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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

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Human immunodeficiency virus (HIV) and simian immunodeficiency virus Nef proteins contain a conserved motif with the minimal consensus (PxxP) site for Src homology region 3 (SH3)-mediated protein–protein interactions. Nef PxxP motifs show specific binding to biotinylated SH3 domains of Hck and Lyn, but not to those of other tested Src family kinases or less related proteins. A unique cooperative role of a distant proline is also observed. Endogenous Hck of monocytic U937 cells can be specifically precipitated by matrix-bound HIV-1 Nef, but not by mutant protein lacking PxxP. Intact Nef PxxP motifs are dispensable for Nef-induced CD4 down-regulation, but are required for the higher *in vitro* replicative potential of Nef$^+$ viruses. Thus, CD4 down-regulation and promotion of viral growth are two distinct functions of Nef, and the latter is mediated via SH3 binding.

Key words: AIDS/host factor/signal transduction/virus replication

Introduction

Human immunodeficiency virus (HIV)-1 Nef is a 27/25 kDa protein encoded by a conserved open reading frame partially overlapping with the 3′ long terminal repeat (LTR) element of human (HIV-1 and HIV-2) and simian immunodeficiency viruses (SIVs; see Guy *et al.*, 1992; Hovanessian, 1992). The function of Nef at the cellular and molecular levels is not understood. An intact Nef reading frame is required for the accumulation of high viral loads and the development of acquired immune deficiency syndrome (AIDS) in SIV_{mac}-infected Rhesus monkeys (Kestler *et al.*, 1991), and was also shown recently to be necessary for *in vivo* replication and pathogenicity of HIV-1 in the SCID-Hu mouse model (Jamieson *et al.*, 1994).

Studies on the role of Nef in HIV replication in established leukemia cell lines have yielded variable results, and reported marked negative effects (Luciw *et al.*, 1987; Niederman *et al.*, 1989), weak (+/−) or no effects (Fisher *et al.*, 1986; Terwilliger *et al.*, 1986, 1991; Ahmad and Venkatesan, 1988; Kim *et al.*, 1989; deRonde *et al.*, 1992), or strong positive effects (Terwilliger *et al.*, 1991; Zazopoulos and Haseltine, 1993; Chowers *et al.*, 1994), apparently depending on the Nef allele and the cell line used. Results from infection of primary cells have been more in agreement, consistently indicating a modest to strong growth advantage for Nef$^+$ viruses (Kim *et al.*, 1989; deRonde *et al.*, 1992; Zazopoulos and Haseltine, 1993; Miller *et al.*, 1994; Spina *et al.*, 1994). The recent studies by Miller *et al.* (1994) and Spina *et al.* (1994) greatly clarified this issue by showing that the magnitude of the virus growth enhancement by Nef depends on the activation state of the infected cells, and that upon infection of primary cells under appropriate culture conditions a pronounced difference in the spread of Nef$^+$ and Nef$^-$ viruses can be consistently observed.

One known consequence of Nef expression is the post-translational down-regulation of the CD4 protein via a mechanism that involves a dileucine endocytosis target motif in CD4 (Guy *et al.*, 1987; Garcia and Miller, 1991; Garcia *et al.*, 1993; Aiken *et al.*, 1994). It has been speculated that CD4 down-regulation by Nef could be important for HIV/SIV pathogenesis by preventing super-infection of a cell with multiple viruses and/or by facilitating the release of progeny viruses. Expression of SIV Nef can indeed render cells resistant to HIV infection *in vitro* (Benson *et al.*, 1993). It should be noted, though, that most HIV-containing cells in infected individuals are CD4-positive (Schnittman *et al.*, 1989). It has also been reported that Nef$^+$ HIV particles appear to be more infectious than Nef$^-$ particles, even if they were produced in cells that do not express CD4 (Miller *et al.*, 1994). Thus, the significance of CD4 down-regulation by Nef in the pathogenesis of HIV infection is unclear.

Despite uncertainties about the role and mechanisms of Nef function, the available data seem consistent with a hypothesis that Nef promotes viral spread by modifying the intracellular milieu of an infected cell such that the early part of the viral life cycle is facilitated and/or the infectivity of the progeny viral particles is increased. To understand the function of Nef, one valuable approach could be to study its ability to communicate with defined cellular processes. In this regard, we were intrigued to notice a conserved proline-containing motif in HIV-1, HIV-2 and SIV Nef proteins which resembles the recently identified site involved in protein–protein interactions mediated by Src homology region 3 (SH3; Mayer and Baltimore, 1993; Pawson and Schlessinger, 1993).

Since the binding site for the SH3 domain of the Abl oncoprotein in the putative signal-transducing protein 3BP-1 was identified (Cicchetti *et al.*, 1992; Ren *et al.*, 1993), similar proline-rich SH3 binding sites sharing the minimal PxxP consensus motif have been discovered in a number of other proteins, many of which are involved in intracellular signaling (Ren *et al.*, 1994; Yu *et al.*, 1994, and references therein). The Src family of non-receptor tyrosine kinases, all of which contain SH3 domains, includes nine characterized members, some of which (Lck,

Fyn, Blk, Fgr, Lyn and Hck) are preferentially expressed in hematopoietic cells and are known to be involved in leukocyte activation via cell-surface receptors (for reviews see Cooper and Howell, 1993; Mustelin and Burn, 1993; Sudol, 1993). Like Nef, Src family kinases are also targeted to the plasma membrane by myristoylation. Therefore we have examined whether the SH3 domains from Src-related kinases might interact with Nef.

In this study we show that HIV-1 Nef binds specifically to the SH3 domains of Hck and Lyn, and that the PxxP motifs of Nef mediate this binding. We tested the significance of SH3 binding for Nef function by examining the ability of wild-type, as well as SH3 binding-negative mutant forms of Nef, to enhance HIV replication in infected primary cell cultures and to down-regulate cell-surface expression of CD4 in a cotransfection assay. These experiments indicate that (i) CD4 down-regulation and promotion of HIV growth are separate functional properties of Nef, and (ii) the SH3 binding site is involved in the latter but not the former function. The interaction between the Nef PxxP motifs and Hck or Lyn, but not Lck or Fyn, represents a very selective PxxP–SH3 interaction, and provides a molecular basis for the enhanced HIV growth by Nef as well as a potential target for intervention.

Results

Hck and Lyn SH3 domains can interact with the conserved Nef PxxP motifs

A characteristic feature of Nef proteins of primate lentiviruses is a region containing proline residues repeated in every third position of the polypeptide (Myers *et al.*, 1991; Shugars *et al.*, 1993). These proline residues are among the most highly conserved amino acids in different virus isolates and occur in HIV-1 as a tetraproline motif, whereas HIV-2 and SIV Nef sequences typically have three such repeated prolines. To determine if the PxxP motifs formed by the tetraproline repeats of HIV-1 Nef could mediate interactions with SH3-containing proteins, the Nef peptide EVGF**P**VT**P**QV**P**LR**P**MTYK (corresponding to amino acids 65–82 of HIV-1 NL4-3) was produced as a bacterial fusion protein with glutathione-S transferase (GST). Because NL4-3 Nef contains a threonine residue at position 71 within this putative SH3 binding site, while patient-derived Nef sequences almost invariably contain a basic amino acid in this position, most often an arginine (Shugars *et al.*, 1993), we also produced a similar GST fusion protein including the Nef peptide EVGF**P**V**R**P**P**QV**P**LR**P**MTYK. These two fusion proteins, GST–PXXP-T and GST–PXXP-R, respectively, were tested in a filter binding assay for their ability to bind to different SH3 domains which were produced as bacterial fusion proteins, purified and biotinylated for their subsequent detection (Figure 1A). Non-recombinant GST protein and GST–BB3-1, a fusion protein containing a 10 amino acid SH3 binding site from the Btk protein (Cheng *et al.*, 1994), were also included in the analysis as negative and positive controls for Src family SH3 domain binding specificity (Figure 1A).

Specific binding of Hck and Lyn SH3 domains with both GST–PXXP-T and GST–PXXP-R proteins was observed, the latter showing somewhat more avid binding. Notably, no specific binding of GST–PXXP-T or GST–PXXP-R proteins to the highly homologous SH3 domains of Lck and Fyn was observed. Also, no binding to the SH3 domains of the less closely related oncoproteins Abl and Crk was seen (data not shown). Thus, GST–PXXP-T and GST–PXXP exhibited highly specific SH3 binding, a pattern even more selective than that of GST–BB3-1 which, as reported previously (Cheng *et al.*, 1994), bound to SH3 domains of Hck, Lyn and Fyn but not Lck.

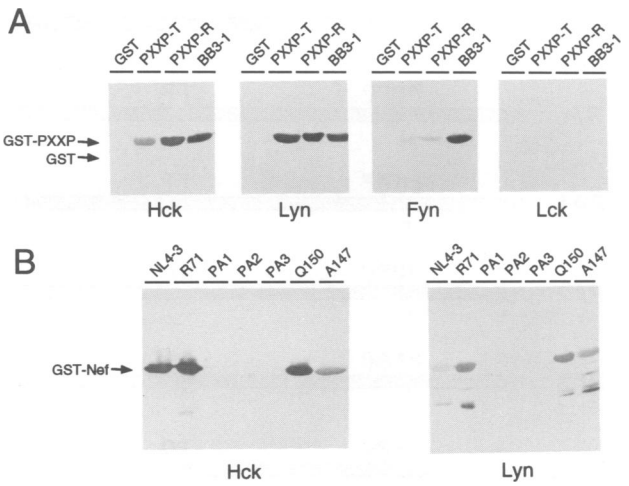

Fig. 1. Binding of Src family SH3 domains to Nef. (**A**) Biotinylated Hck, Lyn, Fyn and Lck SH3-containing proteins were incubated with nitrocellulose filters onto which 5 μg of GST alone or as a fusion protein including the peptides PXXP-T, PXXP-R or BB3-1 (see text) were transferred after SDS–PAGE, as indicated. The binding of different SH3 proteins was visualized by a secondary incubation with alkaline phosphatase-conjugated streptavidin, followed by enzymatic detection. Even loading and transfer of samples on all filters was confirmed before the incubations by transient protein staining. The positions of the fusion proteins (GST–PXXP) and the faster-migrating, non-recombinant GST (GST) on the filters are indicated. (**B**) GST fusion proteins containing the full-length NL4-3 Nef or the indicated altered derivatives were analyzed for their binding to the SH3 domains of Hck and Lyn, as in (A). The different Nef proteins included in these fusion proteins are described in the legend to Figure 2.

Binding of Hck and Lyn SH3 domains to full-length Nef proteins

To study the ability of the Nef PxxP domains to mediate SH3 binding in the context of the full-length Nef protein, the NL4-3 Nef and different derived mutagenized forms were expressed as GST fusion proteins. These differentially altered forms of NL4-3 Nef (R71, PA1, PA2, PA3, Q150 and A147) are illustrated schematically in Figure 2. Both NL4-3 Nef and its derived 'patient sequence-like' derivative Nef-R71 bound well to Hck SH3 domain (Figure 1B). This binding was not contributed by the GST part of the fusion protein, as Nef-R71 cleaved off the fusion protein by thrombin digestion bound Hck with an at least similar or higher affinity (data not shown). When the tetraproline repeat in Nef was disrupted by changing the two internal prolines (amino acids 72 and 75) to alanines, thereby leaving no intact PxxP motifs in this region, the binding of Hck SH3 was abolished (Nef-PA1 in Figure 1B), indicating that this interaction was indeed PxxP-mediated.

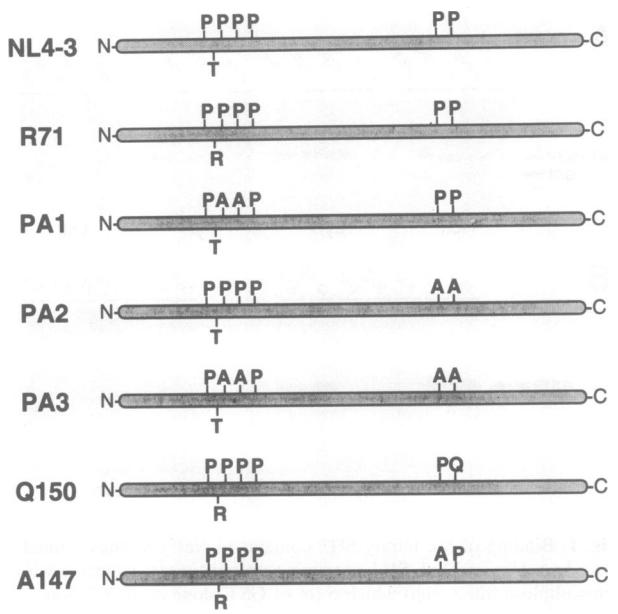

Fig. 2. Altered forms of Nef used in structural and functional SH3 binding studies. The 206 amino acid HIV-1 NL4-3 Nef polypeptide corresponding to the major 27 kDa myristoylated isoform of Nef is shown at the top of the figure, with the proline residues forming the PxxP motifs indicated (positions 69, 72, 75, 78, 147 and 150). A threonine residue in position 71 within the tetraproline repeat region is also indicated, and was changed into an arginine in some constructs to mimic sequences more typical for Nef genes in wild-type HIV-1 strains. Below are shown the specific amino acid changes introduced into these position in the derivatives R71, PA1, PA2, PA3, Q150 and A147, which were produced for *in vitro* binding studies as bacterial GST fusion proteins (Figures 1 and 3) and expressed for functional studies in human cells either from a separate vector (Figure 4) or in the context of infectious HIV-1 NL4-3 (Figure 5).

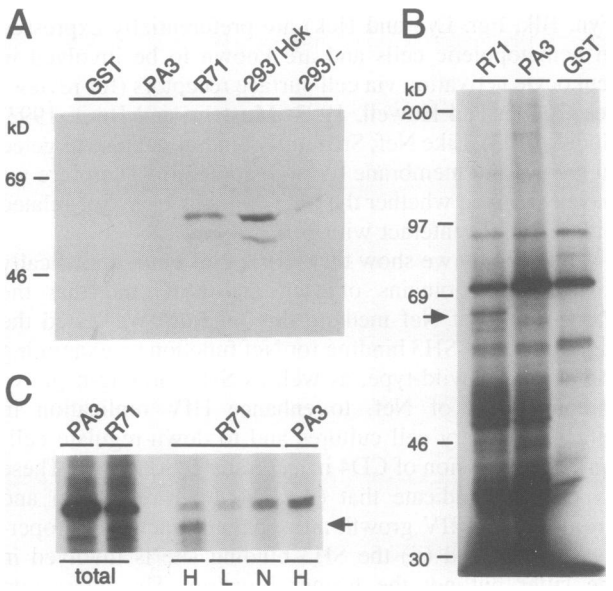

Fig. 3. Hck from U937 cells is specifically precipitated by Nef with intact PxxP motifs. (**A**) Western blot analysis with an anti-Hck antiserum of U937 cell proteins associating with beads coated with non-recombinant GST (GST) or GST−Nef fusion proteins with disrupted (PA3) or intact (R71) PxxP motifs. The two lanes on the right show control extracts from Hck-transfected and untransfected 293 cells. (**B**) Electrophoretic analysis of total phosphorylated proteins associated with the differentially coated beads, as detailed above, after *in vitro* incubation with [γ-^{32}P]ATP. (**C**) Immunoprecipitation analysis of *in vitro* phosphorylated U937 cell proteins associated with GST−Nef-R71 and GST−Nef-PA3 proteins. The two left-hand lanes (total) of the gel were loaded with unselected phosphorylated products, as in the independent experiment shown in (B). The lanes on the right show proteins eluted from these beads and immunoprecipitated with antibodies against Hck (H) or Lyn (L), or with normal rabbit serum (N). The intensely labeled product migrating above Hck appears to bind to the agarose beads used in the precipitation steps and is not Nef-specific.

In contrast to Hck, the binding of Lyn SH3 to full-length Nef was weaker and was dependent on the arginine residue at position 71 to show significant affinity in this assay (Figure 1B). This was unexpected given the avid binding of the Lyn SH3 domain to the Nef PxxP peptide-containing fusion proteins, and suggested that other determinants in Nef may also play a role in Hck SH3 binding. This directed our attention towards another putative SH3 binding site closer to the C-terminus of Nef, where the NL4-3 amino acid sequence contained a single PxxP motif (amino acids 147–150) with surrounding residues potentially favorable for an SH3 interaction. To disrupt this putative cooperative Hck SH3 binding site, these two prolines were also replaced by alanines (Nef-PA2). In addition, we generated a doubly mutated GST−Nef protein which contained both sets of proline → alanine substitutions (Nef-PA3).

We found that the affinity of Hck SH3 binding to Nef-PA2 was reduced below the threshold of detection of the filter binding assay, even though the more N-terminal tetraproline repeat region was intact. Similarly, only weak binding was observed to a full-length Nef protein with a single PxxP-disrupting proline → alanine substitution in position 147 (Nef-A147 in Figures 1B and 2). The weak residual binding to Nef-A147 could be due to its arginine 71-containing tetraproline repeat region. As expected, combination of the two PxxP mutations (Nef-PA3) abolished any detectable binding to Hck SH3.

In contrast to Hck, the weaker binding exhibited by the Lyn SH3 domain was far less affected by the alteration in the C-terminal PxxP motif, and was apparently primarily mediated by the PxxP motif(s) of the N-terminal tetraproline repeat region (compare Nef-R71 and Nef-A147 in Figure 1B). These results suggested that the binding to Hck SH3 domain is coordinated by two non-contiguous proline-containing regions in Nef, and the lack of this synergistic interaction explains the lower affinity of the Lyn SH3 domain to full-length Nef. Although the role of the C-terminal Nef PxxP motif in Hck SH3 binding was critical, it appeared to be strictly cooperative in nature, since a peptide containing the C-terminal PxxP motif (WCYKLV**P**VE**P**DKVEEANK) failed to show any binding to Hck SH3 when tested as an independent GST fusion protein (results not shown).

A survey of different Nef sequences indicated that the second proline in the C-terminal PxxP motif of NL4-3 Nef (amino acid 150) was, however, not strongly conserved among different HIV-1 strains or in HIV-2 and SIV. Since this residue is often a glutamine in patient-derived HIV-1 Nef sequences (Shugars *et al.*, 1993), we also generated a GST−Nef fusion protein with a proline → glutamine substitution in position 150 (Nef-Q150). This altered NL4-3-derived Nef protein bound to Hck SH3 as well as a Nef protein with a proline residue at position 150 (Figure 3),

indicating that the second (non-conserved) proline in the C-terminal PxxP motif can be replaced with other amino acids without losing the ability to cooperate in Hck SH3 binding. In contrast, the importance of the first (conserved) proline (amino acid 147) was indicated by the loss of high-affinity binding of Hck SH3 to GST−Nef-A147 in which this proline residue was replaced by an alanine without changing proline residue 150 (Figures 1B and 2).

Thus, these experiments show that a highly specific SH3 binding interface, formed by the PxxP motif(s) within the conserved Nef tetraproline repeat cooperating with a more C-terminal proline residue, can bind Nef to the protein tyrosine kinase Hck.

Specific PxxP-dependent binding of cellular Hck to Nef

To extend these filter binding results, we examined the binding of soluble cell-derived Hck protein to Nef. Glutathione−agarose beads were coated with GST−Nef-R71, GST−Nef-PA3 or non-recombinant GST and incubated with cytoplasmic extract from the human monocytic leukemia cell line U937. The proteins specifically retained by these coated beads were then examined by Western blot analysis using an anti-Hck antiserum (Figure 3A), and by an *in vitro* kinase reaction followed by electrophoretic analysis of the phosphorylated proteins, either directly (Figure 3B) or after an immunoprecipitation step using antisera against the Hck or Lyn (Figure 3C).

Western blot analysis with an Hck-specific antiserum revealed a polypeptide doublet which comigrated with Hck protein expressed by transient transfection of 293 cells. This doublet was specifically associated with the beads coated with GST−Nef-R71 protein but not with PxxP-defective GST−Nef-PA3 protein or non-recombinant GST (Figure 3A). A faint background band comigrating with the darker Hck band was also evident in the untransfected 293 cells (Figure 3A, right-hand lane). Analysis of the Nef-R71- and Nef-PA3-associated U937 cell-derived proteins that could undergo auto- or trans-phosphorylation *in vitro* revealed a very similar overall pattern of phosphorylated products (Figure 3B), some of which could represent specific Nef-associated cellular proteins such as the serine-phosphorylated polypeptides reported recently by Sawai *et al.* (1994), since they were not precipitated by the beads coated with GST only. However, in addition to these common phosphorylated proteins, distinct differences between the Nef-R71 and Nef-PA3 precipitates were observed in the molecular size range of Hck (arrows in Figure 3). When the *in vitro* phosphorylated proteins were first eluted from the beads and subsequently subjected to an immunoprecipitation analysis, we found that the protein specifically associated with Nef-R71 could indeed be recognized by an anti-Hck antiserum, but not by an anti-Lyn antiserum or normal rabbit serum (Figure 3C). These results indicated that the affinity and specificity of the Nef-PxxP−Hck-SH3 binding was sufficient to select the endogenously expressed Hck protein from U937 cell extract, suggesting that this interaction could occur during HIV/SIV infection and be involved in the pathogenic function of Nef.

SH3 binding by Nef is dispensable for CD4 down-regulation

To examine the possible involvement of SH3 binding by Nef in down-regulation of CD4 expression, we generated

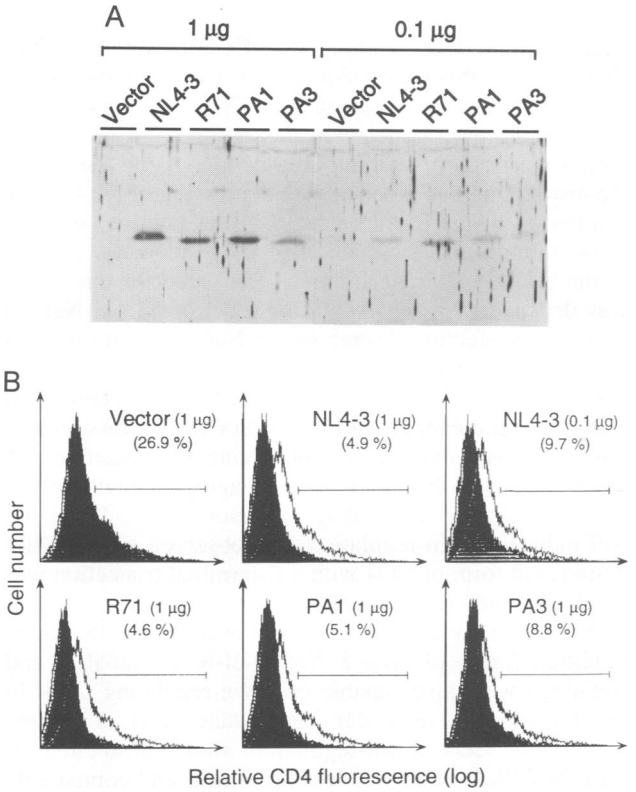

Fig. 4. Nef PxxP motifs are not required for down-regulation of CD4 surface expression. 1.0 or 0.1 µg of vectors expressing the indicated Nef proteins (see Figure 2) or an empty pBabe-puro vector were cotransfected together with a constant amount (2 µg) of expression vectors for human CD4 and CD8 into 293 cells. Two days later the transfected cells were harvested and analyzed for (**A**) Nef expression by Western blotting and (**B**) surface expression of CD4 by fluorocytometry using double staining for CD8 as an internal control for transfection and cell viability.

retroviral vectors expressing the complete reading frame of HIV-1 NL4-3 Nef as well as its mutagenized forms Nef-R71, Nef-PA1 and Nef-PA3. The ability of these different forms of Nef to reduce cell-surface expression of CD4, as tested by flow cytometry, was then examined in transiently cotransfected 293 cells. In addition to the expression vectors for Nef and CD4, we also transfected the cells with an expression vector for CD8. This allowed double staining with antibodies against CD4 and CD8 labeled with different fluorochromes to specifically examine CD4 expression in the transfected (CD8-positive) subpopulation of cells, and to confirm that any negative effect by Nef on CD4 expression was not due to non-specific effects, such as reduced cell viability. At 2 days after cotransfection the cells were harvested and analyzed. Figure 4 shows representative results from one of three independent experiments. Two different dosages of Nef expression vectors (1 µg and 100 ng) were used in the transfections, while the amount of CD4 and CD8 vectors was kept constant. With the exception of Nef-PA3, similar dose-dependent levels of Nef protein expression were detected in all Nef-transfected cells, but not in cells transfected with an empty pBabe-puro vector (Figure 4A).

The FACScan-generated histograms of CD4 surface expression in CD8-positive cells transfected with 1 µg of the Nef expression vectors (and 100 ng in the case of

NL4-3 Nef) are shown in Figure 4B. The top left-hand panel portrays CD4 staining in cells not expressing Nef (Vector), and this expression profile is also shown as an overlay plot in the other panels. The percentage value displayed in each panel indicates the proportion of cells within the brightly staining area delineated by the bar in the histograms. As expected, the expression of NL4-3 Nef resulted in a significant decrease in the intensity of anti-CD4 staining and a reduction of the percentage of cells in the brightly staining fraction. The extent of this effect was dependent on the expression level of NL4-3 Nef, as a lower transfection dosage of the Nef vector resulted in a less dramatic reduction in CD4 expression (compare panels with 1.0 and 0.1 μg NL4-3 Nef). In contrast to CD4, no significant effect by Nef on CD8 expression was observed (data not shown), indicating the specificity of the Nef effect in this assay. Also, in agreement with recent reports (Aiken *et al.*, 1994; Anderson *et al.*, 1994), no Nef-induced down-regulation was observed when a 403 amino acid form of CD4 with a C-terminal truncation was similarly tested (data not shown).

As shown by the data in the lower panels, all three modified forms of NL4-3 Nef (Nef-R71, Nef-PA1 and Nef-PA3) were also capable of down-regulating CD4. In accordance with its lower steady-state level of protein expression, CD4 down-regulation after transfection of 1 μg Nef-PA3 vector was less prominent and comparable with the effect of 100 ng of the NL4-3 Nef vector. These results indicated that SH3 binding by Nef is not required for its ability to induce down-regulation of CD4. Another conclusion from these data is that the proline → alanine mutations introduced into Nef to disrupt its PxxP motifs did not result in an unstructured or non-specifically inactivated protein.

Requirement for Nef SH3 binding motifs for the enhanced growth of Nef$^+$ viruses

To examine whether the SH3 binding-deficient mutant forms of Nef were capable of enhancing HIV replication *in vitro*, fragments containing the specific mutations in Nef-R71, Nef-PA1 and Nef-PA3 were introduced into a complete infectious HIV-1 NL4-3 molecular clone, resulting in the proviral clones NL-R71, NL-PA1 and NL-PA3, respectively. As a control we also generated a Nef$^-$ HIV-1 NL4-3 proviral clone (NL-fs) by introducing a frame shift-generating 4 bp insertion into the N-terminal coding region of Nef. The same strategy has been used previously, and is known to inactivate Nef (Miller *et al.*, 1994). These proviral clones were transfected into 293 cells to produce infectious virus which was collected 2 days later. Small volumes of virus-containing overnight cell culture supernatants from these transfections corresponding to the indicated amounts of p24 antigen were then used to infect resting or prestimulated human peripheral blood mononuclear cells (PBMCs) from two different donors as specified in the legend to Figure 5 and Materials and methods.

In accordance with previous studies (Miller *et al.*, 1994; Spina *et al.*, 1994), we found that when PBMCs were infected with a low input of virus and thereafter incubated for 2 days before a mitogenic stimulation was applied, Nef$^+$ viruses grew significantly better (maximally ~100-fold) than Nef$^-$ viruses (NL4-3 and NL-fs in Figure 5A and C; data not shown). In the experiment using PBMCs from donor no. 1, the cells were stimulated 2 days after infection with interleukin-2 (IL-2), while those from donor no. 2 were similarly treated with IL-2 plus phytohemagglutinin (PHA). In both cases the NL-fs virus exhibited slower growth kinetics than the parental NL4-3 strain, taking more time to reach the peak p24 titers after the cells were stimulated. The maximal p24 titers eventually obtained in infections with NL-fs were also lower than those with NL4-3 in most experiments, probably because the slow-growing NL-fs virus was able to infect fewer cells before viability of the cultures began to decrease. The effect of Nef on virus growth was less evident when a higher virus input and/or a 2 day prestimulation of the cells before the infection was used. By such modifications of the infection conditions the phenotypic effect of Nef could be almost completely abrogated (Figure 5B and D; data not shown).

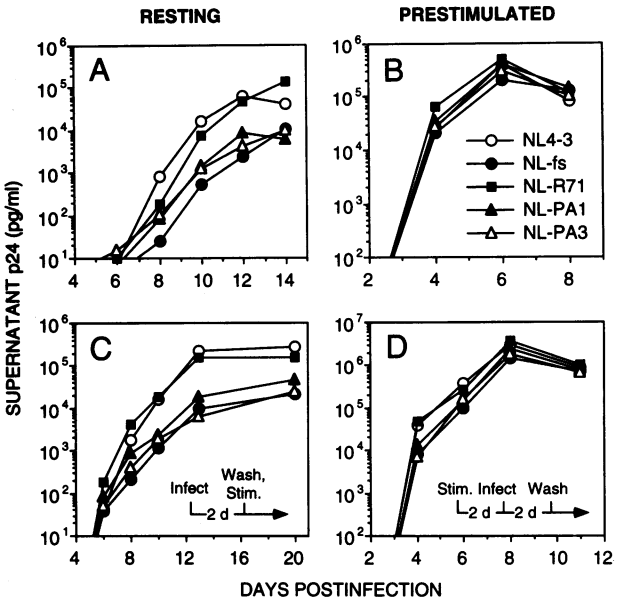

Fig. 5. SH3 binding-deficient forms of Nef fail to enhance HIV growth. Infection of PBMCs from two different donors (donor no. 1, **A** and **B**; donor no. 2, **C** and **D**) with HIV-1 NL4-3 and derived viruses expressing altered forms of Nef under culture conditions which either favor the growth of Nef$^+$ viruses (resting; A and C) or do not reveal a significant phenotypic effect for Nef (prestimulated; B and D). The graphs show on a logarithmic scale the accumulation of p24 antigen in the culture supernatants of the differentially infected PBMCs measured on the indicated days after infection. The symbols corresponding to the different viruses in these graphs are shown in (B). NL4-3 virus is an unmodified HIV-1 NL4-3 strain virus. NL-fs is a derived virus which carries a frame shift mutation resulting in an inactivated Nef gene. NL-R71, NL-PA1 and NL-PA3 are HIV-1 NL4-3 viruses with specific point mutations in their Nef genes expressing modified Nef proteins illustrated in Figure 2. The different infection conditions are summarized schematically in (C) (for 'resting') and (D) (for 'prestimulated'), and are detailed in Materials and methods. The PBMCs of donor no. 1 were in both cases (A and B) infected with 2.5 ng supernatant p24 equivalents of the different viruses. The infection of PBMCs of donor no. 2 was carried out using 0.5 (C) or 5.0 ng (D) p24 equivalents of the viruses.

Comparison of the growth of the viruses carrying altered forms of Nef (NL-R71, NL-PA1 and NL-PA3) with the parental NL4-3 virus and its Nef$^-$ derivative (NL-fs) revealed an absolute requirement for intact PxxP motifs in Nef-mediated enhancement of HIV replication. Under

all tested experimental conditions, the NL-R71 replicated with kinetics very similar to the parental NL4-3 virus, whereas the growth kinetics of the NL-PA1 and NL-PA3 viruses were virtually identical to the NL-fs (Nef^-) virus, exhibiting similar decreased growth when cells were stimulated post-infection (Figure 5; data not shown). It was notable that NL-PA1 and NL-PA3 grew equivalently to wild-type under cell culture conditions which do not favor the growth of Nef^+ viruses (Figure 5B and D). This confirmed that the decreased growth of these mutant viruses in the unstimulated infections was caused by a functional defect in their PxxP-deficient Nef proteins, rather than a generally reduced replicative potential due to their altered genomes.

Discussion

In this study we demonstrate a novel and highly selective SH3-mediated protein−protein interaction involving Nef and Hck, and show that the function of Nef is critically dependent on its SH3 binding site. By suggesting a direct link between Nef and cellular signal transduction, these results provide a molecular framework that could help in the understanding of the elusive function of Nef.

The principal SH3 binding site in Nef is contributed by the PxxP motifs of its conserved tetraproline repeat structure. A short peptide overlapping this region of Nef showed specific binding to Hck and Lyn but not to Lck or Fyn, despite the close similarity of these SH3 domains. While some distinct features unique to the Lck SH3 domain can be identified, the lack of binding to Fyn SH3 domain is even more striking, as it only shows very subtle differences from Hck and Lyn, particularly in regions thought to be involved in target site recognition (Musacchio *et al.*, 1992; Yu *et al.*, 1994). An unprecedented feature of SH3 binding by Nef is the involvement of another distantly located proline residue which appears to contribute significantly to its binding to the Hck SH3 domain. It is possible that the affinity and specificity of other SH3-mediated interactions may also prove to be determined by such additional structures outside the principal proline-rich SH3 binding sites. One potential consequence of such complex binding sites could be an increased flexibility in the conformational regulation of SH3 binding, achieved, for example, by post-translational modification of these proteins.

Interestingly, promotion of HIV growth by Nef, but not down-regulation of CD4, was found to be dependent on intact Nef PxxP motifs, indicating that these are two distinct functions of Nef. Thus, the mechanism by which Nef down-regulates CD4 does not require SH3 binding, and additional mechanisms coupling Nef to cellular processes are therefore likely to exist. The ability to separate by selective mutagenesis the effects of Nef on viral replication and CD4 expression should allow the dissection of the relative contributions of these phenomena to the pathogenesis of AIDS in the SIV/Rhesus monkey system.

Importantly for the present conclusions, the ability of the PxxP-deficient forms of Nef to down-regulate CD4 indicated that these proteins were not generally incapacitated due to the introduced structural alterations. Together with the observation that the slower growth of viruses carrying PxxP-disrupted Nef genes (NL-PA1 and NL-PA3) was only seen under experimental conditions which reveal the phenotypic effect of Nef, these findings strongly suggest that the enhanced replication of Nef^+ viruses is achieved via PxxP-mediated SH3 binding by Nef.

In addition to the remarkable specificity of the Nef−Hck-SH3 interaction in the filter binding assays, PxxP-dependent selection of the endogenous Hck protein from U937 cell extracts by Nef suggest that Hck could indeed be a physiological mediator of Nef function. In cells, this interaction may be transient rather than stable as it has not been possible to directly coimmunoprecipitate Hck with cell-derived Nef (data not shown). Alternatively, the formation of stable complexes involving Hck and Nef might be dependent on association of these myristoylated proteins with the plasma membrane. Additional or even preferential interactions involving Nef and other SH3-containing proteins cannot be ruled out, however, and could be involved in Nef function in other cell types, such as T lymphocytes which express less Hck than cells of the monocyte/macrophage lineage. The present data do not support such a role for Lck or Fyn, the two related Src kinases preferentially expressed in T cells. This may not exclude their involvement, however, and other experimental approaches might be indicated, particularly in the case of the Lck, the SH3 domain of which still awaits the identification of a high-affinity substrate.

The observed requirement for SH3 binding gives distinct insights into the mechanism(s) by which Nef promotes viral growth. Recruitment of Hck by Nef may, for instance, result in modification of the targets of this tyrosine kinase. Conceivably Nef might in this way activate a cellular signal transduction pathway promoting enhanced HIV/SIV replication. However, recent studies have suggested that although Nef itself is not known to be packaged into virions, at least part of the phenotypic effect of Nef can be explained by a higher infectivity of virus particles produced in Nef-expressing cells, rather a higher rate of virus production in infected cells (Chowers *et al.*, 1994; Miller *et al.*, 1994). Thus, it is also possible that the Nef−Hck interaction could target some viral components as substrates of phosphorylation by Hck.

Any functional component that is important for the pathogenicity of HIV represents a potential target for pharmaceutical intervention. In this regard, molecules that could interrupt interactions between cellular and viral factors, such as Hck and Nef, would be particularly attractive, since the conformational restrictions presented by the cellular counterpart would be expected to minimize the ability of the virus to overcome this inhibition by diversifying its structure. Hopefully a more detailed understanding of SH3 binding by the Nef PxxP motifs will allow the design of novel therapeutic strategies targeting this interaction.

Materials and methods

Plasmid constructs and GST fusion proteins

HIV-1 NL4-3-derived Nef genes with mutations resulting in the specific amino acid changes indicated in Figure 2 were generated by two-step overlap PCR mutagenesis using *Pfu* DNA polymerase (Stratagene). The distal set of oligonucleotides covering the complete Nef reading frame included *Eco*RI (5′) and *Sal*I (3′) restriction endonuclease sites for subsequent subcloning. The upstream *Eco*RI site-containing oligo was designed to maintain the reading frame of GST when cloned into the

*Eco*RI–*Xho*I sites of pGEX-1ZT [pGEX-1T (Pharmacia) with a modified polylinker], and to provide a favorable context for translational initiation when cloned into the *Eco*RI–*Sal*I sites of the retroviral vector pBabe-puro (Morgenstein and Land, 1992). The absence of undesired mutations in the resulting modified Nef genes was confirmed by sequencing. Complementary oligonucleotides encoding the peptides PXXP-T and PXXP-R were annealed to create a DNA fragment with cohesive ends compatible with insertion into the *Bam*HI and *Eco*RI sites of pGEX-2T (Pharmacia). The generation and details of the expression vectors for the GST–SH3 fusion proteins (Cheng *et al.*, 1994), as well as their purification and biotinylation (Mayer *et al.*, 1991), have been described previously.

SH3 filter binding assay

After SDS–PAGE and transfer onto nitrocellulose filters, the different GST proteins were visualized by transient staining with Ponceau-S to confirm even loading and transfer of the gels. The derived filter strips were first blocked in TBST (100 mM Tris, pH 8.0, 150 mM NaCl, 0.05% Tween-20) with 2% non-fat dry milk for 30 min. The blocked filters were then incubated for 2–3 h at 4°C with 1 μg/ml of the different biotinylated SH3-containing GST proteins in TBST–milk plus 5 μg/ml unlabeled, non-recombinant GST. After three washes (total ~15 min) with cold TBST, the filters were incubated for 30–45 min at 4°C with 1:500 diluted alkaline phosphatase-conjugated streptavidin (1000 U/ml; Boehringer Mannheim), washed again three times in cold TBST and rinsed with alkaline phosphatase reaction buffer before visualizing the bound SH3 proteins by incubation with Nitro blue tetrazolium and 5-bromo-4-chromo-3-indolyl phosphate (Promega).

Nef-affinity analyses of cell extracts

Logarithmically growing bacterial cultures (200 ml) harboring different GST constructs were induced for 1.5 h with 0.5 mM isopropyl-β-D-thiogalactoside (IPTG), washed in cold water, sonicated briefly on ice in 10 ml PBS, 1% Triton X-100, 20 μg/ml approtinin (Sigma), and the derived soluble fractions incubated with 0.5 ml of glutathione–agarose beads (Molecular Probes Inc.) for 30 min at 4°C. After three washes with the above lysis buffer and one with PBS, 50 μl of these beads were added to 1 ml aliquots of cytoplasmic extract from ~1×10^8 U937 cells. This extract was prepared by lysing PBS-washed cells into 25 mM HEPES, pH 7.4, 10 mM NaCl, 1 mM $MgCl_2$, 0.5% Triton X-100 and 20 μg/ml approtinin, pelleting the nuclei and adjusting the NaCl concentration of the cytoplasmic fraction to 150 mM. After a 4 h incubation at 4°C, three washes with cytoplasmic extraction buffer and one with PBS, the proteins associated with the beads were analyzed as follows.

Half of the beads were boiled in SDS–PAGE sample buffer and used for Western blotting using a rabbit anti-Hck antiserum (Santa Cruz), while the other half were subjected to an *in vitro* phosphorylation reaction. This was performed by first washing the beads once with 20 mM HEPES, pH 7.4, 5 mM $MgCl_2$, followed by a 30 min incubation at room temperature in 30 μl of this buffer supplemented with 2 μl of [γ-^{32}P]ATP (7000 Ci/mmol; ICN). After three washes with PBS, a fraction of the phosphorylated bead-associated products was boiled in SDS–PAGE sample buffer to be directly analyzed by gel electrophoresis and autoradiography. The remaining phosphorylated products were boiled in 0.5% SDS, diluted 10-fold in PBS and subjected to immunoprecipitation before SDS–PAGE. The immunoprecipitation step was carried out by incubation of 3 μl of rabbit antiserum against Hck or Lyn (Santa Cruz), or normal rabbit serum together with 25 μl of protein A–Sepharose beads for 2 h at 4°C mixed with 1 ml aliquots of phosphoproteins in PBS, and 0.05% SDS prepared as described above, followed by three washes in PBS before boiling in SDS–PAGE sample buffer.

CD4 down-regulation assay

293 cells were transiently cotransfected as described previously (Pear *et al.*, 1993) with SV40-driven expression vectors for the full-length or truncated 403 amino acid form of human CD4 (Landau *et al.*, 1988) and a CMV vector for human CD8 (Lenburg and Landau, 1993) together with a pBabe retroviral vector expressing puromycin resistance alone (Morgenstein and Land, 1992) or in combination with different forms of Nef. At 36 h after transfection the cells were treated with trypsin–EDTA, replated as sparse cultures and allowed to recover for 12 h before harvesting as single-cell suspension by pipetting with PBS + 4 mM EDTA. After two washes with cold PBS + 2% calf serum, the cells were stained with FITC-conjugated anti-human CD8 (Pharmingen) and/or PE-conjugated anti-human CD4 (Pharmingen) by a 30 min incubation on ice with 1:200 diluted antibodies in PBS + 2% calf serum. After three washes with cold PBS the stained cells were fixed with 4% paraformaldehyde in PBS, in which they were kept until fluorocytometric analysis by FACScan (Becton Dickinson). Based on forward and side light scatter profiles of the cell samples, as well as fluorescence patterns of the unstained and singly stained (CD4 or CD8 alone) samples, the parameters of the FACScan apparatus were set to quantitate CD4 staining based on the observation of 10 000 CD8-positive single cells derived from each transfection. The HIV-1 BH-10 rabbit anti-Nef antiserum used in the Western blotting analysis of cells used in these experiments was derived from the AIDS Research and Reference Reagent Program, NIAD, NIH.

Production of HIV-1 NL4-3-derived viruses

The *Pml*I site present in the 5′ LTR of HIV-1 NL4-3 was first disrupted by the insertion of an 8 bp *Not*I linker, resulting in the proviral plasmid HIV-1 NL4-3N with a unique *Pml*I site in the C-terminal coding region of Nef (but yet producing wild-type HIV-1 NL4-3 progeny). *Xho*I–*Pml*I fragments containing the R71, PA1 and PA3 Nef mutations were then transferred into HIV-1 NL4-3N from the respective pBabe-puro vectors resulting in the HIV-1 proviral clones NL-R71, NL-PA1 and NL-PA3. To produce a frame shift mutation in the Nef gene, *Xho*I-linearized HIV-1 NL4-3N plasmid was treated with Klenow DNA polymerase and religated. Thirty μg of HIV-1 NL4-3N and the derived proviral plasmids were transfected into 293 cells on 10 cm dishes (Pear *et al.*, 1993). At 36 h after the transfection the medium was replaced with 8 ml of fresh DME + 10% FCS; 12 h later the virus-containing supernatants were harvested, aliquoted and frozen at −70°C. The supernatant p24 concentrations were determined by an antigen capture assay (Abbot Laboratories); they were relatively high and of similar magnitude (150–500 ng/ml) in all duplicate transfections with each of the proviral constructs.

PBMC infections

PBMCs were prepared by Ficoll-Hypaque (Pharmacia) gradient centrifugation immediately after 250 ml of heparinized blood were collected from healthy volunteers. After three washes in PBS, 2×10^6 PBMC were aliquoted into 24-well tissue culture plates in 1.5 ml of RPMI + penicillin/streptomycin and 25 mM HEPES, pH 7.4, supplemented with 10% autologous serum obtained from the donors. Half of the plates (prestimulated) were also supplemented with 2 μg/ml PHA, whereas the other half of the plates (resting) were infected immediately by the addition of small volumes of virus-containing 293 cell supernatants corresponding to the amount of p24 antigen indicated in the text. Two days later the cells on the unstimulated plates were washed three times with RPMI, resuspended into 2 ml RPMI (supplemented as above) plus 2 μg/ml PHA and 10 U/ml IL-2, followed by the removal of 0.5 ml of the supernatant as a 0-sample. At the same time the prestimulated plates were infected by the similar addition of viruses, and supplemented with 10 U/ml IL-2. After a further 2 days the prestimulated plates were washed, 0-samples collected and the cells resuspended to the same media. After the 0-samples were collected (which contained <20 pg/ml of p24 in all cultures), 0.5 ml fractions of the supernatants on both plates were collected every second day and replaced with RPMI + penicillin/streptomycin, 25 mM HEPES, pH 7.4, 10% FCS and 10 U/ml IL-2. These infection procedures are illustrated schematically in Figure 5.

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