) for 42 randomly selected HIV and SIV Nef sequences (only 9 are shown) in all cases predicted an α-helical structure for residues 14–21 (boxed). For this region no deletions or insertions occur in any of the Nef amino acid sequences. Owing to the conserved spacing of the hydrophobic residues 16 and 20 (bold), the amphipathic character of the predicted helix is retained in all Nef proteins. Another feature of region 14–21 in all Nef sequences is its overall basic charge. In addition, we show the sequences of Nef.4 and SIV_{mac239}.

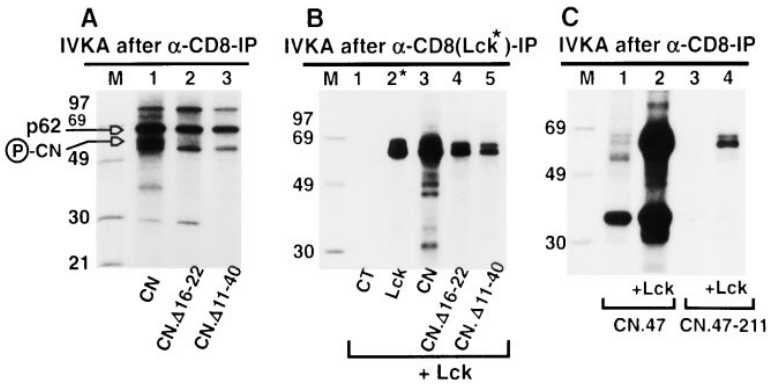

Figure 6. The N-Terminal Nef Mutants Bind p62 but Not the NAKC

(A) In vitro kinase assay (IVKA) of CN and mutant CN chimeras with N-terminal deletions (CN.Δ16–22 and CN.Δ11–40) after anti-CD8 immunoprecipitation (IP) from COS cells. CN (lane 1), but not mutant CN chimeras (lanes 2 and 3), were phosphorylated (P-CN and arrow) and associated with additional phosphorylated proteins. In contrast, all CN constructs associated with p62 (arrow).

(B) IVKA after anti-CD8 immunoprecipitation of control CT (lane 1), CN (lane 3), and CN mutant chimeras (CN.Δ16–22 and CN.Δ11–40, lanes 4 and 5) cotransfected with Lck into COS cells. For comparison, Lck was immunoprecipitated (lane 2). The mutant CN chimeras showed a reduced binding to Lck (lanes 4 and 5).

(C) IVKA after anti-CD8 immunoprecipitation of CN.47 and CN.47–211 cotransfected with (lanes 2 and 4) or transfected without Lck (lanes 1 and 3) into COS cells. In comparison with the N-terminus (CN.47, lane 2), the C-terminus of Nef (CN.47–211, lane 4) showed a significantly reduced association with Lck.

a primary Nef variant was used, which was obtained from uncultured PBMC (Figure 7A, nef.11) and repeatedly showed a reduced ability to down-regulate CD4 in transient assays. Finally, both mutants were cloned into a proviral background (NL4-3). The resulting clones, along with a positive (wild-type SF2/NL4-3) and a *nef* negative control (N-terminal deletion in *nef*), were transfected into Jurkat cells and infectious supernatants were collected. Resting PBMC were infected with an infectious dose equivalent to 2 ng of p24. After 2 days the cells were stimulated with OKT3, and culture supernatants were collected for 14 days and analyzed for RT activity. In comparison with the positive control both mutants showed a delay in detectable viral replication of about 4 days (Figure 7B), which was an intermediate phenotype between the wild type and the *nef* negative control. Thus, an intact N-terminus of Nef appeared to be required for optimal viral infectivity.

Discussion

In this study we present evidence in support of an interaction of Nef with a serine and a tyrosine kinase activity. Binding of both kinases was colocalized to the far N-terminus of the viral protein, suggesting the formation of a multiprotein complex (NAKC). Whereas the tyrosine kinase could be identified as Lck, the serine kinase was found to phosphorylate Nef as well as Lck in vitro. The NAKC could be demonstrated in COS cells, in infected cells, and in vitro by incubating recombinant Lck and Nef with Jurkat cytoplasmic lysates. Finally, we presented evidence that the NAKC interacted with a conserved amphipathic α-helix in the N-terminus of Nef and was necessary for optimal infection of resting PBMC.

The NAKC is clearly distinct from a second set of cellular factors (p72/74 and p62), including a serine kinase, reported previously to associate with Nef of HIV and SIV (Sawai et al., 1994). For binding to Nef the latter factors require the conserved central domain (Sawai et al., 1995; see also Figure 1), whereas the kinase complex reported here binds to the far N-terminus of Nef. In addition, Nef is not phosphorylated by the serine kinase described by Sawai et al., and differential binding of both protein complexes to Nef could be demonstrated in our report (note that the presence of p72/74 and p62 is best observed in T cells). Even after overexpression in COS cells, it took a sensitive detection system such as the in vitro kinase assay to demonstrate an association between Lck and Nef. This could be explained by a transient, low affinity interaction of both partners, a rate-limiting modification step, or a third or bridging protein being the limiting factor in these assays. As shown with

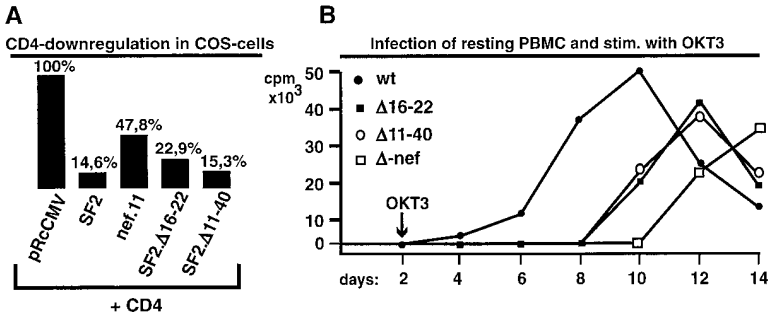

Figure 7. The N-Terminal Nef Mutants Show a Reduced Viral Infectivity but Maintain the Ability to Down-Regulate CD4

(A) CD4 expression on the surface of COS cells after cotransfection of empty vector DNA (pRcCMV), untagged SF2-Nef (SF2), a primary Nef variant (nef.11), mutant SF2-proteins (SF2.Δ16–22 and SF2.Δ11–40), and a βGal-expression plasmid (pSVβGal). Values on top of the bars represent the CD4 expression in percent of the negative control (CD4 plus pRcCMV), representative of two independent experiments with a CD4 surface expression of at least 60% in the negative control.

(B) HIV replication after infection of resting PBMC with NL4-3 (wild type [wt]) serving as positive control, NL4-3.Δ-nef (Δ-nef) serving as negative control, and NL4-3 *nef* mutants (Δ16–22 and Δ11–40). At 2 days postinfection, the PBMC were stimulated with OKT3 for 2 days. Data presented are averages of RT values, assessed in the culture supernatants and obtained from two independent infections.

recombinant proteins, Lck did not bind directly to Nef but required a third factor present in Jurkat but not Sf9 cytoplasmic lysates. This again suggested the formation of a protein complex at the N-terminus of Nef. The experiment with the recombinant proteins also demonstrated that the NAKC could be formed independently of Nef myristoylation and membrane targeting, but did not rule out that association of Nef with the plasma membrane favored formation of the complex.

Despite the considerable sequence heterogeneity in the N-terminus of all HIV/SIV Nef proteins, secondary structure predictions revealed a 7 amino acid overall basic amphipathic α-helix, which appeared to be conserved in all Nef proteins (Figure 5). Mapping of the binding site for the NAKC (Figure 2) was leading to the same motif. This could explain why, despite the sequence heterogeneity, both the N-termini of SIV and HIV-1 Nef bound Lck and were phosphorylated probably by the same serine kinase. Deletion of the IRERMRR motif in the full-length Nef protein abolished phosphorylation of Nef and reduced binding of Lck. In addition, several phosphorylated bands, usually observed in kinase assays with Nef proteins, were absent with the mutants. This further suggested that a protein complex associated with the N-terminal α-helix. Preliminary data indicate that one of these "disappearing" bands of higher molecular mass (between 70 and 80 kDa) is the second kinase itself (Baur et al., 1996). In line with our results, Bodeus et al. (1995) reported the association of a serine kinase with short N-terminal fragments of Nef, expressed as GST proteins and incubated with cytoplasmic lysates from Jurkat cells. These authors also described the coprecipitation of a phosphorylated 60 kDa protein, probably representing Lck but not investigated in detail.

Deletion of the N-terminal α-helix did not completely abolish binding of Lck. A direct comparison of kinase assays with CN.47 and CN.47–211 in COS cells indicated that regions other than the N-terminus bound to Lck, albeit to a much lesser extent (Figure 6). A similar observation was made by Collette et al. (1996), who found a direct binding of recombinant expressed SH2/SH3 domains of Lck to tyrosine-phosphorylated Nef and a proline-rich motif (PxxP motif) in the conserved central domain. It is not unlikely that several domains in Nef are involved in the binding of Lck, as reported for the binding of Hck to Nef (Saksela et al., 1995). We have observed that in COS cells Nef is hyperphosphorylated on tyrosine residues (data not shown). Therefore at least in COS cells phosphorylated tyrosine residues, which are absent in the N-terminus, could interact with the SH2 domain of Lck. In T cells (Jurkat cells) we did not find Nef to be phosphorylated on tyrosine. This would explain why in Jurkat cells an interaction of CN.47–211/CN.47–100 with Lck was not observed (Figures 1 and 2). In line with the latter result, we could not confirm a direct association of recombinant Nef and Lck proteins. A similar observation was also reported by Saksela et al. (1995).

Both N-terminal Nef mutants (Δ16–22 and Δ11–40) retained their ability to down-regulate CD4 and bind p62. This was not surprising since the N- and C-terminus of Nef define distinguishable domains (Freund et al., 1994a, 1994b; Lee et al., 1996) and both p62 and CD4 appear to bind the more conserved C-terminal portion (Sawai et al., 1995; Grzesiek et al., 1996). However, the mutant viral clones displayed a reduced capacity to infect resting PBMC. This suggested that the association of the NAKC with the N-terminal α-helix is important for Nef function. We did not observe a significant difference between the 7 (Δ16–22) and the 29 (Δ11–40) amino acid deletion. Therefore, the α-helix may be the critical interacting domain.

Since phosphorylation of the HIV matrix protein p17 greatly increases viral infectivity (Gallay et al., 1995; Bukrinskaya et al., 1996), it is reasonable to speculate that the kinase complex described here could be required for this effect. Like p17, Nef is myristoylated, associates with the plasma membrane, and is incorporated into the virion. This assumption, however, awaits further confirmation. Besides increasing viral infectivity, Nef was also found to increase viral transcription/replication through the induction of T cell activation (Baur et al., 1994; Du et al., 1995). It is possible that Lck, a critical second messenger of T cell activation signals, is also involved in this Nef-mediated effect. Therefore, the NAKC may support viral replication beyond the stage of proviral integration.

Experimental Procedures

Plasmid Constructions

The CD8–Nef (SF2) fusion protein was generated by a two-step PCR procedure and cloned into the pRcCMV expression vector (pCN) as described previously (Baur et al., 1994). To clone shorter forms of Nef upstream of CD8, a BglII site was introduced into pCN between truncated CD8 and Nef. Resulting constructs were designated pCN (for CD8–Nef) plus the number of amino acids fused to CD8 (for example pCN.94). N-terminal deletions (Δ16–22 and Δ11–40) were introduced in SF2-Nef by a two-step PCR procedure as described above. Resulting constructs were cloned into the pRcCMV vector either with or without the CD8 tag. Resulting clones were designated CN.Δ16–22 and CN.Δ11–40 (with CD8 tag) or SF2.Δ16–22 and SF2.Δ11–40 (without CD8). The *nef* alleles *nef.4* and *nef.11* were obtained from a single HIV-1-infected individual by nested PCR from uncultured PBMC. C-terminal-tagged Nef proteins were constructed by PCR amplification, including the sequence of the "AU-1" epitope (N-GAC ACC TAT CGC TAT ATA-C) into the 3′ primer. The PCR product was cloned into the pRcCMV vector. To express an N-terminal fragment of Nef in infected cells, an oligonucleotide coding for an AU-1 tag (see above) followed by a stop codon was introduced into the Xho site of the *nef* gene of the NL4-3 proviral construct. The resulting recombinant viral clone was designated NL4-3.nef.37.AU. To introduce the N-terminal Nef deletions into the proviral NL4-3 clone, first a MluI restriction site was introduced in between the *env* and *nef* open reading frames by PCR mutagenesis. Subsequently, the first 231 bp of NL4-3-Nef (MluI–KpnI) were removed and replaced with the corresponding region of SF2-Nef and SF2-Nef mutants (Δ16–22 and Δ11–40), resulting in a chimeric *nef* gene of SF2 and NL4-3. The KpnI site is conserved in most *nef* sequences. A proviral construct without the MluI–KpnI insert, resulting in a N-terminal *nef* deletion, served as negative control. All chimeric constructs and mutations were verified by dideoxynucleotide sequencing. Expression plasmids for Lck and Fyn were provided by Joseph Bolen. All tyrosine kinases were expressed in the pSV7C (Pharmacia) vector.

Cells, Antibodies, and Recombinant Proteins

Jurkat cell lines constitutively expressing truncated CD8 or CD8–Nef fusion proteins (SF2) were cultured in RPMI 1640 medium, 10% fetal calf serum (FCS), geneticin at 500 μg/ml (G418; GIBCO BRL, Eggenstein, Federal Republic of Germany). COS-7 cells were grown in Dulbecco's minimal essential medium (DMEM) containing 10% FCS. Jurkat, J.CaM.1, and J.CaM.1/lck cells were provided by Arthur

Weiss. The anti-CD8 monoclonal antibody (MAb) OKT8, obtained from American Type Culture Collection, recognizes an extracellular epitope of CD8 and was used at a concentration of 1 μg/ml. The rabbit anti-SF2 Nef serum, raised against recombinant SF2-Nef protein, was provided by Patricia Olson (Chiron Corp., Emeryville, CA) and used at a 1:500 dilution. The anti-SIV_{mac239}-Nef serum was provided by Paul Luciw. The anti-human Lck (IF-6, ascites; used at $1:10^5$) antibody, as well as the recombinant GST–Lck, was a gift from Joseph Bolen. The GST proteins were used at a concentration of 1.0 μg/ml. The MAb against phosphotyrosine (4G10) was obtained from UBI (Lake Placid, NY) and used as recommended by the manufacturer. The MAb against the AU-1 epitope was purchased from Hiss-Diagnostics (Freiburg, Federal Republic of Germany).

Transfections into T and COS Cells

The pCN expression plasmids were stably established in Jurkat cells (clone E6) as described previously (Baur et al., 1994), using neomycin (G418; 2 mg/ml medium; GIBCO BRL) for selection. To designate the resulting cell clones, a J. (for Jurkat) was added to the respective name of the construct. For example, pCN.94 expressed in Jurkat cells resulted in a cell line termed J.CN.94. For transient transfections, 5×10^5 COS-7 were plated into 10 cm culture dishes (Falcon). The next day, cells were washed once in PBS and incubated for approximately 2 hr in 4 ml of Opti-Mem low serum medium (GIBCO BRL) containing 5–10 μg of DNA (each plasmid) and 200 μg of DEAE–dextran. After transfection, the cells were washed once in PBS before 10 ml of DMEM containing 10% FCS was added. Following 48 hr of culture, the cells were harvested for in vitro kinase activity. For the CD4 down-regulation assay, 3 μg of pSVCD4 (expression plasmid for CD4 obtained from Waldemar Kolanus) along with 3 μg of the respective pRcCMV-Nef expression plasmid or the empty vector and 1 μg of pSVβGal expression plasmid (Clonetec) were cotransfected into COS cells. Cells were harvested after 2 days by PBS/EDTA and subsequently stained with FITC-conjugated anti-CD4 by standard procedure and analyzed by FACS as described previously (Baur et al., 1994). One portion (about 10%) was used to prepare cell lysates for βGal assays to verify comparable transfection efficiency.

Protein Expression Assays

Metabolic labeling and immunoprecipitation were performed as described previously (Baur et al., 1994). For the metabolic labeling with [^{35}S]methionine (Translabel, ICN, Costa Mesa, CA), 2×10^7 to 3×10^7 cells were used. The cells were lysed (EB or extraction buffer: 1% NP-40, 150 mM NaCl, and 50 mM Tris–HCl [pH 8]) in the presence of protease inhibitors phenylmethylsulfonyl fluoride (PMSF; 1 mM), leupeptin (10 μg/ml), and aprotinin (500 U/ml). The immunoprecipitates were washed four times (WB or wash buffer: 1% NP-40, 450 mM NaCl, 50 mM Tris–HCl [pH 8], 1 mM EDTA) before being resolved by 10% or 12% SDS–PAGE. For immunoprecipitations preceding the in vitro kinase assay, 1×10^7 Jurkat cells were used, washed once in PBS, and lysed in 1 ml of EB (see above). After the cellular extraction and before the kinase assay, the immunoprecipitates were washed three times with WB (see above) and one time with the kinase activation buffer (KAB: 50 mM HEPES [pH 8.0], 150 mM NaCl, 5 mM EDTA, 0.02% Triton X-100, and 10 mM $MnCl_2$ or $MgCl_2$). Unless indicated in the figure legends and the text, Mn^{2+} cations were used in the KAB. For the in vitro kinase assay, the immunoprecipitates were resuspended in 100 μl of KAB containing 10 μCi of γ^{32}P, incubated for 10 min at room temperature, and subsequently washed again three times with WB before being analyzed by 10% or 12% SDS–PAGE and autoradiography. In one immunoprecipitation (see Figure 4), the GST–Lck immunoprecipitations were performed under mild conditions, meaning that the EB contained 1% Brij instead of 1% NP-40. In this experiment, the precipitates were washed in the same Brij-EB (instead of WB) before and after the IvK reaction. Jurkat and Sf9 cells were collected in Eppendorf tubes (5×10^7 in 500 μl PBS) before they were lysed by repeated freeze/thaw. The cytoplasmic lysates were cleared from cellular debris and nuclei by two centrifugation steps (10,000 × g). Cytoplasmic lysates equivalent to 2×10^7 were used per reaction in a final volume of 500 μl. For reimmunoprecipitations, the washed immunoprecipitates were resuspended in 50 μl of elution buffer (25 mM Tris–HCl [pH 7.5], 100 mM NaCl, 2 mM EDTA, 0.4% SDS, 2 mM 2-mercaptoethanol) and incubated at 95°C for 2 min, causing the disruption of the protein–antibody complexes. The protein A–Sepharose was then pelleted, the supernatant was transferred to a new tube, and iodoacetamide was added to a final concentration of 10 mM and incubated at 30°C for 30 min in the dark. Triton X-100 was added to final concentration of 2% and finally anti-Lck antibodies and protein A–Sepharose. The reimmunoprecipitates (final volume of 250 μl) were rotated for 1 hr at 4°C and subsequently processed as a regular immunoprecipitation (see above) before resolution on a SDS–polyacrylamide gel. For immunoblot analysis, immunoprecipitates were transferred to nitrocellulose followed by Western blotting as described previously (Baur et al., 1994). To visualize reactive bands, an enhanced chemiluminescence detection system was used (Amersham, Arlington Heights, IL). To determine the identity of the phosphorylated residues, phosphoamino acid analyses were done on the phosphorylated substrates from the in vitro kinase assay, as described previously (Cooper et al., 1983). The *nef* genes *nef.4* and an unrelated protein (up; representing an unknown protein from a two-hybrid screen) were cloned into a pET vector (pET15b), expressed in E. coli (BL21), and purified under native conditions through a histidine tag as described by the manufacturers (Novagen, Heidelberg; Clontech, Heidelberg). For purification the Talon affinity resin was used (Clontech).

Virus Stocks and Infectivity Assay

Jurkat cells (2×10^6) were transfected by electroporation with 20 μg of linearized proviral DNA. After 7 days of culture, supernatants were assayed for RT activity. All proviral clones used yielded comparable RT values in the culture supernatant, which were subsequently filtered and assayed/standardized for p24 antigen concentration. For infection of resting PBMC, 2×10^6 PBMC from an adult donor were infected, in duplicates, with 2 ng p24 of wild-type and mutant viruses. The infected cells were then cultured for 2 days and subsequently stimulated for 2 days with OKT3 (anti-CD3) at a concentration of 0.2 μg/ml. Thereafter, the cells were washed and culture supernatants were collected every second day for 2 weeks and subsequently tested for RT activity in 10 μl of supernatant.

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