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SH3-Domain Binding Function of HIV-1 Nef Is Required for Association with a PAK-Related Kinase

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HIV-1 Nef has previously been shown to bind to Src homology-3 (SH3) domains of a subset of Src family tyrosine kinases. In addition, Nef has been reported to coprecipitate with a serine/threonine kinase activity termed NAK (for Nef-associated kinase). The identity of NAK remains uncertain, but it has been suggested to represent a novel member of the p21-activated kinase (PAK) family. We report here that NAK autophosphorylation is increased not only by an activated form of the p21-family GTPase cdc42 but also by a plasma membrane-targeted fragment of the adapter protein Nck, thus providing further evidence that NAK is related to PAKs. A detailed structure-based mutational analysis of Nef revealed that all amino acid changes that inhibited the Nef/Hck-SH3 interaction, as measured by surface-plasmon resonance, also abolished coprecipitation of NAK. As PAK family proteins do not contain SH3 domains, these observations are best explained by a protein complex in which Nef, NAK, and an SH3-protein all contact each other. In addition, a number of conserved amino acids in Nef that are not involved in SH3 binding were also found to be crucial for association with NAK. Molecular modeling suggests that these residues are involved in formation of an adjacent binding surface for NAK or another critical component of the NAK/Nef complex.

Key Words: HIV; AIDS; Nef; SH3; PAK; NAK; cdc42; Nck; protein kinase.

INTRODUCTION

Nef is a 27- to 34-kDa myristoylated protein of primate lentiviruses that is indicated as a critical factor in development of high viremia and AIDS but is not absolutely required for the viral life cycle (reviewed in Harris, 1996; Saksela, 1997). Findings on physical and functional interactions between Nef and various protein kinases involved in cellular signal transduction pathways have implicated modulation of host cell signaling cascades as a potentially important function of Nef (reviewed in Saksela, 1997; Sawai *et al.,* 1997).

A structural basis for a Nef/kinase-interaction was provided by the observation that a highly conserved amino-acid sequence in Nef, consisting of repeated proline residues in a Pro-x-x-Pro configuration (PxxP motif), can bind with high affinity to the Src homology-3 (SH3) domain of the Src-family tyrosine kinases Hck and Lyn (Saksela *et al.,* 1995). Mutations that disrupt the Nef PxxP motif abolish its ability to increase the replicative potential of HIV and block a number of effects of Nef on cellular signaling but do not prevent Nef-induced down-modulation cell-surface CD4 expression (Goldsmith *et al.,* 1995; Saksela *et al.,* 1995; Iafrate *et al.,* 1997). Binding of Nef potently stimulates the catalytic activity of Hck (Moarefi *et al.,* 1997), and their coexpression can activate cells and even lead to malignant transformation (Briggs *et al.,* 1997), suggesting a functional role for this interaction in HIV-infected cells that express Hck, such as macrophages. Although interactions between Nef and the SH3 domains of the T-cell Src-kinases Lck and Fyn have been noted (Lee *et al.,* 1995; Collette *et al.,* 1996; Greenway *et al.,* 1996; Arold *et al.,* 1997), an SH3-domain protein expressed in T lymphocytes and showing Hck-like avid binding to Nef remains to be identified.

In contrast to the restricted expression pattern of Hck, a 62-kDa serine kinase coprecipitating with Nef has been demonstrated in a wide variety of cell types, including T cells (Sawai *et al.,* 1994, 1997). The identity of this kinase, termed NAK (for Nef-associated kinase), has remained unclear, but a number of observations suggest that it may belong to the family of p21-activated protein kinases (PAKs) (Lu *et al.,* 1996; Nunn and Marsh, 1996; Sawai *et al.,* 1996). These observations include cross-reactivity with certain anti-PAK antibodies and the activation of NAK (as evidenced by its increased autophosphorylation) by dominantly active forms of cdc42 and Rac, two small p21-GTPases that regulate the PAKs (reviewed in Sells and Chernoff, 1997). However, it appears that NAK represents none of the three known members

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of the mammalian PAK family (Lu *et al.,* 1996; Nunn and Marsh, 1996).

The structural determinants required for NAK-association have been mapped to multiple regions in Nef, such as a highly conserved di-arginine (RR) motif in the central core domain of Nef (Sawai *et al.,* 1995). Interestingly, it has been reported that mutations disrupting the HIV-1 Nef PxxP motif also abolish coprecipitation of NAK (Wiskerchen and Cheng-Mayer, 1996). More recently, it was also noted that a number of amino-acid changes within the SH3-ligand region of SIVmac239 Nef similarly abrogate its association with NAK (Khan *et al.,* 1998). However, because of certain ostensibly contradictory observations, most notably the inhibitory effect on NAK association by mutations in the PxxP region of Nef that are not thought to be involved in SH3 binding (Wiskerchen and Cheng-Mayer, 1996), Nef/NAK complex formation has been considered unrelated to the SH3-binding function of Nef.

In this study we have performed an extensive structure-based mutational analysis of Nef, examined the quantitative effects of these changes on the SH3-binding capacity of Nef using a Biacore biosensor apparatus and correlated these data with the ability of the corresponding mutants to coprecipitate NAK. Based on these results we argue that an SH3 protein plays a critical role in the assembly of the NAK/Nef complex. In addition, a number of other Nef residues, including some in the proline-repeat region of the Nef polypeptide, appear to be important for NAK association by forming an adjacent but distinct binding surface, which may be a docking site for NAK. In addition, we provide evidence further strengthening the idea that NAK is indeed related to the PAK family kinases.

RESULTS

NAK is activated by a membrane-tagged SH3 domain of Nck

To reproduce earlier results, which demonstrated an association of HIV-1 Nef with a cdc42-regulated cellular kinase, we transfected human embryonic 293 cells with expression vectors for the NL4–3 R71 Nef with or without a dominantly active mutant of cdc42 (cdc42^{V12}). As shown in Fig. 1, we were able to coprecipitate using an anti-Nef antiserum a 62-kDa protein that could be phosphorylated in an *in vitro* autokinase assay (IVKA) and in further discussion is referred to as NAK. NAK signals from cells transfected with R71 Nef were typically faint and sometimes even difficult to detect over the background signal. However, cotransfection of cdc42^{V12} resulted in a robust increase in the NAK signal (Fig. 1). Slightly less Nef protein was seen by Western blotting in cells transfected with Nef (plus an empty control vector) (Fig. 1, lane A) than in cells transfected with cdc42^{V12} (lane C) of myrNck-SH3–2 (lane B, see discussion below). However, this small difference in Nef expression was negligible as compared to the large difference in the NAK signal from these cells and was not observed in a number of other experiments in which a similar increase in coprecipitating NAK activity was seen.

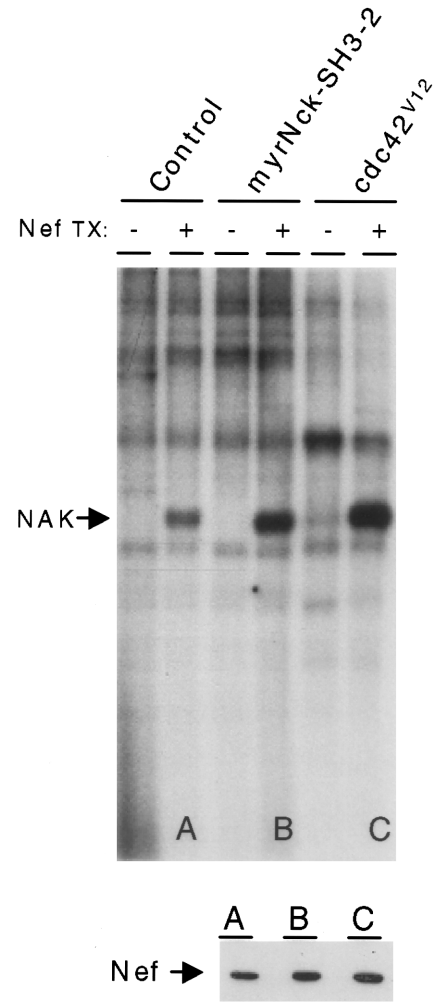

FIG. 1. NAK activity coprecipitating with Nef is increased by coexpression of a constitutively active form of cdc42 as well as a membrane-targeted Nck-SH3 protein. (Top) IVKA analysis of anti-Nef immunocomplexes from 293 cells transfected with 10 μg of an Nef expression vector (Nef TX: +) or an empty control vector (Nef TX: −) alone or in the presence of 5 μg of expression vectors for cdc42^{V12} or a myristoylated second SH3 domain of Nck (myrNck-SH3–2) or 5 μg of the relevant empty control vector (Control). (Bottom) Anti-Nef Western blot analysis demonstrating similar amounts of Nef protein in all immunocomplexes from cells transfected with Nef (corresponding lanes in both panels marked A, B, and C.

To examine other possible similarities between NAK and PAKs, we tested whether NAK can also be activated by an Nck-SH3-mediated targeting to the plasma membrane. It has previously been shown that expression of a protein consisting of a 14-aa v-Src myristoylation motif and the second (of three) SH3 domains of the adapter protein Nck can activate PAK1 in 293 cells by binding to a PxxP motif in the amino terminus of PAK1 (Lu *et al.,*

TABLE 1

HIV-1 Nef Variants Used in This Study

Category	Variant
A	Parental pNL4-3 Nef (T71) and its "wild-type" derivative: R71 T71, R71
B	Changes in SH3-contacting PxxP-motif residues in the PPII helix region: P72A, P75A, P72A; P75A (PA-1), V74D, R77E, V74D;R77E
C	Changes in non-SH3-contacting residues in the PPII helix region: P69A, Q73P;L76A
D	Changes in SH3-contacting residues outside of the PPII helix F90R
E	Changes in non-SH3-contacting residues outside of the PPII helix: R106A, L112R, F121R

1997). As shown in Fig. 1, co-expression of this membrane-targeted Nck-SH3 protein (myrNck-SH3–2) with Nef also resulted in a clear increase in NAK signal in an IVKA analysis of anti-Nef immunoprecipitates (IPs) from the cotransfected cells. This observation suggests that like PAK1, NAK can be activated by relocation to the plasma membrane and that NAK also contains a functional binding site for the second SH3 domain of Nck. Because of the lack of reagents to directly detect NAK, it was not technically feasible to formally demonstrate that the activation of NAK was indeed due to its relocation to the plasma membrane by myrNck-SH3–2. However, this assumption was strongly supported by the observation that cotransfection of an identical construct expressing a nonmyristoylated form of Nck-SH3–2 had no effect on NAK activity (data not shown). Since it would formally also be possible that myrNck-SH3–2 could increase coprecipitation of NAK by binding to and targeting Nef (rather than NAK) to the plasma membrane, we tested whether recombinant Nck produced in bacteria could bind to Nef. However, under conditions when strong *in vitro* binding to PAK1 was seen, no evidence of an interaction between Nck and Nef could be observed (data not shown).

Thus the observations that NAK can be activated by the p21-family GTPase cdc42 as well as by plasma membrane-targeted Nck-SH3 agree with and provide new support for the idea that NAK is a member of the PAK kinase family.

Structure-based mutagenesis of residues in the core domain of Nef

To examine the role of SH3-binding of Nef in association with NAK, a number of new mutants were constructed into R71 Nef background (Table 1). R71 is a derivative of the original HIV-1 NL4–3 Nef allele (termed T71 in this study) and has an arginine residue in position 71 to mimic the sequence of most naturally occurring Nef sequences (Shugars *et al.,* 1993; Saksela *et al.,* 1995). In "the resting PBMC assay" for Nef function, HIV strains carrying R71 or T71 Nef alleles grow with similar wild-type kinetics (Saksela *et al.,* 1995). Binding of R71 Nef with the SH3 domain of the tyrosine kinase Hck has been previously characterized in detail (Lee *et al.,* 1995, 1996). This high-affinity interaction has an equilibrium dissociation constant (K_D) of approximately 250 nM, measured based on surface plasmon resonance (SPR; Biacore) and confirmed by an independent method (isothermal titration calorimetry; K_D 190 nM). In addition to the novel Nef mutants, a previously described Nef allele with a double-mutation affecting both of the PxxP-defining prolines (P72A;P75A in T71 background; PA-1) was also used. PA-1 Nef has been shown by multiple methods to have lost all measurable affinity to Hck-SH3, and viruses carrying the PA-1 allele replicate with delayed kinetics identical to those of an isogenic Nef(−) strain (Saksela *et al.,* 1995).

The classification of the mutations engineered based on the available Nef/SH3 cocrystal and Nef NMR structures (Lee *et al.,* 1996; Grzesiek *et al.,* 1997) is shown in Table 1, and the rationale behind constructing these is explained in the following. Mutations in category B involve residues that constitute the minus-orientation SH3 ligand consensus sequence PXØPXR, in which X is any amino acid, and Ø is a (typically small) hydrophobic residue (Feng *et al.,* 1994; Lim *et al.,* 1994). This pattern is conserved in the vast majority, if not all, available HIV-1 Nef sequences and corresponds to the residues $P^{72}Q^{73}V^{74}P^{75}L^{76}R^{77}$ in T71 and R71. In the Nef/SH3 cocrystal (Lee *et al.,* 1996), as well as in structures of a number of linear peptides complexed with their cognate SH3 domains (for a review, see Mayer and Gupta, 1998), these consensus residues make important contacts with the PPII helix-accommodating binding surface of SH3. By contrast, although located in the PPII helix region of Nef, the mutated residues in category C are not part of the SH3-ligand consensus sequence and occupy positions that are not intimately involved in SH3 binding. The double mutant Q73P;L76A carries a change in both of the residues within the Nef PxxP motif, which are not predicted to be critical for SH3 binding. The P69A mutant was made because this change, which affects a residue immediately adjacent to the SH3-binding site of Nef, has been previously reported to abolish NAK binding (Wiskerchen and Cheng-Mayer, 1996).

The F90R mutation in category D was engineered based on the structure of the Nef/SH3 complex (Lee *et al.,* 1996). Although the Phe^{90} residue is relatively distant from the canonical PxxP/SH3 contact region, this change was predicted to affect binding of Nef to Hck, and probably also to other SH3 domains, because of the important role of Phe^{90} in coordinating a tertiary (non-PxxP) interaction between Nef and the SH3 domain RT-loop (Lee *et al.,* 1996; Lim, 1996). Finally, the mutations in the

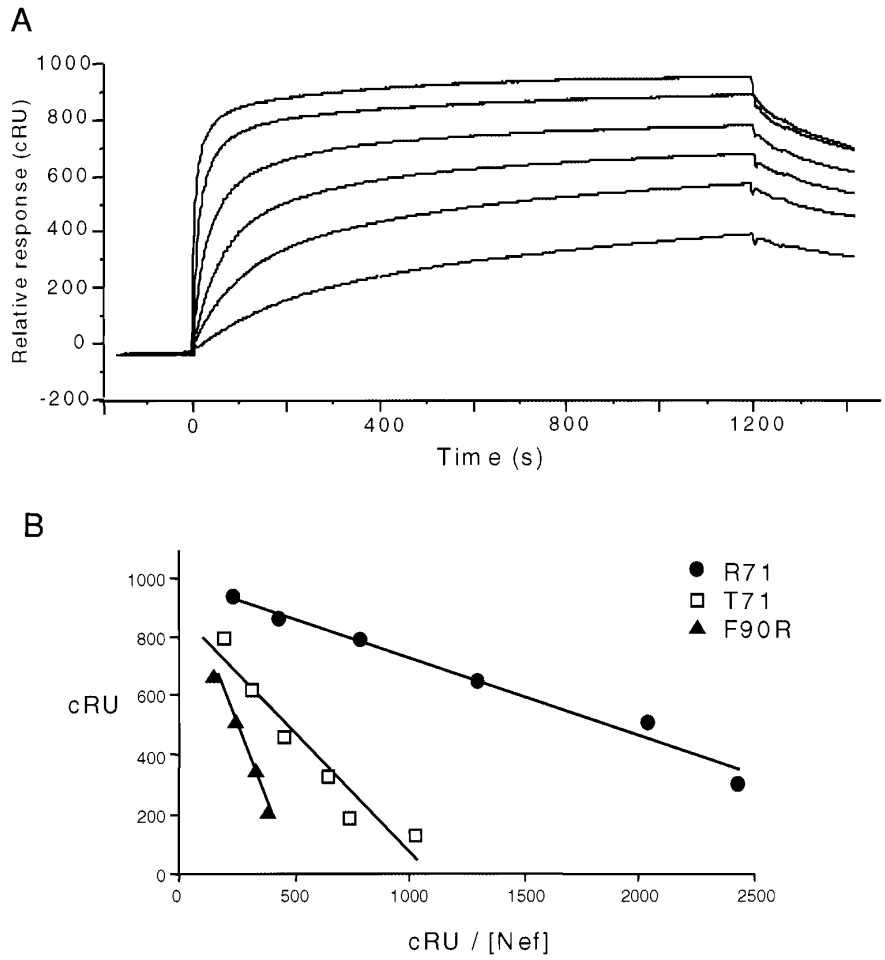

FIG. 2. Biacore analysis of the effects of mutations in Nef on binding to Hck-SH3. (A) An overlay of sensorgrams of specific SPR signals (cRU; bulk refractive index effects recorded on a parallel control channel have been subtracted) illustrating association and dissociation of R71 Nef protein (analyte) to Hck-SH3 (ligand) immobilized on a biosensor chip. The time point zero corresponds to the start of six different injections of twofold serial dilutions of R71 Nef ranging from 4.0 (top) to 0.125 μM (bottom). Part of the slow dissociation phase starting after the end of the 20-min (1200-s) Nef injection can also be seen. (B) A Scatchard plot analysis for estimating the equilibrium dissociation constants (K_D) for three different Nef variants (R71, T71, and F90R) based on SPR data obtained as shown in (A) for R71 Nef. The corrected relative resonance unit values (cRU) of each protein dilution at the end of the 20-min injection were plotted as shown, and the affinity of the interaction was estimated from the slope of the line that best fitted to these data points. The coefficients for determination (R^2) for these analyses, together with the complete data on all the Nef variants tested are shown in Table 2.

category E involve residues that are not located in the PPII helix region nor have been implicated in SH3 binding. These mutations were made either because of published inhibitory effects on NAK association (R106A) (Sawai *et al.,* 1995; Wiskerchen and Cheng-Mayer, 1996) or guided by our initial results on inhibition of NAK binding by mutations in category C.

Effect of mutations in Nef on association with Hck-SH3

To quantitatively assess the SH3-binding capacity of the different modified forms of Nef, we produced these in *E. coli* as glutathione-S-transferase (GST) fusion proteins and examined by a Biacore biosensor apparatus their binding to the Hck SH3 domain, a known high-affinity ligand for Nef. Hck-SH3 was produced in bacteria fused to the maltose-binding protein (MBP) to avoid artifacts arising from GST homodimerization. For the same reason, GST was removed from the Nef proteins by thrombin cleavage before SH3-binding analysis to avoid possible GST-directed complex formation on top of Nef-SH3 heterodimers on the biosensor chip. The completeness of this cleavage, as well the spectrophometrically estimated Nef protein concentrations, were confirmed by SDS–PAGE before the Biacore analysis (data not shown). The MBP-SH3 protein was biotinylated and attached to a streptavidin-coated biosensor chip to give a relatively high MBP-SH3 surface density. The second channel on the same biosensor chip was coated with an equal molar amount of similarly biotinylated plain MBP. The injected Nef proteins passed through both of these channels, and the bulk refractive index effects on the SPR signal recorded on the MBP-only channel (in

TABLE 2

Summary of SPR Studies on SH3 Binding of Nef Variants

Nef protein	Category in Table 1	Concentration range (μM)	Number of data points	Coefficient of determination (R^2)	Affinity K_D (μM)
R71	A	4.0–0.125	6	0.97	0.26
T71	A	4.0–0.125	6	0.93	0.81
P72A	B	4.0–1.0	3	0.99	4.18
P75A	B	4.0–1.0	3	0.94	5.12
P72A;P75A	B	4.0	1	—	—
V74D	B	4.0	1	—	—
R77E	B	4.0	1	—	—
F90R	C	4.0–0.50	4	0.99	1.99
P69A	D	4.0–0.125	6	0.95	0.22
Q73P;L76A	D	4.0–0.125	6	0.97	0.18
R106A	E	4.0–0.25	5	0.97	0.28
F121R	E	4.0–0.125	6	0.99	0.32

which no binding was observed) were subtracted from the RU values of the MBP-Hck channel (shown in Fig. 2A for R71 Nef).

As noted before (Lee *et al.,* 1995), the association rate of R71 Nef with Hck-SH3 was too rapid to be reliably estimated from the shape of the SPR curve, and the affinities (equilibrium dissociation constants; K_D) of the Nef/SH3 interactions were therefore derived by Scatchard analyses of the cRU values at the end of a long injection of serial twofold dilutions of the Nef proteins. The primary SPR data (sensorgrams) regarding such serial injections of R71 Nef are shown as an overlay plot in Fig. 2A. These curves appear very similar to the sensorgrams we have previously recorded using an R71 Nef preparation that was highly purified for crystallographic purposes. The K_D values (260 nM) derived from the Scatchard analysis of the R71 Nef data in Fig. 2B also agree well with the affinities previously recorded based on SPR (250 nM) and ITC (190 nM), thus validating the present experimental approach.

Figure 2B shows a composite of Scatchard plots based on the R71 Nef sensorgrams (Fig. 2A) together with similar data on T71 and F90 Nef. A summary of all Biacore experiments and the K_D values obtained are presented in Table 2. As predicted by previous structural studies, the Arg^{71} residue in R71 Nef contributes to the high affinity of its interaction with Hck-SH3 (K_D 260 in the present study), as indicated by the threefold lower affinity measured for T71 Nef (K_D 810 nM). Although, we haven't noted a significant difference in the replicative potential of HIV strains carrying the R71 *vs* T71 Nef alleles in the available cell culture assays (Saksela *et al.,* 1995), it is possible that under some conditions the more avid SH3 binding of the "patient-like" R71 Nef provides such viruses a growth advantage over viruses resembling the original HIV-1 NL4–3 strain (T71) in this regard.

As seen in various instances before (Lee *et al.,* 1995; Saksela *et al.,* 1995), the PA-1 mutant showed no measurable SH3-binding affinity (Table 2). Change of either one of the PxxP-defining proline residues alone into an alanine (P72A or P75A) also resulted in a large reduction in binding affinity but did not completely abolish binding to Hck-SH3 (Table 2). The residual binding of these single-proline mutants (K_D 4.18 μM for P72A and K_D 5.12 μM for P75A) was not surprising considering the multiple stabilizing non-PxxP interactions between Nef and Hck-SH3 and that another hydrophobic residue was used to replace the prolines. The Nef residue Phe^{90} plays an important role in the aforementioned non-PxxP interactions by participating in accommodation of the SH3 RT-loop (Lee *et al.,* 1996; Lim, 1996). Accordingly, the F90R mutation decreased binding to Hck-SH3 almost 10-fold (K_D 1.99 μM). Also, in good agreement with the current structural understanding on SH3/PxxP binding, replacement of either the hydrophobic (V74D) or the positively charged (R77E) residue in the PxxP-consensus sequence of Nef with an acidic residue completely abolished SH3 binding (Table 2). Thus as also indicated by the PA-1 mutation, the stabilizing tertiary (non-PxxP) interactions are not sufficient to mediate SH3 binding in the absence of a functional PxxP motif.

In contrast to the inhibitory mutations described above, changes involving residues in the PPII helix region of Nef that were not predicted to participate in SH3 binding (P69A and Q73P;L76A) did not decrease, or even improved, the affinity toward Hck-SH3. The slight increase in SH3 binding of the Q73P;L76A mutant may be due to stabilization of the PPII fold conformation in the PxxP-region of Nef and highlights the fact that these nonconsensus residues do not directly participate in SH3 binding. As expected, mutations located outside of the PPII helix region and affecting residues that have not been implicated in SH3 binding by structural studies did

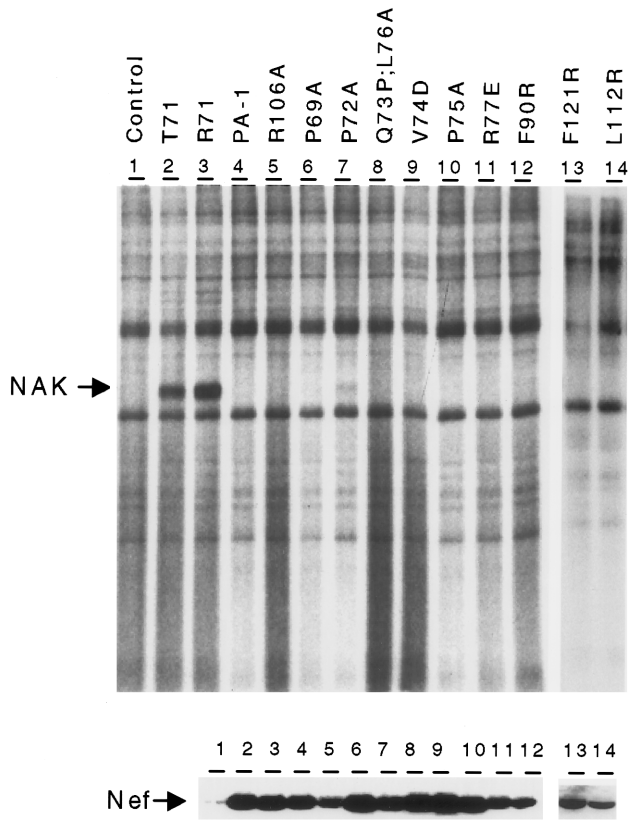

FIG. 3. Effect of different Nef mutations on NAK/Nef complex formation. Ten micrograms of expression vectors for each of the indicated wild-type or mutant forms of Nef were transfected into 293 cells together with the NAK-activating myr-Nck-SH3–2. Equal amounts of protein lysates normalized for their protein concentration were subjected to anti-Nef immunoprecipitation, followed by IVKA (top) or anti-Nef Western blotting (bottom). The order and numbering of the lanes are the same in both panels. In lane 1 an empty vector without a Nef-insert was transfected as a control.

not significantly interfere with the interaction of these variants (R106A, L112R, and F121R) and Hck-SH3.

Effect of mutations in Nef on coprecipitation of NAK

To examine the dependence of NAK association on SH3-binding capacity of Nef we cotransfected all the Nef variants listed in Table 1 into 293 cells together with the NAK-activating myristoylated Nck-SH3 protein (myrNck-SH3–2). We chose to use myrNck-SH3–2, instead of $cdc42^{V12}$ for enhancing the NAK signals because it appeared to be a more specific activator of NAK. In prolonged exposures of IVKA gels of anti-Nef IPs, we typically observed a phosphoprotein comigrating with NAK in cells transfected with $cdc42^{V12}$ but not expressing Nef (faintly visible in Fig. 1), probably representing a nonspecifically precipitating abundant kinase regulated by cdc42. However, when NAK was activated by myrNck-SH3–2, this NAK-sized phosphoprotein was not observed even in very long exposures, unless the 293 cells were also transfected with Nef (Fig. 3 and data not shown), thus providing a clean system for evaluation of the inhibitory effects of the different mutations introduced in Nef.

As evident from Fig. 3, all Nef mutants that failed to bind to Hck-SH3 (PA-1, V74D, and R77E) were also completely negative for NAK association. Also, no NAK could be coprecipitated with the single-proline Nef mutant P75A. A faint NAK signal could in most experiments be observed in P72A-Nef-transfected cells in accord with its slightly better SH3-binding capacity (see Table 2). Also, the threefold decrease in the SH3-binding capacity of T71 Nef was proportionally reflected by a similar diminution in the amount of coprecipitating NAK signal. Instead, F90R Nef, a mutant with low but apparent SH3-binding potential, was negative for NAK association. It should be noted, however, that since the Phe^{90} residue is not part of the idiotypic PxxP/SH3 interface of Nef, mutation in this residue could affect binding of another SH3 domain even more severely than that of Hck. In conclusion, we found that without exception, mutations that affected SH3 binding of Nef also resulted in decreased or abrogated NAK association.

As expected from the literature, no NAK association was seen in cells transfected with the R106A Nef mutant (Sawai *et al.,* 1995; Wiskerchen and Cheng-Mayer, 1996). In our hands this mutant had a slightly decreased stability (see Western blot in Fig. 3) as compared to the other Nef variants tested but was clearly expressed in sufficient levels to allow the conclusion that it suffered from a specific defect in NAK binding. Also in agreement with published data (Wiskerchen and Cheng-Mayer, 1996), the P69A mutant was equally negative for NAK association. A similar result was also seen with the double-mutation affecting the two nonconsensus residues within the PxxP motif of Nef (Q73P;L76A).

To understand why the P69A and Q73P;L76A Nef proteins did not associate with NAK, despite their undiminished ability to bind to Hck-SH3 (Table 2), we carefully examined the atomic organization of this region in the Nef/SH3 cocrystal and Nef NMR structures (Lee *et al.,* 1996; Grzesiek *et al.,* 1997). Both Gln^{73} and Leu^{76} are on the surface of the core domain of Nef, even when it is bound to an SH3 domain (Fig. 4). Together with the neighboring residues provided by the α-helical part of Nef (such as Phe^{121} and Leu^{112}), Gln^{73} and Leu^{76} form an exposed surface, which could well serve as an interaction site for NAK (or another necessary component of the NAK/Nef/SH3 complex). Pro^{69} can not be seen in the available Nef/SH3 cocrystal structures (Lee *et al.,* 1996; Arold *et al.,* 1997), and its position is relatively variable among the 40 calculated structures from which the NMR model of Nef was derived (Grzesiek *et al.,* 1997). However, it is the most amino-terminal residue of the well-ordered core domain of Nef and is probably important for providing a correct backbone angle to properly position the residues extending further toward the amino termi-

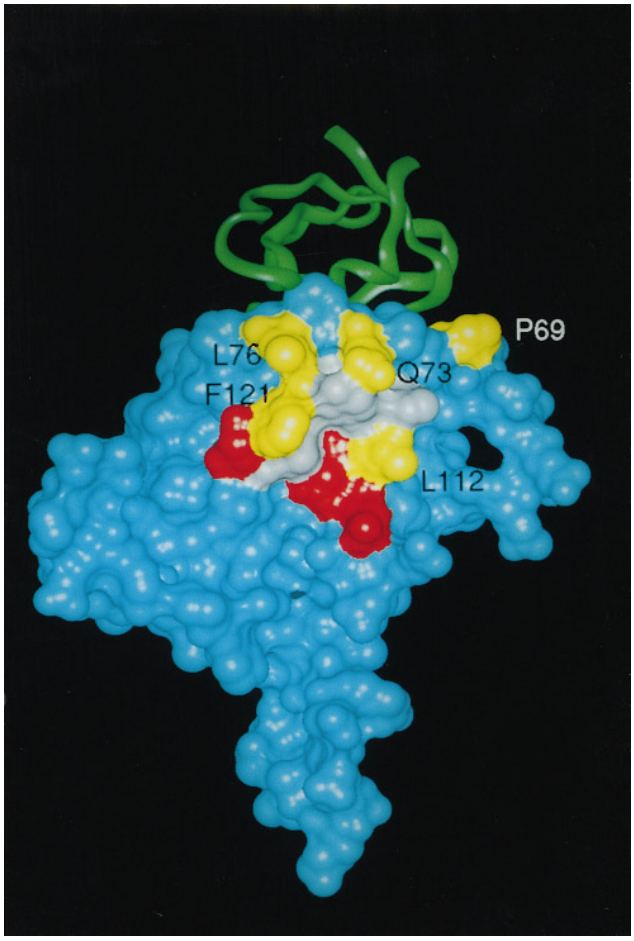

FIG. 4. Putative NAK binding site on the surface of Nef. Surface presentation of the core regions of Nef bound to an SH3 domain (green ribbon). The corresponding parts of the crystal (Lee *et al.,* 1996) and NMR (Grzesiek *et al.,* 1997) structures were superimposed to position the SH3 domain correctly. Residues mutated and found to affect NAK binding are shown in yellow and indicated by one-letter symbols (P69, L76, L112, and F121) on the surface of Nef. Three aspartates (residues 108, 111, and 123; colored red) form a negatively charged patch around residues Leu^{112} and Phe^{121}. Changing the latter into arginines (in L112R and F121R mutants) therefore alters the electrostatic properties of this surface. Note that although the Gln^{73} and Leu^{76} residues are components of the peptide backbone of the SH3-binding PPII helix, their hydrophobic side chains extend away from the SH3 domain sitting on the other side of Nef and participate in formation of the putative NAK-binding surface. Other Nef residues that can also be seen to contribute to this hydrophobic surface patch (Tyr^{115}, His^{116}, and Pro^{122}) but were not targeted by mutagenesis in this study are indicated by gray color. The exact positions of Pro^{69} and the residues aminoterminal to it (the "arm-like" structure below P69) are not well defined by the available structural data. However, as discussed in the text, the role of Pro^{69} in associating with NAK is more likely to be the positioning of the Nef amino terminus, rather than direct involvement in accommodating NAK.

nus, which in turn, may be important for allowing NAK to bind (see Discussion)

To experimentally test the idea that the surface in Nef adjacent to the SH3-binding region would be important for NAK binding, as hypothesized above, we produced mutations in the residues Leu^{112} (L112R) and Phe^{121} (F121R), thus bringing positive charge to this hydrophobic region, which on one side is neighbored by Gln^{73} and Leu^{76} and on the other side by a negatively charged patch formed by three highly conserved aspartate residues (Asp^{108}, Asp^{111}, and Asp^{123}) (Fig. 4). In agreement with our model, both of these new Nef variants (F121R and L112R) were also completely defective in binding to NAK.

DISCUSSION

In this study we show that a cellular SH3-protein is likely to play a critical role in association of HIV-1 Nef with a kinase known as NAK. In addition, by demonstrating that a plasma-membrane targeted Nck-SH3 protein can activate NAK, we provide further evidence that this kinase is a member of the PAK family.

Since the known PAKs do not contain SH3 domains, it appears likely that the NAK/Nef complex contains an additional SH3-containing protein. The Nck adapter protein would be an attractive candidate for this role, as we found that it can interact with NAK via its second SH3 domain and contains two additional SH3 domains that could bind to the SH3-ligand site in Nef. However, we have not been able to show any affinity between a recombinant full-length Nck and Nef, suggesting that another protein, perhaps similar to Nck, is serving this function.

We confirmed the previously reported finding (Wiskerchen and Cheng-Mayer, 1996) that mutation of the Pro^{69} residue, which precedes the PPII helix region of Nef but is not a component of the SH3-ligand motif, also resulted in a loss of NAK association. Pro^{69} is in a position where it is likely to govern the orientation of the region located amino-terminally relative to the Nef core domain (see Fig. 4). Thus careful coordination of the positioning of this region appears to be important for NAK association. If misplaced, these residues could interfere with the interaction of NAK and its adjacent putative binding surface. Alternatively, the residues in this region might fold to form a part of this putative NAK-binding surface. The latter possibility (active participation of the region amino terminal to the PPII helix) appears more likely in the light of the results of a deletion analysis undertaken by Sawai *et al.* (1995). They reported that while the first 44 amino acids of HIV-1 SF2 Nef could be deleted without losing NAK binding, provided that membrane-targeting of Nef was preserved, but extension of the deletion to the SF2 Nef Val^{70} residue (corresponding to Val^{66} in R71) resulted in a complete loss of NAK coprecipitation. Thus although it is not possible to conclude with certainty which residues in Nef are directly contacting NAK, our results suggest that a complex binding surface contributed by Gln^{73}, Leu^{76}, Leu^{112}, and Phe^{121} (Fig. 4), and possibly residues located between

the positions 40 and 69 in the Nef polypeptide, may be involved.

Identification of the SH3-protein involved in the NAK/Nef complex, and of course, molecular characterization of NAK itself represent important goals to better understand the role of NAK in HIV infection. The fact that NAK has remained unidentified so far might be related to the multiprotein nature of the cellular NAK/Nef complex, in which direct interactions between these two proteins might be weak and therefore difficult to detect in simplified experimental systems. Our observation that an SH3 domain of Nck can interact with NAK could provide a useful new approach for cloning this elusive kinase. On a cautious note, however, it may be worth pointing out that, although the current and previous data clearly demonstrate that NAK is regulated like a PAK, and despite the apparent cross-reactivity of certain anti-PAK antisera with NAK (Nunn and Marsh, 1996; Sawai *et al.,* 1996), it may still not be possible to formally exclude the possibility that NAK could also be an unrelated downstream kinase, regulated by one of the known PAKs.

The relevance of NAK for the virulence of HIV or SIV is currently not clear. The study by Wiskerchen and Cheng-Mayer (1996) concluded, based on mutations affecting the RR and PxxP motifs, as well as other residues (such as Pro^{69}), that NAK association is important for Nef-induced infectivity enhancement of HIV-1 particles in a single-round infection assay, by observing an intermediate phenotype between that of Nef(+) and Nef(−) HIV-1 strains. In contrast, it has been reported, based on transfer of a NAK-negative phenotype into SF2 Nef by substituting its carboxyl-terminal third with a sequence from another molecular clone of HIV-1, that NAK binding would be dispensable for Nef function in the same assay (Luo *et al.,* 1997).

Luciw and colleagues have addressed the pathophysiologic relevance of the Nef/NAK complex by infecting a large number of rhesus macaques with SIV strains carrying mutations in the double-arginine motif (RR-to-LL) (Sawai *et al.,* 1996) or in the PxxP motif (PxxP-to-AxxA) (Khan *et al.,* 1998). In both cases, the infection of most animals initially closely resembled that by a Nef-deleted strain, and high viral loads and disease progression seen later in some animals were associated with genotypic and phenotypic (NAK positive) reversion of the mutations. However, it is easy to imagine how the effects of these mutations on Nef function could be more pleiotropic than anticipated. On the other hand, contradicting results were observed in another study, which reported disease progression in two macaques before a significant proportion (>10%) of the input virus showed reversions of the PxxP-to-AxxA mutations in their Nef genes (Lang *et al.,* 1997). Furthermore, it is not clear how directly the results from the SIV/rhesus macaque model can be applied to the role of Nef in the pathogenesis of HIV-1 infection. For example, it has been noted in studies addressing the effects of Nef on cellular signal transduction pathways that HIV-1 Nef is functionally more strictly dependent on its PxxP motif than SIV Nef (Iafrate *et al.,* 1997).

Nevertheless, despite such contradictory observations, it can be concluded that NAK association represents a potentially important property common to many divergent Nef isolates and a possible link to important cellular signaling cascades and therefore clearly warrants more a detailed characterization by future investigations.

MATERIALS AND METHODS

Cells and DNA transfection

293T, a human embryonic kidney fibroblast-derived cell line (ATCC), was grown in Dulbecco's modified Eagles's medium (DMEM, Hyclone) containing 10% fetal calf serum (FCS), 2 mM L-glutamine, and 100 units/ml of penicillin and streptomycin. Plasmid DNA to be transfected was mixed with 62 μl of 2 M $CaCl_2$ and sterile water up to 500 μl. An equal volume of 2× HEPES solution [280 mM NaCl, 1.5 mM Na_2HPO_4, 55 mM N-2-hydroxyethylpiperazine-*N′*-2-ethanesulfonic acid (HEPES), pH 7.0] was added, followed by immediate dispersed onto 293 cells (splitted 1:3 24 h earlier) growing in complete medium on a 10-cm dish. Fresh medium was changed for the cultures 16 h after the transfection.

Plasmids and mutagenesis

The eukaryotic expression plasmids for the previously described (R71, T71, PA-1; Saksela *et al.,* 1995) or new Nef mutants were all constructed by inserting Nef allele sequences between the *Eco*RI and *Sal*I sites of the CMV-driven vector pcDNA3 (Invitrogen). The new mutant alleles were derived by standard PCR-based techniques using Pfu polymerase (Stratagene) and specific oligonucleotides (Gibco-BRL) and confirmed by sequencing (ABI Prism 310). The MBP-Hck-SH3 plasmid was made by inserting a fragment encoding for a 56-aa Hck polypeptide fragment (starting: IVVA . . . , ending: . . . VDSL) into pMAL-S, a modified version of pMAL-c2 (New England Biolabs) with Stop codons after the polylinker. Plasmids for expression of $cdc42^{V12}$, Nck, myrNck-SH3–2 have been described before (Lu *et al.,* 1997).

Recombinant protein production

Expression and purification of GST and MBP fusion proteins in *Escherichia coli* BL21 were carried out as suggested by the suppliers of these systems (Pharmacia and New England Biolabs, respectively). After specific elution from their respective affinity beads, the fusion proteins were concentrated and changed into the desired buffers by successive rounds of microconcentration using Centrex UF2 columns (Schleicher & Schuell).

The buffer of the GST-Nef proteins was changed to HBS (10 mM HEPES, pH 7.4; 150 mM NaCl; 3 mM EDTA) containing 0.005% (v/v) surfactant P20 and 1 mM dithiotreitol (DTT), (HBS-plus) in which they were subjected to thrombin cleavage (1 U thrombin/0.2 mg fusion protein) for 4–6 h at 37°C in HBS. The MBP proteins were biotinylated using the EZ-Link Sulfo-NHS-LC-Biotin reagent, as suggested by the manufacturer (Pierce), except for using less biotin (molar ratio of protein:biotin was 1:2), and subjected to multiple additional rounds of microconcentration to remove any free biotin.

Biacore measurements

Surface plasmon resonance (SPR) experiments were carried out using a Biacore X apparatus (Pharmacia Biosensor, Uppsala). An SA biosensor chip (Pharmacia Biosensor) with pre-immobilized streptavidin was coated with biotinylated MBP (to the reference channel) or MBP-Hck-SH3 (to the test channel) proteins (100 ng/ml in HBS-plus) by serial short injections (flow rate of 5 μl/min). The attachment of the ligands was monitored by the changes in the refractive index and was set to $\sim$2200 (MBP-Hck-SH3) and $\sim$2000 (MBP) response units (RU), corresponding to the relative difference in their molecular weights. After immobilization of the biotinylated ligands, the chip was subjected to three rounds of preregeneration cycles, which were subsequently applied once between each Nef injection. One regeneration cycle consisted of successive 1-min pulses (flow rate of 5 μl/min) of pH 2.2 glycine buffer, 0.05% SDS, and 4 M urea. Some loss of the refractive index was observed during the first and the second cycles but no longer during the third cycle of this preregeneration treatment. The injections of different Nef protein were done using concentrations ranging from 4.0 to 0.0125 μM, with a flow rate of 5 μl/min at 25°C in HBS-plus. Each chip was used for approximately 50 Nef injections, during which no loss of the immobilized ligand or the capacity of the chip to bind to a standard solution of R71 Nef was observed. The sensorgrams, in which the refractive index values from the reference channel were subtracted [to give corrected relative resonance units (cRU)], were analyzed using BIAevaluation (v3.0) software (Pharmacia Biosensor). The Scatchard plots and line fitting was done with Excel (Microsoft) and were based on values from the sensorgrams at a 20-min postinjection timepoint.

Immunoprecipitation, IVKA, and Western blotting

The sheep polyclonal anti-Nef antiserum was kindly provided by Mark Harris (University of Leeds, UK). Mouse monoclonal anti-Nef antibodies (2A3, 3A2, 6.2, 2H12, 3E6, 3F2.1, 3D12, and 2F2) directed against different regions of Nef were kindly provided by Kai Krohn from our institute. The rabbit polyclonal anti-PAK antibody (N-20) raised against the N-terminal region of rat PAK1 was purchased from Santa Cruz Biotechnologies Inc. (Santa Cruz, CA).

Forty-eight hours after transfection the cells were washed once with ice-cold phosphate-buffered saline (PBS) and lysed in kinase assay lysis buffer (50 mM HEPES, pH 7.4; 150 mM NaCl; 10% glycerol; 1% Triton X-100; 1 mM EGTA; 1.5 mM $MgCl_2$; 10 mM NaF; 1 mM sodium orthovanadate; 1 mM phenylmethyl-sulfonyl fluoride (PMSF); and 10 μg/ml approtinin). Three microliters of the anti-Nef serum or 0.3 μg of the anti-PAK antibody was incubated with the lysates (1 mg of total cellular protein) for 2 h at $+4$°C, followed by 30-min agitation in the presence of protein A–Sepharose beads (Sigma). The beads were subsequently washed three times with kinase assay lysis buffer and splitted for IVKA and immunoblotting.

For IVKA, the beads were subjected to two additional washes with IVKA buffer (50 mM HEPES, pH 7.4, and 5 mM $MgCl_2$). ^{32}[P]-γ-ATP (2.5 μCi) was added to the bead-bound immunocomplexes (in 100 μl of IVKA buffer) and incubated at $+37$°C for 20 min. The beads were washed with ice-cold PBS and boiled for 2 min in SDS–PAGE sample buffer. The phosphorylated proteins were separated by SDS–PAGE, and visualized by autoradiography.

For immunoblotting, the beads were boiled in Laemmli sample buffer, the associated proteins separated by SDS–PAGE, and transferred onto nitrocellulose membranes. The membranes were blocked for 45 min in PBS–Tween (PBS containing 0.05% Tween 20), plus 3% bovine serum albumin (BSA), and 0.05% NaN_3, followed by a 2-h incubation with a mixture of monoclonal mouse anti-Nef antibodies in PBS–Tween. After three washes with washing buffer (PBS plus 0.2% Tween 20), the membranes were incubated for 30 min with 1:3000 anti-mouse IgG (DAKO) in PBS–Tween, washed three times, incubated for 20 min with streptavidin-conjugated horseradish peroxidase (Amersham) diluted 1:5000 in PBS–Tween, washed three times again, and developed by ECL (Amersham) according to manufacturer's instructions.

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