Activation of PAK by HIV and SIV Nef: importance for AIDS in rhesus macaques

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Background: The primate lentiviruses, human immunodeficiency virus types 1 and 2 (HIV-1 and HIV-2) and simian immunodeficiency virus (SIV), encode a conserved accessory gene product, Nef. *In vivo*, Nef is important for the maintenance of high virus loads and progression to AIDS in SIV-infected adult rhesus macaques. In tissue culture cells expressing Nef, this viral protein interacts with a cellular serine kinase, designated Nef-associated kinase.

Results: This study identifies the Nef-associated kinase as a member of the p21-activated kinase (PAK) family of kinases and investigates the role of this Nef-associated kinase *in vivo*. Mutants of Nef that do not associate with the cellular kinase are unable to activate the PAK-related kinase in infected cells. To determine the role of cellular kinase association in viral pathogenesis, macaques were infected with SIV containing point-mutations in Nef that block PAK activation. Virus recovered at early time points after inoculation with mutant virus was found to have reverted to prototype Nef function and sequence. Reversion of the kinase-negative mutant to a kinase-positive genotype in macaques infected with the mutant virus preceded the induction of high virus loads and disease progression.

Conclusions: Nef associates with and activates a PAK-related kinase in lymphocytes infected *in vitro*. Moreover, the Nef-mediated activation of a PAK-related kinase correlates with the induction of high virus loads and the development of AIDS in the infected host. These findings reveal that there is a strong selective pressure *in vivo* for the interaction between Nef and the PAK-related kinase.

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Introduction

The *nef* gene of human immunodeficiency virus types 1 and 2 (HIV-1 and HIV-2) and simian immunodeficiency virus (SIV) encodes a 27–34 kDa, myristylated and phosphorylated protein that is expressed in the early stage of the viral replication cycle (reviewed in [1–3]). Although Nef is dispensable for productive infection in T-cell lines, this viral accessory protein plays an important role in the induction of acquired immune deficiency syndrome (AIDS) in SIV-infected rhesus macaques [4]. Studies addressing Nef function in tissue culture cells [5–7], transgenic mice [8] and SIV-infected macaques [9] suggest that this viral protein alters a cell signalling pathway(s). In T-cell cultures, Nef decreases the levels of the CD4 antigen on the cell surface [10–12] and increases virion infectivity [13–15] (reviewed in [1–3]). In transgenic mice expressing HIV-1 Nef, thymocytes display altered cell activation responses [8]. Rhesus macaques infected with SIVmac239nefYE, a clone containing two point mutations that generate a novel SH2-like domain near the amino terminus of Nef, exhibit an acute, fatal, gastrointestinal tract disease accompanied by T-cell proliferation in several lymphoid organs [9]. This mutant *nef* also transforms murine NIH3T3 cells *in vitro* [9]. Taken together, these observations imply that Nef has a role in perturbing a cell-activation pathway(s) which is presumed to influence the level of viral replication in the host and/or cause dysfunction of cells in the immune system.

An important approach to study Nef function(s) has been to identify and characterize cellular proteins that interact with this viral protein. Accordingly, Nef has been reported to bind CD4 [7,16], Lck [7,17], p53, mitogen-activated protein (MAP) kinase [7], the SH3 domains of Hck and Lyn [18,19], c-Src [9] and β-COP [20]. However, most of these reports lack experiments demonstrating that Nef and these various cellular proteins form complexes in permissive cells infected with virus; they also lack genetic analysis using viruses bearing mutations in *nef* that would establish the significance of such protein–protein interactions. To address these limitations, we have previously shown that Nef expressed in HIV-1-infected lymphocytes associates with a cellular serine kinase (Nef-associated kinase) which phosphorylates two cellular proteins, p62 and p72, that co-immunoprecipitate with Nef [21]. Additionally, mutation

analysis demonstrated that amino-terminal myristylation and the central conserved domain of Nef were important for the Nef-associated kinase activity [22].

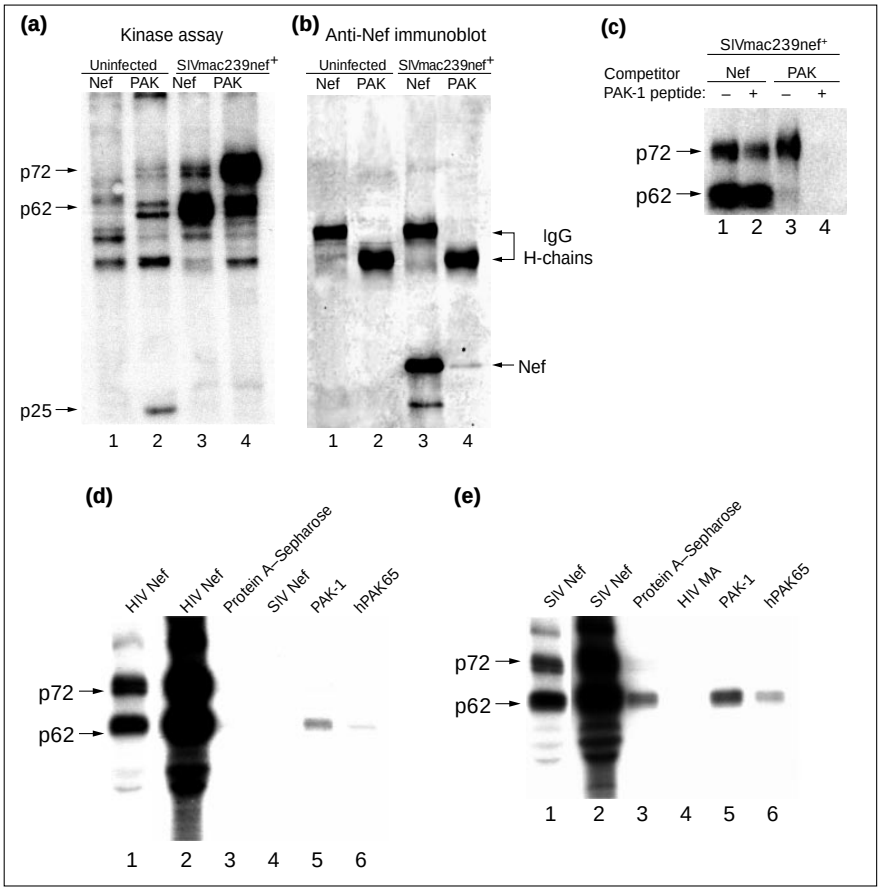
Results

Characterization of the Nef-associated kinase

To identify the cellular kinase activity that associates with Nef, we screened numerous commercially available antibodies to known signal transduction molecules for their ability to recognize two cellular proteins, p62 and p72, that coimmunoprecipitate with Nef from lymphoid cells infected with HIV-1 or SIV. During this screening process, an antibody directed against a p21-activated kinase (PAK) was found to immunoprecipitate proteins of 62 and 72 kDa in an *in vitro* kinase assay. The PAKs are highly conserved, ubiquitous serine kinases important for the transduction of external signals to the nucleus [23–28]. These kinases, ranging in size from 62 to 70 kDa, become activated and autophosphorylate after they bind the p21 Rho-like GTP-binding proteins, Rac-1 and Cdc42Hs [23–31].

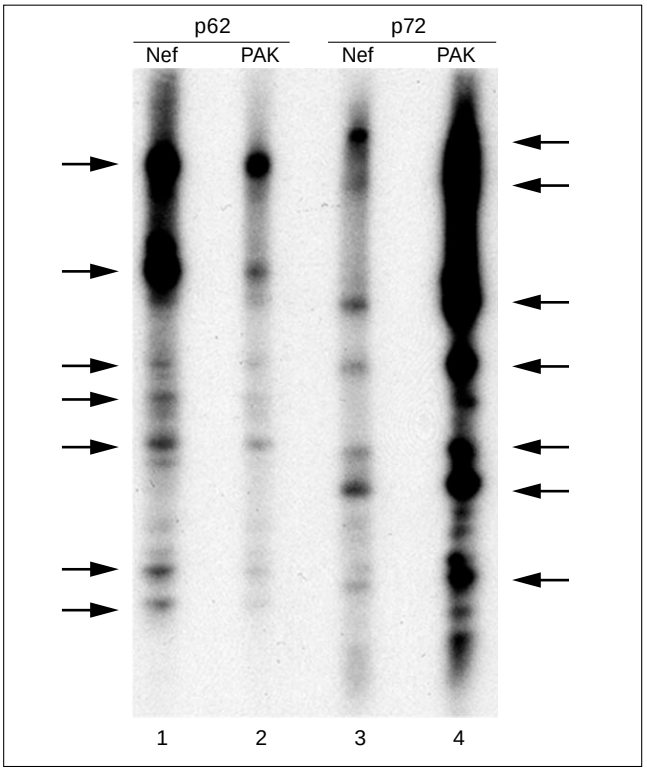
A polyclonal antiserum specific for the amino-terminal 20 amino acids of PAK-1 from rat brain was used to immunoprecipitate PAK from uninfected CEMx174 cells and cells chronically infected with SIVmac239nef$^+$ (a molecular SIV clone containing a prototype *nef* gene). *In vitro* kinase assays performed on these immunoprecipitates from infected cells revealed two phosphorylated proteins of 62 and 72 kDa

Figure 1



Association between Nef and a PAK-related kinase. (a) An *in vitro* kinase assay was performed on anti-SIV Nef (lanes 1 and 3) and anti-PAK (lanes 2 and 4) immunoprecipitates from uninfected (lanes 1 and 2) and SIVmac239nef$^+$-infected (lanes 3 and 4) CEMx174 extracts, as previously described [21]. Nef was immunoprecipitated with a monoclonal antibody directed against SIV Nef (17.2) and PAK was immunoprecipitated with a polyclonal serum directed against the amino-terminal 20 amino acids of rat PAK-1. Proteins were separated by SDS–PAGE on a 12 % gel and transferred to PVDF membranes. Radiolabeled proteins were visualized by autoradiography. The positions of p62, p72 and p25 are indicated by arrows on the left. Phosphorylated bands in the 46 kDa range were non-specific in these immunoprecipitations. (b) Immunological detection of Nef in anti-PAK immunoprecipitates. The PVDF filter from (a) was probed with the anti-Nef monoclonal antibody (17.2) as described in Materials and methods. The positions of Nef and immunoglobulin G (IgG) heavy (H) chains are indicated on the right. (c) Specificity of the anti-PAK antibody. Kinase assays were performed on anti-Nef (lanes 1 and 2) and anti-PAK (lanes 3 and 4) immunoprecipitates that had been preincubated with (+, lanes 2 and 4) or without (–, lanes 1 and 3) a competitor peptide corresponding to the amino terminus of PAK-1 that is recognized by the anti-PAK antiserum. (d) Re-immunoprecipitation of PAK from HIV-1 Nef immunoprecipitates. A kinase assay was performed on a Nef immunoprecipitate from HUT78 cells chronically infected with the HIV-$1_{SF11114}$ strain (lanes 1 and 2). Proteins associated with Nef were released from the Nef immunoprecipitates with kinase extraction buffer [21] containing 0.1 % SDS. The supernatant was subjected to two successive incubations with protein A–Sepharose; lane 3 represents the pellet from the second protein A–Sepharose clearance. The supernatant was split three ways and immunoprecipitated with anti-SIV Nef control (lane 4) antibodies, anti-PAK-1 (lane 5) antibodies, or anti-human PAK65 (hPAK65, lane 6) antibodies. Lanes 2–6 represent a 5 day autoradiographic exposure; lane 1 represents an overnight exposure of lane 2. (e) Re-immunoprecipitation of PAK from SIV Nef immunoprecipitates. A PAK re-immunoprecipitation analysis was performed on a SIV Nef immunoprecipitate from CEMx174 cells chronically infected with SIVmac239nef$^+$ as described in (d): lanes 1 and 2, SIV Nef immunoprecipitates; lane 3, second protein A–Sepharose clearance; lane 4, anti-HIV-1 matrix protein (MA) control; lane 5, anti-PAK-1; lane 6, anti-human PAK65 (hPAK65). Lanes 2–6 represent a 5 day autoradiographic exposure; lane 1 represents an overnight exposure of lane 2.

Figure 2



Phosphopeptide maps of p62 and p72. Partial chymotryptic digestion was performed on bands corresponding to p62 (lanes 1 and 2) and p72 (lanes 3 and 4) from SIV Nef (lanes 1 and 2) and PAK (lanes 2 and 4) immunoprecipitates. The arrows indicate the positions of the major partial peptide cleavage products for p62 (left side) and p72 (right side).

(Fig. 1a, lane 4), which are hyperphosphorylated forms of the 62 and 72 kDa proteins observed in PAK immunoprecipitates from uninfected cell lines (Fig. 1a, lane 2). Importantly, these phosphorylated proteins were similar in size to the two proteins (p62 and p72) that co-immunoprecipitated with Nef (Fig. 1a, lane 3). In the anti-Nef immunoprecipitate, p62 and p72 were the major and minor phosphorylated species, respectively (Fig. 1a, lane 3), whereas p72 was the major and p62 was the minor phosphorylated species in the anti-PAK-1 immunoprecipitate (Fig. 1a, lane 4). In uninfected CEMx174 cells, a phosphorylated protein migrating slightly faster than p62, and a phosphorylated protein of 25 kDa (p25) were detected in the anti-PAK-1 immunoprecipitate (Fig. 1a, lane 2). Neither PAK nor p25 was found in immunoprecipitates from infected cells (Fig. 1a, lane 4). The identity and significance of p25 for Nef-mediated PAK activation remain to be elucidated.

To determine whether the hyperphosphorylation of p72 in SIV-infected lymphocytes was PAK-specific, kinase assays were performed on Nef and PAK immunoprecipitates using antibodies that were preincubated with a PAK-1 competitor peptide (Fig. 1c). This peptide represents the epitope recognized by the anti-PAK-1 antibody. Because p72 was not phosphorylated after anti-PAK sera was preincubated with the PAK-1 competitor peptide, the hyperphosphorylation of p72 in SIV-infected cells was indeed PAK-specific.

Identification of the Nef-associated kinase as a PAK-related kinase

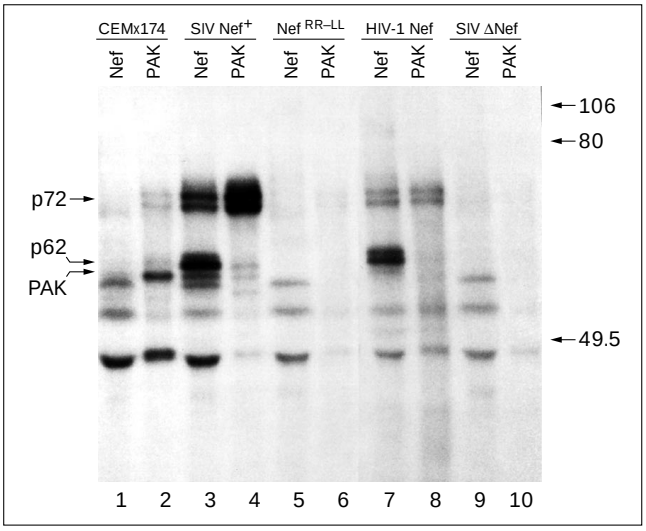
To determine whether Nef and PAK associate in infected cells, an immunoblot analysis (Fig. 1b) using a Nef-specific antibody probe was performed on immunoprecipitates produced with anti-Nef and anti-PAK-1 antibodies (Fig. 1a). Nef was detected in both the anti-Nef and anti-PAK-1 immunoprecipitates from cells infected with SIVmac239nef$^+$ (Fig. 1b, lanes 3 and 4). Nef was not detected in uninfected CEMx174 cells (Fig. 1b, lanes 1 and 2). Using the PAK-1-specific antibody to probe the anti-Nef and anti-PAK-1 immunoprecipitates described above, we detected p62 in the anti-PAK-1 immunoprecipitates from uninfected and infected cells (data not shown). However, it was difficult to demonstrate the presence of p62 in the anti-Nef immunoprecipitate of cells infected with SIVmac239nef$^+$ (data not shown); this could result from the small proportion of PAK-1 (or a PAK-related protein) that is associated with Nef in infected cells. These results indicate that Nef forms a complex with PAK-1 (or a PAK-related protein) in infected lymphoid cells.

To confirm that Nef associates with a PAK-related kinase, a re-immunoprecipitation analysis was performed on a HIV-1 Nef immunoprecipitate (Fig. 1d). Anti-PAK antibodies recognized p62, which was released from the HIV-1 Nef immunoprecipitates (Fig. 1d,e). Similar results were obtained with SIV Nef immunoprecipitates (Fig. 1e). Note that p62 was not observed in the lane containing the non-specific antibody control (Fig. 1d,e; lane 4). These results confirm data from a recent study [32] showing that a HIV-1 Nef fusion protein expressed by a retroviral vector in T cells associates with a member of the PAK family of proteins. Taken together, these studies show that Nef associates with a PAK-related protein in cells infected with HIV-1 or SIV.

Comparison of p62 and p72 by analysis of phosphopeptide maps

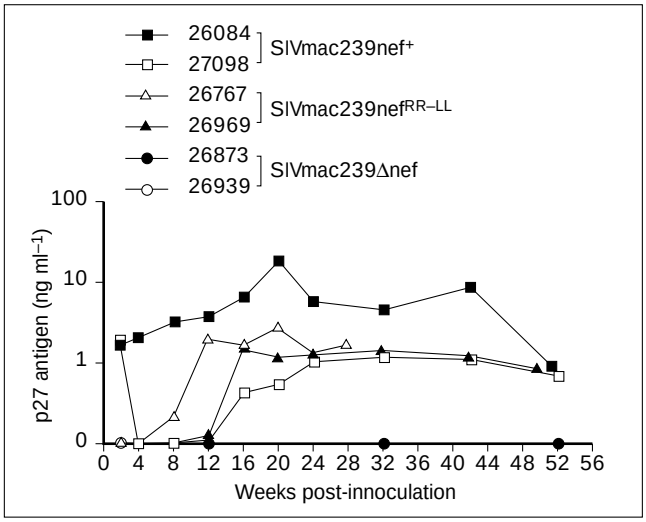
The relationship between p62 and p72 from anti-Nef and anti-PAK-1 immunoprecipitates was investigated by phosphopeptide mapping. Proteins immunoprecipitated with anti-Nef and anti-PAK-1 antibodies were radiolabeled in an *in vitro* kinase assay and subjected to SDS–PAGE; ^{32}P-labeled bands corresponding to p62 and p72 were isolated and subjected to partial protease cleavage with chymotrypsin. The phosphopeptide maps of p62 from anti-SIV Nef and anti-PAK-1 immunoprecipitates were similar,

Figure 3



Analysis of HIV and SIV Nef mutants for activation of PAK. Kinase assays were performed on anti-SIVmac239 Nef (Nef, lanes 1,3,5 and 9), anti-HIV-1_{SF2} Nef (lane 7) and anti-rat PAK-1 (PAK, lanes 2,4,6,8 and 10) immunoprecipitates from uninfected CEMx174 cells (lanes 1 and 2), CEMx174 cells chronically infected with SIVmac239nef^{+} (SIV Nef^{+}, lanes 3 and 4), SIVmac239nef$^{RR–LL}$ (Nef$^{RR–LL}$, lanes 5 and 6) or SIVmac239Δnef (SIV ΔNef, lanes 9 and 10) or HUT78 cells chronically infected with HIV-1_{SF2} (HIV-1 Nef, lanes 7 and 8). The positions of migration of PAK from uninfected cells, and of p62 and p72, are indicated on the left.

Figure 4



Viral loads in SIV-infected rhesus macaques. The levels of SIV p27gag were measured by enzyme-linked immunosorbent assay (ELISA) from the plasma of monkeys infected with SIVmac239nef^{+} (26084 and 27098), SIVmac239nef$^{RR–LL}$ (26767 and 26969), or SIVmac239Δnef (26873 and 26939) at the indicated weeks post-inoculation.

indicating that p62 associated with SIV Nef and PAK-1 was highly related (Fig. 2, lanes 1 and 2). Additionally, the phosphopeptide maps of p72 from anti-SIV Nef and anti-PAK-1 immunoprecipitates were also similar (Fig. 2, lanes 3 and 4). However, it is important to note that the partial proteolytic cleavage maps for p62 and p72 are distinct (Fig. 2). Similar results were obtained when phosphopeptide maps of p62 and p72 from cells infected with SIV or HIV-1 were compared after partial proteolysis with staphylococcal V8 protease (data not shown). Taken together, these results indicate that p62 and p72 are different molecules.

Mutants of Nef defective for kinase association do not activate PAK

Previously, we have shown that mutation of two highly conserved arginine residues at positions 137 and 138 of Nef from SIVmac239 abrogates association with the Nef-associated kinase in transient expression assays [22]. These point mutations and a large deletion of *nef* [4] were introduced into the molecular clone of SIVmac239nef^{+} to produce the two clones SIVmac239nef$^{RR–LL}$ (in which the arginine residues are replaced by leucines) and SIVmac239Δnef, respectively. The synthesis and stability of the $R^{137}R^{138}$–LL mutant Nef in cells infected with SIVmac239nef$^{RR–LL}$ were indistinguishable from those of the prototype Nef in cells infected with SIVmac239nef^{+} (data not shown), but the ability of the mutant Nef to associate with and activate PAK was abrogated (Fig. 3, compare lanes 3 and 4 with lanes 5 and 6). The cellular proteins p62 and p72 were phosphorylated by the Nef-associated kinase in cells chronically infected with SIVmac239nef^{+} (Fig. 3, lanes 3 and 4) but not in cells infected with SIVmac239nef$^{RR–LL}$ (Fig. 3, lanes 5 and 6) or SIVmac239Δnef (Fig. 3, lanes 9 and 10). Nef was not detected by immunoblot analysis of PAK immunoprecipitates from cells infected with SIVmac239nef$^{RR–LL}$ or SIVmac239Δnef (data not shown). Immunoblot experiments using anti-PAK-1 antibodies revealed that equal amounts of PAK were detected in all PAK immunoprecipitates (data not shown). Interestingly, infection of CEMx174 cells with Nef mutants results in hypophosphorylation of p72 and a loss of basal PAK activity (compare lanes 6 and 10 with lane 4). The mechanism by which this loss of PAK activation occurs during virus infection has not been elucidated. Nevertheless, the hyperphosphorylation of p72 is a marker for the Nef-mediated activation of the PAK-related kinase. These data, based on a combination of genetic and biochemical experiments, demonstrate that the ability of Nef to activate the PAK-related kinase in productively infected lymphoid cells is linked to its ability to bind the PAK-related kinase.

Because Nef from T-cell lines infected with HIV-1 strain SF2 (HIV-1_{SF2}) interacts with the Nef-associated kinase [21], we examined the activation of the PAK-related kinase in cells infected with HIV-1 (Fig. 3, lanes 7 and 8). *In vitro* kinase assays revealed that the PAK-related kinase

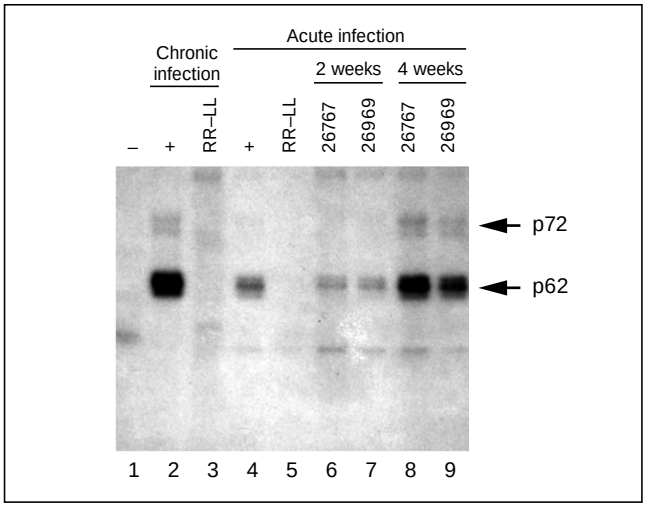
was activated in the T-cell line HUT78 chronically infected with HIV-1_{SF2}. Thus, the property of Nef-mediated activation of the PAK-related kinase in lymphoid cells is shared by HIV-1 and SIV.

In vivo analysis of SIV Nef mutants

To examine the role of the association between Nef and the PAK-related kinase in viral pathogenesis, we compared virus load and disease potential of SIVmac239nef$^{RR–LL}$ with those of the non-pathogenic clone SIVmac239Δnef and the pathogenic clone SIVmac239nef^{+} in juvenile rhesus macaques. Figure 4 shows that plasma virus load in macaques infected with SIVmac239nef^{RR-LL} (macaques 26767 and 26969) or the non-pathogenic clone SIVmac239Δnef (macaques 26873 and 26939) was below the level of detection in the first 4 weeks after infection, whereas viral antigen was readily detected in this acute stage of infection in plasma from macaques inoculated with SIVmac239nef^{+} (macaques 26084 and 27098). After 4 weeks, there was an increase in the level of viral antigen in plasma from macaques inoculated with SIVmac239nef$^{RR–LL}$. However, in macaques infected with SIVmac239Δnef, plasma antigen remained below the level of detection (Fig. 4).

The increase in plasma virus load in the two macaques after 4 weeks of infection with SIVmac239nef$^{RR–LL}$ suggested that the mutation in *nef* may have reverted. At 2 and 4 weeks after inoculation, Nef was analyzed from virus isolated from the peripheral blood mononuclear cells (PBMCs) of both of these animals. Immunoprecipitation studies revealed a low level of Nef-associated kinase activity from virus recovered at 2 weeks (Fig. 5a, lanes 6 and 7). A striking enhancement in the ability of Nef to associate with the kinase was observed from virus collected at 4 weeks (Fig. 5a, lanes 8 and 9). Thus, the ability of Nef to associate with the kinase had been restored during the acute stage of infection in the two animals inoculated with SIVmac239nef$^{RR–LL}$. Furthermore, both animals exhibited a progressive increase in virus load (Fig. 4) and developed clinical signs of disease (Table 1). To determine the nature of the genetic changes in the viruses showing this phenotypic reversion, DNA was isolated from PBMCs collected from the two SIVmac239nef$^{RR–LL}$-infected macaques at 2 and 4 weeks after inoculation, and the *nef* gene was amplified by the polymerase chain reaction (PCR) and sequenced. All *nef* clones from the 2 week samples had the mutant leucine codons at positions 137 and 138. In contrast, the leucine (mutant) codon at position 138 had been replaced with a codon for arginine in most (19/20 PCR clones) *nef* sequences from both animals at 4 weeks (Table 2). Moreover, both leucine (mutant) codons were replaced by arginine codons at positions 137 and 138 in several (6/11) of the *nef* clones from macaque 26767. By 8 weeks, almost all (15/16) of the *nef* sequences had the prototype

Figure 5



Functional and genetic changes in Nef from macaques infected with SIVmac239nef$^{RR–LL}$, showing phenotypic reversion. A polyclonal anti-SIV Nef antibody was used to immunoprecipitate cellular extracts from uninfected CEMx174 cells (–, lane 1), or from CEMx174 cells that were either chronically infected (lanes 2 and 3) or acutely infected (lanes 4 and 5) with SIVmac239nef^{+} (+, lanes 2 and 4), SIVmac239nef$^{RR–LL}$ (RR–LL, lanes 3 and 5), or with virus recovered 2 or 4 weeks after inoculation from the PBMCs of macaques 26767 (lanes 6 and 8) or 26969 (lanes 7 and 9). *In vitro* kinase assays were performed on all of the immunoprecipitates.

Table 1

Clinical summary

Monkey	Virus	Time of death	Number of CD4^{+} cells	Pathological findings
26767	SIVmac239nef$^{RR–LL}$	28 weeks	Decreased	Multiple malignant abdominal and spinal lymphonas, lymphadenopathy and pneumonia (*Pneumococcus carinii*)
26969	SIVmac239nef$^{RR–LL}$	50 weeks	Decreased	Wasting, diarrhea, lymphadenopathy, splenic lymphoid hyperplasia and multiple opportunistic infections (*Trichonomas*, *Giardia*, *Blastocystis hominis* and *Entamoeba coli*)
26084	SIVmac239nef^{+}	51 weeks	Decreased	Wasting, diarrhea, lymphadenopathy and pneumonia
27098	SIVmac239nef^{+}	87 weeks	Decreased	Wasting, diarrhea and lymphadenopathy

Table 2

Analysis of viral DNA sequences in macaque PBMCs

	Macaque 26969		Macaque 26767	
Time after inoculation	Number of clones	Sequence	Number of clones	Sequence
2 weeks	8	CTA CTA = L,L	8	CTA CTA = L,L
4 weeks	8	CTA CGA = L,R	4	CTA CGA = L,R
	1	CTA CTA = L,L	6	CGA CGA = R,R
			1	CCA CGA = P,R
8 weeks	7	AGA AGA = R,R	1	CGA CGA = R,R
	1	CTA CTA = L,L	7	AGA AGA = R,R

nef genes from macaques (26767 and 26969) infected with SIVmac239nef^{RR-LL} were sequenced from DNA isolated from PBMCs collected at 2, 4 and 8 weeks post-inoculation. The codons for amino-acid residues 137 and 138 are shown, and the amino acids they encode are indicated in single-letter code. Numbers indicate the number of *nef* clones with the respective sequence. The input virus, which was negative for cellular kinase association had the sequence CTA CTA, which encodes leucine (L) residues, at positions 137 and 138, whereas the corresponding sequence of the SIVmac239nef^{+} prototype virus was AGA AGA, which encodes arginine (R) residues.

Nef amino acids, with arginines at positions 137 and 138. Interestingly, the reversion of leucine at position 138 to arginine occurred before the leucine to arginine reversion at position 137 (Table 2). This pattern of genetic change suggested that the arginine at position 138 in the di-arginine motif is more important for Nef function. This finding is consistent with previous mapping studies with HIV-1_{SF2} Nef, in which the second arginine of the di-arginine motif (positions 109 and 110) was shown to be critical for the association between Nef and the cellular kinase [22]. Although the first arginine residue of the di-arginine motif was not critical for the Nef–kinase association (see macaque 26969 at the 4 week time point in Fig. 5a and Table 2), this residue may be important for maintaining an optimal structure for kinase association.

Clinical signs of disease were monitored to assess the pathogenic potential of these viruses. Macaques 26767 and 26969, which were inoculated with SIVmac239nef^{RR-LL}, and macaques 26084 and 27098, which received the prototype virus SIVmac239nef^{+}, developed simian AIDS and were euthanized at 28, 50, 51 and 87 weeks after infection, respectively (Table 1). Analysis of the cell-associated virus load in PBMCs and lymph nodes, and measurements of cell-free virus in plasma, revealed that all four animals contained high amounts of infectious virus in the period immediately prior to and at the time of necropsy (data not shown). Pathological and histopathological examination of several organs collected at necropsy confirmed that these macaques had signs of simian AIDS, including lymphadenopathy and infection with one or more opportunistic pathogens (Table 1). Control macaques inoculated with SIVmac239Δnef had low viral loads and no clinical abnormalities for an observation period of over one year (see also [4]). Taken together, the above results demonstrate that there is strong selective pressure for restoring the ability of Nef to activate PAK *in vivo*, and that activation of a PAK-related kinase correlates with the induction and maintenance of high viral loads and disease progression.

Discussion

Nef activates a PAK-related kinase

In this study, Nef from lymphoid cells infected with HIV-1 or SIV was shown to associate with and activate a cellular serine kinase in the PAK family. Nef forms a complex with a PAK or PAK-related protein in infected lymphoid cells, as demonstrated by the co-immunoprecipitation of Nef with PAK in PAK immunoprecipitates (Fig. 1b), the re-immunoprecipitation of PAK released from Nef immunoprecipitates (Fig. 1d,e), and the proteolytic mapping studies of p62 and p72 from Nef and PAK immunoprecipitates (Fig. 2). Furthermore, these findings are supported by genetic studies using a transdominant mutant of PAK65 that blocked Nef-mediated PAK activation in a transient expression assay (data not shown). Recently, a highly conserved motif in the amino-terminus of PAK65 has been shown to be responsible for interacting with the Rac1/Cdc42 GTPases; this regulatory domain is found in a number of cellular kinases [33]. Thus, it is possible that the Nef-associated kinase is either a known PAK, or a kinase that shares the amino terminus of PAK-1. Further studies are required to determine the exact relationship of p62 and p72 with the PAK family of serine kinases.

Nef-associated kinase and viral pathogenesis

This study demonstrates that a mutation in Nef, which abrogates the association with, and activation of, a PAK-related kinase, rapidly reverts to a PAK activation-positive phenotype and genotype *in vivo* (Fig. 5). Importantly, this reversion correlates with the induction of high viral loads (Fig. 4) and progression of infected macaques to disease (Table 1). Although a previous study demonstrated that reversion of a premature stop codon in the SIV Nef translation frame correlated with increased virus load and development of disease [4], our report links a specific biochemical function — the ability of Nef to associate with and activate a PAK — to induction of high viremia and pathogenesis. The precise relationship between virus load and the rate of disease progression in macaques infected with molecular clones of SIVmac is not clear. In a previous study, macaques infected with SIVmac239/nef-stop, a clone with a premature translational termination codon in *nef*, showed a similar range of times until death as macaques infected with SIVmac239nef^{+} [4,34]. Complex host factors (such as the genetics of the host's immune responses) as well as potential cofactors (such as opportunistic pathogens) can influence virus load and disease progression.

Activation and Nef functions

Perturbation of a key cell-activation pathway(s) could explain the seemingly disparate roles attributed to Nef, such as downregulation of cell-surface CD4 and enhancement of virion infectivity (reviewed in [1–3]). The effects of Nef on cellular activation may influence the levels of CD4 surface expression and/or transcription of lymphoid cell genes; such changes mediated by Nef may lead to dysfunction of helper T cells and subsequent immunodeficiency. The effects of Nef on infectivity may be a consequence of changes in phosphorylation of a viral structural protein(s) during particle assembly, which is a process presumed to involve components of intracellular architecture. For example, Nef activation of PAK may influence, either directly or indirectly, phosphorylation of the matrix protein (MA) in the viral core during virion morphogenesis. Modification of MA by phosphorylation is important for viral infectivity [35–37]. Additionally, the enhancement of virion infectivity and transcriptional activation of the provirus are expected to produce high virus load, which is associated with the depletion of CD4 cells and disease progression [38].

Nef structure and function

The di-arginine motif, which reverts *in vivo*, is located within the conserved central domain of Nef that is important for kinase association. The importance of the conserved central region of Nef for viral pathogenesis has also been highlighted by the finding that SIVmac clone 8, which encodes a Nef protein with a four amino-acid deletion, reverts from an attenuated to a pathogenic phenotype by the duplication of sequences flanking the deletion [39]. Another intriguing report, which focuses attention on the conserved central region of Nef, describes a group of long-term nonprogressors infected with a strain of HIV-1 that contains a deletion in this region ([40], see also [41]). Further studies are required to precisely define the domain of Nef responsible for PAK activation in the context of the structural model recently deduced by nuclear magnetic resonance spectroscopy [42] and X-ray crystallography [43]. It is intriguing to note that the X-ray crystallographic structure of Nef [43] suggests the importance of the second arginine in the central di-arginine pair of the conserved domain for both HIV-1 [22] and SIVmac239 (this study). Accordingly, knowledge of the structure and function(s) of the PAK(s) interacting with Nef will not only help to elucidate the key intracellular signalling events in the mechanism of viral pathogenesis, but also may be used to design novel drugs that inhibit virus replication and block progression to AIDS.

Conclusions

This study demonstrates that both HIV-1 and SIV Nef associate with and activate a PAK-related kinase in productively infected lymphocytes. Furthermore, mutations of Nef that abrogate cellular kinase association do not activate PAK. Using the SIV/rhesus macaque model for AIDS pathogenesis, we found that there is a strong selective pressure for the ability of Nef to associate with the PAK-related kinase. Moreover, the reversion of Nef from a PAK activation-defective to a PAK activation-positive phenotype correlates with the induction of high virus loads and disease progression in infected macaques. These results should facilitate the development of novel therapeutic approaches to inhibit HIV replication and prevent AIDS progression.

Materials and methods

Cells, virus and antibodies

CEMx174, a T-/B-hybrid human lymphoid cell line, permissive for replication of SIVmac clones, was obtained from J. Hoxie. CEMx174 cells chronically infected with SIVmac clones and HUT78 cell cultures chronically infected with HIV-1_{SF2} were derived as outgrowths of acute infection with the respective virus and were cultured as described previously [21]. Human rhabdomyosarcoma cells (RD-4), obtained from the ATCC, were maintained in Dulbecco's minimal essential medium containing 10 % fetal bovine serum (FBS), 1 % glutamine and 1 % penicillin–streptomycin (complete DMEM). HIV-$1_{SF11114}$, a primary isolate from a patient classified as a rapid progressor, was kindly provided by J.A. Levy. Polyclonal antisera directed against SIVmac239 Nef was prepared in rabbits (Babco) using recombinant Nef protein produced in *Escherichia coli* (a generous gift from C. Morrow). A monoclonal antibody directed against SIV Nef (17.2) was generously provided by K. Krohn. The rabbit anti-HIV-1_{SF2} Nef antibody was described previously [21]. The anti-rat PAK-1 (PAK) polyclonal antibody, recognizing the amino-terminal 20 amino acids, and the corresponding amino-terminal PAK-1 competitor peptide was purchased from Santa Cruz Biotechnology. The polyclonal antisera to human PAK65 (hPAK65) was generously provided by A. Abo [24]. A monoclonal antibody directed against HIV-1 MA, was obtained from the ATCC.

Construction of SIVmac239 proviral clones containing mutations in Nef

The $R_{138}R_{139}$–LL mutation, which substitutes arginine codons at positions 137 and 138 of Nef from SIVmac239 for leucine codons [22], and the Δnef mutation [4] were subcloned into the 3′ half of the SIVmac239 provirus. Recombinant SIVmac239nef$^{RR–LL}$ was produced by cotransfecting the 5′ and 3′ proviral halves of SIVmac239 into RD-4 cells and co-cultivating them with CEMx174 lymphoid cells as previously described [44]. Virus released into the culture supernatant was recovered after 7 days and titered by p27gag ELISA (Coulter).

Kinase assay, phosphopeptide mapping and immunoblot analysis

Cell extraction, immunoprecipitation, SDS–PAGE and the *in vitro* kinase assay were performed as described previously [21]. Procedures for partial proteolytic cleavage and phosphopeptide mapping have been described previously [45]. Chymotryptic digestions were performed on ^{32}P-labeled bands corresponding to p62 and p72 from Nef and PAK immunoprecipitates. For the immunoblot analyses, PVDF filters were washed four times with blocking buffer (Tris-buffered saline containing 0.1 % NP40, 0.1 % Tween-20 and 0.25 % gelatin) and probed with the anti-SIV Nef monoclonal antibody (17.2). After four washes with blocking buffer, the membranes were incubated with a secondary goat anti-mouse antibody conjugated with alkaline phosphatase (Southern Biotechnology Associates), washed four times with blocking buffer, and twice with 100 mM Tris–HCl (pH 9.5). A BCIP/NBT detection kit (Vector Laboratories) was used for detection.

PAK re-immunoprecipitation analysis

An *in vitro* kinase assay was performed on HIV-1 Nef immunoprecipitates from HUT78 cells chronically infected with HIV-$1_{SF11114}$ or on SIV

Nef immunoprecipitates from CEMx174 cells chronically infected with SIVmac239nef$^+$. The immune complexes were incubated with kinase extraction buffer (KEB, [21]) containing 0.1 % SDS for 1 h. The pellet was washed three times with KEB and resuspended in SDS-gel loading buffer (DB). To clear the supernatant of released IgG, the supernatant was incubated twice with protein A–Sepharose. The pellet from the second protein A–Sepharose clearance was washed twice with KEB and resuspended in DB. The supernatant from the second protein A–Sepharose clearance was divided equally into three tubes and incubated with either anti-SIV Nef (control), anti-PAK-1 or anti-hPAK65 antibodies. Immunocomplexes were collected after incubation with protein A–Sepharose, washed three times with KEB, and resuspended in DB.

Infection of rhesus macaques

All rhesus macaques (*Macaca mulatta*) in this study were housed in accordance with the American Association for Accreditation of Laboratory Animal Care Standards (A3433-01) at the California Regional Primate Research Center (CRPRC). Two colony-bred juvenile rhesus macaques free of simian type D retroviruses, SIV, and STLV were inoculated intravenously with 1000 $TCID_{50}$ ($TCID_{50}$: 50 % of the tissue culture infectious dose) of either SIVmac239nef$^+$ or the mutant viruses SIVmac239Δnef or SIVmac239nef$^{RR–LL}$. The titer of each virus in this study was measured in CEMx174 cells by end-point dilution as previously described [46]. Animals were monitored for weight loss, lymphadenopathy, splenomegaly and opportunistic infections; complete differential blood counts included enumeration of CD4$^+$ and CD8$^+$ T lymphocytes by flow cytometry. Infected macaques that became seriously ill were humanely euthanized and necropsied. Post-mortum examination included histological analysis of all organ specimens. Detection and quantitation of plasma antigenemia (levels of p27gag measured by ELISA), viremia and cell-associated virus load were performed as described previously [46,47].

Characterization of virus recovered from monkeys

Acute infections of CEMx174 cells (1×10^6) were performed using 1.0 ml of virus recovered from each monkey (macaques 26767 and 26969) at 2 and 4 weeks post-inoculation. After 5 days, infected cells were extracted for immunoprecipitation with an anti-SIV Nef serum and kinase assays were performed. DNA was isolated and purified from 400 μl of peripheral blood drawn from macaques 26767 and 26969 at 2, 4 and 8 weeks after infection with SIVmac239nef$^{RR–LL}$. This DNA served as a template for amplification of *nef* by PCR. In the first round (30 cycles) of PCR amplification the following 5′ and 3′ primers were used for *nef*: 5′–CCAGAGGCTCTCTGCGACCCTAC and 5′–AGAGGGCTTTAAGCAAGCAAGCGTG, respectively. For the second round (30 cycles), the 5′ primer was the same and the 3′ primer (5′–GCCTCTCCGCAGAGCGACTGAATAC) was used. PCR amplifications were carried out in a DNA Thermal Cycler (Perkin-Elmer Cetus) with *Taq* polymerase (Perkin-Elmer). A final product of 992 base-pairs that contained the full-length *nef* gene was produced. The PCR product was cloned into pCRII (Invitrogen) and the DNA was sequenced.

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