The Human Immunodeficiency Virus Type 1 NEF Protein Binds the Src-Related Tyrosine Kinase Lck SH2 Domain Through a Novel Phosphotyrosine Independent Mechanism

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Primate lentiviruses encode for an unique nef gene with an essential function in both viral replication and pathogenicity in the host. The molecular basis for this function remains however poorly defined. Several Nef-binding cellular proteins are thought to be instrumental in its function. Indeed, Nef contains a proline-rich motif implicated in the binding to the Src-like tyrosine kinase Hck and also to a Ser/Thr kinase of molecular weight 62 kDa. The disruption of this motif affects the binding to both these kinases as well as viral replication. Whereas Hck is expressed in the myeloid lineage and hence may account for the nef function in infected monocytes, we and others have reported previously that Nef also interacts with the T-lymphocyte Src-kinase Lck, leading to specific cell signaling impairment. This interaction occurs through the binding of Nef to both Lck SH2 and SH3 domains. Both the proline motif and phosphorylation of Nef on tyrosine residue were proposed to account for these interactions. Here, we investigate the mechanism of Lck SH2 binding by HIV-1 Nef. Using recombinant fusion proteins to precipitate lysates, we show that although SH2 binding is dependent on phosphorylation events, it occurs in a tyrosine independent manner because it requires neither tyrosine residues in Nef nor the phosphotyrosine binding pocket from the Lck SH2 domain, hence suggesting a role for a phosphoserine or a phosphothreonine residue. Further, we show that Hck SH2 does not interact with Nef, indicating that Hck SH3 binding is sufficient for Nef binding, whereas Lck SH2 cooperates together with SH3 to allow Nef binding to a level similar to Hck SH3. Together, our results establish different mechanisms for Hck and Lck binding by HIV-1 Nef protein, and identify a novel mechanism for Src-like tyrosine kinase targeting by a viral protein. © 1998 Academic Press

INTRODUCTION

Besides genes encoding for structural proteins, the genome of the primate lentiviruses (HIV-1, HIV-2, and SIV) also encodes for proteins with essential function at different stages of the viral life cycle (Trono, 1995). One of these, nef, expresses a myristoylated 25- to 34-kDa membrane-associated protein (Franchini et al., 1986; Guy et al., 1987). In the simian model of acquired immunodeficiency syndrome (AIDS), the nef gene is essential for high virus titer replication and pathogenesis (Kestler et al., 1991). The requirement of HIV-1 nef for in vivo replication and pathogenicity was similarly established in the murine model of hu-SCID mice (Jamieson et al., 1994; Aldrovandi and Zack, 1996; Gulizia et al., 1997). Further, mice expressing a lymphoid targeted nef transgene develop a severe T cell depletion with altered T cell functions (Brady et al., 1993; Skowronska et al., 1993; Lindemann et al., 1994), which indicate that nef can affect both viral and cellular functions. In vitro, nef enhances viral replication (Miller et al., 1994; Spina et al., 1994) and increases the infectivity of viral particles (Chowers et al., 1994; Aiken and Trono, 1995). Nef also downregulates cell-surface expression of CD4 and MHC class I through increased endocytosis (Guy et al., 1987; García and Miller, 1991; Aiken et al., 1994; Schwartz et al., 1996) and was found to regulate cell signaling (Baur et al., 1994; Collette et al., 1996b; Iafrate et al., 1997), leading to a specific impairment of cytokine production (Luria et al., 1991; Collette et al., 1996a). While membrane-receptor downregulation and viral replication promotion appear independent functions of nef (Goldsmith et al., 1995; Legall et al., 1997), a correlation was established between Nef-cellular protein kinases interaction and increase in viral infectivity and replication (Goldsmith et al., 1995; Saksele et al., 1995; Wiskerchen and Cheng, 1996; Swingler et al., 1997). Nef was found to form complexes with cellular kinases known for their essential function in cell signaling, including a protein related to the family of the p21-activated kinases (PAK) whose identity remains to be formally defined (Cullen, 1996; Nunn and Marsh, 1996; Sawai et al., 1994, 1996), the serine/threonine kinases MAPK/Erk2 (Greenway et al., 1995, 1996) and PKCθ (Smith et al., 1996), as well as members of the Src family of tyrosine kinases: Hck and Lyn (Saksele et al., 1995), Lck (Greenway et al., 1995; Collette et al., 1996b), and Fyn (Greenway et al., 1995).

Src tyrosine kinases are involved in signaling pathways that modulate growth, differentiation, and mature
cell function and have been implicated in tumorigenesis, inherited immunodeficiencies as well as in viral diseases (Notarangelo, 1996; Bolen and Brugge, 1997; Collette and Olive, 1997; Messerschmitt et al., 1997). Lck and Fyn are expressed in T lymphocytes and regulate thymocyte development, induction of cytotoxicity, and induction of interleukin-2 (IL-2) as well as T cell receptor (TcR), interleukin-2 receptor and CD28 signaling (Rudd et al., 1994; Howe and Weiss, 1995). Hck and Lyn have a tissue distribution restricted to haemapoietic cells from the monocyte-macrophage lineage and have been implicated in phagocytosis, Fc receptor and integrin signaling. In addition to the Src-homology (SH)-1 region, which forms the catalytic domain, Src kinases share common structural features and are formed by an N-terminal region which features modification by myristoylation and/or palmitoylation involved in membrane targeting followed by SH3 and SH2 domains. Both these domains mediate protein-protein interactions and contribute to substrate recruitment, subcellular localization, and regulation of kinase activity (Pawson, 1995). SH3 domains bind target proteins through specific proline sequences (Ren et al., 1993), while SH2 domains bind specific tyrosine-phosphorylated containing sequences (Songyang et al., 1993).

Nef contains a proline motif (Pxx)₄ with striking similarities with Src-homology (SH)-3 binding motifs (Shugas et al., 1993). This (Pxx)₄ motif is well conserved in all known primate lentiviruses (SIV and HIV-1 and -2), it adopts a polyproline type II helix structure, and it binds directly and specifically to recombinant proteins containing the SH3 domain from the Src kinases Hck, Fyn or Lck, yet with different affinity (Lee et al., 1995; Saksela et al., 1995; Collette et al., 1996b; Greenway et al., 1996). The crystal structure determination of the core region of Nef complexed with a Src family SH3 domain has reveal that the Nef-SH3 interface implicates more than the (Pxx)₄ motif and induces local rearrangements in the SH3 domain (Lee et al., 1996; Arold et al., 1997). Both in vitro and in vivo data have demonstrated that the Nef protein can modulate Lck (Collette et al., 1996b; Greenway et al., 1996), and Hck (Briggs et al., 1997; Moarefi et al., 1997) function, which implicates SH3 binding (Collette et al., 1996b; Greenway et al., 1996; Briggs et al., 1997) and can lead to fibroblast transformation upon coexpression with Hck (Briggs et al., 1997). Disruption of the (Pxx)₄ motif also reduces viral replication and infectivity in vitro (Goldsmith et al., 1995; Saksela et al., 1995; Wiskerchen and Cheng, 1996).

Similarly, the Nef protein encoded by the SIVmac239 and pbj14 isolates were found to interact with Src in transfected COS cells and to be phosphorylated on tyrosine residue(s) upon coexpression with Src (Du et al., 1995). These SIV Nef proteins contain YxxL motifs(s) in the N-terminus which upon phosphorylation of the tyrosine residue may represent a putative Src family SH2-binding motif. Indeed, this motif was found to be phosphorylated on tyrosine residues by Lck and to allow association with ZAP-70, a T cell specific tyrosine kinase (Luo and Peterlin, 1997). Although HIV-1 Nef does not contain a similar YxxL motif, we previously reported a weak phosphorylation of HIV-1 Nef on tyrosine residues (Collette et al., 1996b). Furthermore, in addition to Lck SH3 binding, we showed that cell-derived Nef can interact with Lck SH2 domain but not with other SH2 domains (Collette et al., 1996b). SIV and HIV-1 Nef might thus have evolved an additional binding motif to interact with Src family tyrosine kinases through SH2 binding.

Here, we investigate the mechanism of this interaction of HIV-1 Nef protein with Lck SH2 domain. Using nef-transfected cells, we show that Nef binds Lck but not Hck SH2 domain and that this interaction compensate the low affinity of Lck SH3, allowing an interaction of Nef with Lck SH2 + SH3 similar to Hck SH3 binding efficiency. Surprisingly, however, our results demonstrate that Nef binds Lck SH2 through a novel mechanism depending on phosphorylation events but which requires neither tyrosine residues from Nef nor the phosphotyrosyl binding pocket from Lck SH2.

RESULTS

Differential Src-homology (SH)-domain binding usage by HIV-1 Nef

To determine the region for binding to HIV-1 Nef, we used Glutathione-S-Transferase recombinant protein fused to SH2 and/or SH3 domains from Hck and Lck tyrosine kinases as an affinity matrix to precipitate lysates from the Jurkat lymphoblastoid T cell line transiently transfected by a nef-containing plasmid. The precipitated proteins were analyzed by Nef Western blotting. As shown in Fig. 1A, and following densitometric determination, the Hck SH3 recombinant protein allowed efficient precipitation of Nef from cell lysates (15% of total amount of Nef present in cell lysates was precipitated by Hck SH3), whereas the Lck SH3 was much less efficient even at saturating doses (2%, Fig. 1B). The affinity for Nef was, however, much increased when the Lck SH2 domain was added as an adjacent domain to Lck SH3, allowing precipitation of Nef to a level similar to Hck SH3 (ranging from 10 to 16%). The Lck SH2 domain precipitated Nef alone, even with unsaturating doses of recombinant proteins (Fig. 1B). Although Nef was precipitated very efficiently by Hck SH3, it was not by Hck SH2 (Fig. 1C), despite comparable ability of Lck and Hck SH2 recombinant proteins to interact with phosphotyrosine proteins (Fig. 1D).

We conclude from these results that Hck SH3 binding was sufficient for Nef binding, whereas Lck SH2 cooperated together with SH3 to allow Nef binding at a level similar to Hck SH3 alone. These results also extend...
FIG. 1. Src-homology (SH)-domain binding usage by HIV-1 Nef. Jurkat cells were transfected with pcDNA3-nef or mock transfected. Cell lysates (500 μg of total protein) were precipitated with GST recombinant protein containing the indicated Hck and Lck SH domain and analyzed by Western blot for Nef binding (A, B, and C) or antiphosphotyrosine Western blotting (D). In (A, C, and D), 20 μg of recombinant GST were used, while in (B), increasing amount were used ranging from 5 to 30 μg of GST recombinant proteins. Total lysates (TL) were included as controls (25 μg of total protein). Position of molecular weight markers are indicated on the left in kDa.
further the degree of specificity with which Nef interacts with Src kinase SH2 domains (Collette et al., 1996b).

Characterization of Lck SH2:Nef interaction

To characterize the mechanism of Lck SH2 binding by Nef, we next compared it with previously described Lck SH2 substrates. Both Vav, the guanine nucleotide exchange factor for Rho-related proteins, and ZAP-70, a tyrosine kinase thought to play a critical role in T-cell receptor signal transduction, were efficiently precipitated from cell lysates using the Lck SH2 recombinant proteins (Fig. 2A). Similarly to Vav and ZAP-70, the precipitation of Nef by Lck SH2 was unaffected by denaturation of lysates in presence of 0.1% SDS followed by boiling but was inhibited by higher SDS concentrations (Fig. 2A). The competitive effect of a tyrosine phosphorylated peptide derived from the murine polyomavirus mT antigen (Y(p)EEI) and known to bind strongly and specifically to Lck SH2 (Payne et al., 1993) were also examined. A dose-dependent reduction of binding of both tyrosine phosphorylated proteins and Nef to Lck SH2 was observed (Fig. 2B).

Most SH2 domain associations are dependent on tyrosine-phosphorylated residues (Koch et al., 1991; Straus et al., 1996). To determine whether the Lck SH2:Nef interaction was similarly dependent on phosphorylation events, we performed association assays using phosphatase-treated lysates from nef-transfected cells. As shown in Fig. 3B, phosphatase treatment of cell lysates abrogated the precipitation of Nef by Lck SH2 and largely reduced that by Lck SH2 (SH3 and SH2) to a level similar to Lck SH3 precipitation of Nef. The decrease in association was not due to proteolysis of Nef because the Lck SH3:Nef interaction was not affected by phosphatase treatment (Fig. 3B) and equivalent amounts of Nef were present in all samples following treatment (not shown). Similar results were observed with known specific substrates of Lck SH2 (data not shown). The interaction between Nef and Lck SH2 is thus dependent on phosphorylation.

Nef binds Lck SH2 in a phosphotyrosine independent manner

SIV Nef and HIV-1 Nef were found to be phosphorylated on tyrosine residues when co-expressed with the Src tyrosine kinase in COS cells (Du et al., 1995). Similarly, we have previously reported on the weak phosphorylation of HIV-1 Nef in transfected lymphoblastoid Jurkat cells as detected by antiphosphotyrosine Western blotting of Nef immunoprecipitates (Collette et al., 1996b). HIV-1 Nef protein contains seven tyrosine residues, although none of these forms a predicted Lck SH2 binding sequence (Fig. 4A). The solvent accessibility of tyrosine side chains was derived from the available crystal structures of HIV-1 Nef (Lee et al., 1996; Arold et al., 1997). Tyr81 and Tyr202 are on the surface of the core domain of Nef, Tyr115, Tyr120, Tyr127, and Tyr135 are partly exposed, whereas Tyr143 is found buried. To identify tyrosine(s) residue(s) potentially implicated in Lck SH2:Nef interaction, we performed site directed mutation of each tyrosine residues as well as a mutant lacking all tyrosine residues (D Tyr mutant, see Fig. 4A). These construct were verified by DNA sequencing and transferred in the mammalian expression vector pCDNA3. The ability of these various mutant proteins to interact with Lck SH2 was examined by transfection of Jurkat cells followed by
precipitation with the Lck recombinant fusion proteins. Surprisingly, all these mutants, including the ΔTyr mutant (Fig. 4B), interacted with Lck SH2. Despite a slightly reduced level of expression, the ΔTyr mutant was precipitated by Lck SH2 with an efficiency similar to Nef wild type (Fig. 4B). Similar results were obtained using Lck SH2+SH3 (data not shown). Binding of Nef to Lck SH2 is thus tyrosine independent.

High resolution crystallographic analysis of Src-like SH2 domains complexed with phosphopeptide have revealed the particular importance of conserved arginine residues (Arg134 and Arg154 for Lck-SH2) (Eck et al., 1993) in the phosphotyrosine binding pocket. These arginine residues provide hydrophobic, electrostatic, hydrogen-bonding, and amino-aromatic interactions with the phosphotyrosine side chain (Waksman et al., 1992; Eck et al., 1993). The substitution of these residues with serine and glutamine residue, respectively, abolishes the high-affinity binding of SH2 domains for phosphotyrosyl proteins (Straus et al., 1996). To define further the interaction of Nef with Lck SH2, a recombinant protein containing a double mutation of these arginine residues (Lck-SH2 R134S-R154Q) was produced in bacteria and used to precipitate lysates from pervanadate-stimulated Jurkat cells. As previously described (Straus et
antiphosphotyrosine immunoblotting of these precipitates revealed that the precipitation of phosphotyrosyl proteins by Lck SH2 R_{134S-R_{154Q}} was greatly impaired as compared with Lck SH2 (Fig. 5A). More specifically, Lck SH2 but not the Lck SH2 R_{134S-R_{154Q}} mutant precipitated tyrosine phosphorylated ZAP-70 (Fig. 5B). Lysates from nef-transfected Jurkat cells were similarly analyzed. Strikingly, anti-Nef immunoblotting revealed that Lck SH2 R_{134S-R_{154Q}} precipitated Nef as efficiently as Lck SH2 (Fig. 5C). We conclude that Nef interacts with the Lck SH2 domain in a novel, phosphotyrosine-independent fashion.

**DISCUSSION**

We and others have reported previously that the nef gene product from primate lentiviruses can interact with

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**FIG. 4.** Nef binding does not require Lck SH2 phosphotyrosyl binding pocket. Jurkat cells were stimulated for 5 min. at 37°C with 50 μM pervanadate and cell lysates were precipitated with 20 μg of Lck SH2 or Lck SH2 R_{134S-R_{154Q}} (see Materials and Methods) followed by antiphosphotyrosine Western blotting in (A) or ZAP-70 Western blotting in (B). Alternatively, lysates from nef-transfected Jurkat cells were precipitated (500 μg of total protein) with the indicated Lck SH recombinant protein (20 μg) followed by Nef Western blotting (C). Total lysates (TL) were included as controls (25 μg of total protein).
Src family tyrosine kinases (Du et al., 1995; Greenway et al., 1995; Saksela et al., 1995; Collette et al., 1996b). This interaction, seen both in vitro (Greenway et al., 1995; Saksela et al., 1995; Collette et al., 1996b) and in intact cells (Du et al., 1995; Collette et al., 1996b; Briggs et al., 1997), results in the modulation of the catalytic activity of the enzyme (Collette et al., 1996b; Greenway et al., 1996; Briggs et al., 1997; Moarefi et al., 1997) and can lead to fibroblast transformation (Du et al., 1995; Briggs et al., 1997). The precise role of the Src kinases in the viral life cycle remains to be established, yet a correlation was noted between Src kinase interaction and viral replication (Goldsmith et al., 1995; Saksela et al., 1995; Wiskerchen and Cheng, 1996). A proline motif in Nef is determinant in these interactions (Saksela et al., 1995; Briggs et al., 1997) and allows binding to the SH3 domain of Src kinases, yet with different affinity (Lee et al., 1995; Saksela et al., 1995). In addition to SH3 binding, we also showed that HIV-1 Nef can interact in a specific manner with Lck SH2 domain (Collette et al., 1996b), and a putative SH2-binding motif was identified in SIV Nef (Du et al., 1995). The present study focused on SH2 binding by HIV-1 Nef.

We first compared binding of Nef to Lck and Hck recombinant SH3 and SH2 domains (Fig. 1). A marked preference for Hck SH3 binding was observed when compared with Lck SH3. In a previous report, using Nef fragments containing the proline motif, only Hck SH3 binding was detected in a filter binding assay with no detectable binding of Lck SH3 (Saksela et al., 1995). Here, using the full-length Nef protein expressed in transfected cells, a low but significant interaction with Lck SH3 can be detected that is competed by a consensus proline motif peptide and disrupted following mutation of the proline residues 72 and 75 (data not shown). The higher affinity of Lck SH3 for full-length Nef as compared with Nef fragments most probably reflects the role of additional residues of Nef in the interaction with SH3 domain, as visualized recently by crystallographic analysis (Lee et al., 1996; Arold et al., 1997). The high-
affinity binding of Nef to Hck-SH3 is dependent on residue Ile96 present in its RT loop, which is proposed to contact Nef and to mediate the selective recognition of Nef by Hck-SH3 (Lee et al., 1995). Lck SH3 has a serine residue instead of Ile96. One can propose that this residue could explain the weaker affinity of Lck SH3 than Hck-SH3 for Nef proline-rich region. It is of interest that in addition to the proline-rich motif, a second binding motif may stabilize the interaction of Nef to Lck. Similar cooperation between SH2 and SH3 domains have already been described (Panchamoothy et al., 1994). Interestingly, the binding of Tip, a Herpes Saimiri product that associates with Lck, requires at least two Lck binding motifs (Jung et al., 1995a,b). The first motif is a proline motif identical to the one identified in Nef (Collette and Olive, 1997), required for the interaction with Lck SH3 but is not on its own sufficient for Lck binding. A second motif has been identified that shares homology with the C-terminus of Src Kinase (Jung et al., 1995b). This motif, together with the proline motif allows for Lck binding, yet through an unknown region. Nef binds Lck SH2 but neither Hck SH2 (Fig. 1) nor other additional SH2 domains related or not to Src family tyrosine kinases (Collette et al., 1996b), indicating a high degree of specificity for this interaction.

We next sought to determine the mechanisms of Nef binding to Lck SH2 and unexpectedly found that it occurs in a phosphotyrosine independent manner. Our conclusion was drawn from several complementary observations: (i) SH2 still interacts with the product encoded by nef gene with all tyrosines mutated to phenylalanine residues (Fig. 4); (ii) as compared with tyrosine phosphorylated substrates, Lck SH2 binding is only partly competed by the Y(p)EEI peptide, a highly specific substrate peptide for Src kinase's SH2 domains (Payne et al., 1993; Songyang et al., 1993), which fully competed for Lck SH2 binding of phosphotyrosine substrates (Fig. 2); and (iii) mutations that completely disrupt the well-defined phosphotyrosine binding pocket within the Lck SH2 domain (Waksman et al., 1992; Eck et al., 1993) do not alter Nef binding (Fig. 5). Importantly, we made similar observations using the SIVmac239 nef gene product (H. Dutartre, unpublished results). These results hence contrast to the conventional view of SH2-mediated interactions that involve recognition of the phosphotyrosine residue in the context of three to five residues carboxy-terminal to the tyrosine (Songyang et al., 1993). There is a growing number of such phosphotyrosine-independent interactions described in the literature. The BCR (the Philadelphia chromosome breakpoint cluster region gene product) was found to bind the SH2 domain from this translocation partner c-Abl (Pendergast et al., 1991), as well as to a limited set of SH2 domains ( Muller et al., 1992; Raffel et al., 1996), through phosphotyrosine-independent mechanisms. The SH2 domain from the protein-tyrosine phosphatase SHPTP2 binds its own catalytic domain in the absence of phosphotyrosine (Dechert et al., 1994). Even more relevant to the present study, the SH2 domain from Src tyrosine kinases Src and Fyn was found to interact with the serine kinase Raf (Cleghon and Morrison, 1994), and the Lck SH2 domain interacted in a phosphotyrosyl-independent manner with a recently cloned ubiquitin-binding protein (Park et al., 1995; Vadalami et al., 1996) and possibly the tyrosine phosphatase CD45 (Ng et al., 1996). In most of these studies, the conclusion that SH2 binding occurred in a phosphotyrosine-independent manner was essentially drawn from the absence of detectable tyrosine phosphorylation of the SH2 substrate and/or the lack of tyrosine residue(s) in the mapped binding region. Here by systematic disruption of the seven tyrosine residues present in HIV-1-BRU Nef protein, we formally demonstrate that the Lck SH2 domain can interact with a viral protein independently of tyrosine residues, and hence independently of tyrosine phosphorylation.

Crystal and solution structures have been determined for different SH2 domains, both as isolated domain and in complex with high- or low-affinity peptide substrate (Booker et al., 1992; Overduin et al., 1992; Waksman et al., 1992; Eck et al., 1993; Pascal et al., 1994). These studies have determined the ability of a group of independent pockets in the SH2 domain to accommodate peptide side chains. Because binding of Nef to Lck SH2 is partly competed by the specific Y(p)EEI peptide yet the phosphotyrosyl binding pocket in the SH2 domain is dispensable for this interaction, one can propose that phosphotyrosine-independent binding similarly implicates a group of binding pockets, some of which being shared by both phosphotyrosine-dependent and -independent mechanisms. Although tyrosine phosphorylation is not implicated in binding of Nef to Lck SH2, Pase treatment reduces the binding of Nef to Lck SH2 and Lck SH2+3, demonstrating that phosphorylation events other than tyrosine phosphorylation regulate this interaction. In the previously reported cases of phosphotyrosyl-independent binding of proteins to SH2 domains, serine phosphorylation was suspected to be important (Pendergast et al., 1991; Cleghon and Morrison, 1994; Malek and Desiderio, 1994; Raffel et al., 1996). Nef is predominantly phosphorylated on serine residues in vitro (Bodeus et al., 1995; Coates and Harris, 1995) and also in vivo (Coates et al., 1997; Luo et al., 1997), most probably on several serine residues (Bodeus et al., 1995). Nef can associate with different serine kinases including the p62 PAK-related serine kinase (Nunn and Marsh, 1996; Sawai et al., 1996) and the PKC ε isoform (Smith et al., 1996). However, PKC inhibitors only partially reduce the phosphorylation of Nef (Coates et al., 1997; Luo et al., 1997), suggesting that PKC might not be the only kinase responsible of Nef phosphorylation. Recently, Baur et al. showed an interaction of NH2-terminal Nef fragments with a complex containing Lck and an unidentified serine kinase, pro-
posed to be implicated in the phosphorylation of Nef (Baur et al., 1997). The kinase responsible for Nef phosphorylation remains to be determined and together with the identification of Nef phosphorylated serine residues may provide important tools in the elucidation of the Nef-Lck SH2 interaction.

The function of the phosphotyrosyl-independent interaction of SH2 domains is not well understood, but it is striking that two viral proteins, Nef and v-Abl, direct such interactions. It has been suggested that the binding of Shc to v-Abl SH2 domain (Raffel et al., 1996) might compete for phosphotyrosyl proteins leading to inappropriate signaling through the Ras pathway. The competition between Nef and the Y(p)EEI peptide described here (Fig. 2) suggested that a similar signaling alteration following binding of Nef to Lck SH2 might occur in vivo. Indeed, the presently described binding of HIV-1 Nef to Lck SH2 and SH3 domains could represent some viral adaptation to elude normal protein–protein interaction leading to the disruption of cellular signaling.

MATERIALS AND METHODS

Plasmids and constructions

The GST Lck SH3, SH2 and SH2+3 constructs were previously described (Collette et al., 1996b). The GST Hck SH3 and SH2 were kind gifts from R. Benarous (ICGM, Paris), and the Lck GST-SH2 R 134S-R154Q was constructed by PCR amplification of the pmNC Lck-R134S-R154Q plasmid (kindly provided by M. Marsh, London), using the following oligonucleotides: 5′ primer: 5′AGTCAGAATTCTGTTCTTCAAGAATCTGAGC3′ containing an EcoRI restriction site and 3′ primer: TCATCAGTCCAGCAAGGCTCAACTT3′ containing a Sall restriction site. The PCR product was purified by gel extraction (GeneClean, Clontech), restricted by EcoRI and Sall restriction enzyme, and subcloned in pGEX5 vector (Pharmacia). The construct was verified by sequencing.

The HindIII/XbaI fragment containing the nef gene from HIV-1 Bru/Laβ was subcloned under the transcriptional control of the human CMV IE promoter in the pCDNA3 vector (INVITROGENE). HIV-1 nefBru site-directed mutagenesis were performed using the Transformer Site Directed Mutagenesis Kit (Clontech) according to the manufacturer’s instructions. Briefly, the nef gene contained in the pUC18 backbone was a HindIII-XbaI fragment was mutated by simultaneous annealing of the selection primers Trans Oligo Ndel/Ncol or Switch Oligo Ncol/Ndel provided by the manufacturer (Clontech) and the mutagenesis primer, which introduces the desired mutation to one strand of the denatured double-stranded plasmid. The nef ΔTyr was constructed by sequential mutagenesis of Tyr residues 81, 115, 120, 127, 135, 143, and 202 of HIV-1 nefBru which were substituted each by a Phe residue. Introduction of desired substitution and the integrity of the nef sequence were verified by sequencing, followed by subcloning of mutated nef gene in pCDNA3 vector in the BamHI site.

Cells, cell culture, and transfections

The Jurkat cell clone JH6.2 (20) was cultured in RPMI supplemented with 10% fetal calf serum (FCS, Gibco-BRL) and 1 mM L-glutamine.

For transient transfection, 10⁷ of exponentially growing cells were resuspended in 500 μl of culture medium containing 20 μg plasmid DNA and electroporated at 250 V and 960 μF using a BIORAD GENE PULSER. Electroporated cells were washed once in 10-ml culture medium and cultured further at 37°C, 5% CO₂. To increase the expression of genes placed under the transcriptional control of the CMV promoter, culture of electroporated cells were stimulated by PMA (Sigma; 20 to 50 ng/ml or 20 ng/ml) and ionomycin (Sigma; 2 or 1 to 2 μg/ml) for 5 to 16 h at 37°C, as mentioned in figure legends.

Preparation of cell extract

Cells (2×10⁷)/ml were washed once in PBS and lysed in lysis buffer (1% Triton X-100, 25 mM HEPES pH 7.8, 150 mM NaCl, 10 mM EDTA, 1 mM EGTA supplemented with 1 mM PMSF, 0.1 mM Na Vanadate, 10 μg/ml Leupeptin, 10 μg/ml Aprotinin and Pepstatin). After 15 min on ice, cell extracts were centrifuged at 4°C for 10 min at 13,000 rpm in a Biofuge and postnuclear supernatants were collected and used for precipitation.

Precipitation and immunoblotting

The bacterial host Escherichia coli DH5a, transformed with GST fusion plasmids, was cultured and GST fusion proteins were purified according to the manufacturer’s instructions (Pharmacia Biotech Inc.). Briefly, IPTG-induced bacteria cultures were disrupted by sonication, followed by lysis in Triton-X100 and centrifugation. Soluble proteins were incubated with Glutathione Sepharose 4B to purify GST fusion proteins followed by extensive washing of the protein complexes. To yield highly purified GST recombinant proteins, proteins bound to Glutathione Sepharose 4B were eluted with reduced Glutathione and coupled to Glutathione Sepharose 4B.

Cell extracts were precipitated overnight at 4°C under agitation with GST fusion protein as indicated in figure legends. For peptide competition, the (p)YEEI peptide derived from the Hamster polyomavirus middle T antigen (kindly provided by O. Acuto, Inst. Pasteur, Paris) was added to the cell lysate during the precipitation step. Precipitated proteins were washed three times with 1 ml of lysis buffer. Bound proteins were eluted with 20 μl 3× Laemmli buffer (Biolabs) and boiling, separated by SDS-
PAGE, followed by wet-electrotransfer to polyvinyl difluoride membrane in 10 mM CAPS pH 11, 10% Methanol. Membranes were blocked overnight at 4°C under agitation in 5% non fat milk in TBST buffer (10 mM Tris pH 7.4, 100 mM NaCl and 0.1% Tween 20), or in TBST/5% Bovine Serum Albumin (BSA, Boehringer) for anti-PTyr immunoblotting.

Nef protein was detected using the MATG 0020 mAb (Transgene, Strasbourg; 1 μg/ml in TBST) followed by a POD-linked anti-mouse antibody (Dako). After extensive washing in TBST, bound proteins were detected by chemiluminescence (ECL, Amersham). ZAP-70, Vav and phosphotyrosine proteins were similarly detected using a ZAP-70 rabbit polyclonal antisera (a kind gift from B. Malissen, Marseille), a Vav monoclonal antibody (UBI, Lake Placid) and the monoclonal antibody 4G10 (UBI, Lake Placid), respectively.

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