Human Immunodeficiency Virus Type 1 Nef Associates with a Member of the p21-Activated Kinase Family

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Although human immunodeficiency virus (HIV) Nef is essential for the induction of AIDS, its biochemical function has remained an enigma. In this study, HIV Nef protein is shown to associate with a serine-threonine kinase that recognizes histone H4 as a substrate, is serologically related to rat p21-activated kinase (PAK), and is specifically activated by Rac and Cdc42. These characteristics define the Nef-associated kinase as belonging to the PAK family. PAKs initiate kinase cascades in response to environmental stimuli, and their identification as a target of Nef implicates these signaling molecules in HIV pathogenesis and provides a novel target for clinical intervention.

While lentiviral Nef is not essential for viral replication, it dramatically influences the clinical outcome of infection of macaques by simian immunodeficiency virus and possibly of humans by human immunodeficiency virus (HIV). Nef expression is required for the development of immunodeficiency in the simian immunodeficiency virus infection of adult macaques (27), and several patients who have become infected with a Nef-deleted HIV appear to be free of AIDS (14, 29). As an early viral gene, *nef* is expressed at high levels in infected cells, representing nearly three quarters of total viral transcripts (48). In vitro, increased HIV replication correlates with *nef* expression (16, 38, 51, 58), a positive effect which can be provided in *trans* (3, 39) and suggests a role for Nef in the late viral process of HIV particle maturation. Expression of Nef in cells causes several phenotypic changes, including the downmodulation of CD4 (20, 22), inhibition (34, 43) or induction (5, 46, 50) of a T-cell-activated state, inhibition of inositol triphosphate receptor function (13), and either inhibition (1, 33, 44), no effect on (23, 28), or activation (42) of the HIV long terminal repeat. This diverse array of effects suggests that Nef can affect multiple cellular pathways, perhaps in a manner specific for a given cell type. Although the biochemical function of Nef remains unclear, it has been shown that Nef is a myristoylated protein associated with the cytoplasmic face of the plasma membrane (17) and that this modification, and presumably the membrane association, is essential for its biological activity (2, 58).

Two previous reports have demonstrated that Nef associates with a serine kinase (45, 49), although further characterization of the Nef-associated kinase has remained elusive. In agreement with this previous finding, we report here that a novel SF2 Nef fusion protein associates with an approximate 65-kDa protein that serves as a substrate for the Nef-associated kinase activity in T cells. Furthermore, we demonstrate that the 65-kDa protein is an ATP-binding protein, that the kinase activity is inhibited by staurosporine but not other serine-threonine or tyrosine kinase inhibitors, that the kinase activity has specificity for histone H4, and that the 65-kDa protein is serologically and functionally related to the p21-activated kinases (PAKs), known mediators of cellular signal transduction.

MATERIALS AND METHODS

Cells and DNAs. The human T-cell lymphoma line VB was obtained from the AIDS Research and Reference Reagent Program (Division of AIDS, National Institute of Allergy and Infectious Diseases, National Institutes of Health), and the amphotropic retrovirus packaging cell line PA317 was obtained from the American Type Culture Collection (CRL 9078). Nef-FLAG DNA was generated by PCR from a plasmid DNA clone of HIV-1 SF2 (provided by J. Levy, University of California San Francisco) by using synthetic primers corresponding to DNA sequences upstream of *nef* and the carboxy terminus of *nef*, to include sequences encoding the FLAG peptide sequence, DYKDDDDK, following the *nef* terminal cysteine codon. After digestion with the restriction endonuclease *Bgl*II, which cuts at a site corresponding to the native HIV sequence 5' to *nef* and at a sequence incorporated into the synthetic C-terminal primer, this DNA was cloned into the retroviral expression vector pLXSN (20). Recombinant amphotropic virus and transduced VB cells were generated as described previously (47). G418-resistant VB cells expressing Nef-FLAG were enriched by removing residual CD4 positive cells by using anti-CD4 coated magnetic beads (Dynal Co., Lake Success, N.Y.).

Plasmid DNAs encoding activated forms of the small GTPases RhoA, Rac1, and Cdc42Hs in the pcDNA3 expression vector (Invitrogen, San Diego, Calif.) were provided by S. Gutkind, National Institutes of Health (12).

Cell lysis and immunoprecipitation. VB cells and VB cells transduced with Nef-FLAG (2×10^6) were labeled for 3 h with Trans 35 S-label (ICN, Costa Mesa, Calif.) as described previously (47) and extracts were prepared in lysis buffer {1% Triton X-100, 10 mM PIPES [piperazine-*N,N'*-bis(2-ethanesulfonic acid)] [pH 7.2], 120 mM KCl, 30 mM NaCl, 5 mM MgCl₂, 10% glycerol, protease inhibitors} as described elsewhere in the analysis of Nef-associated proteins in vitro (24). FLAG-epitope tagged proteins were immunoprecipitated from pre-cleared lysates by incubating 1 h at 4°C with monoclonal antibody M2 coupled to agarose beads (10 μ l) (Eastman Kodak, Rochester, N.Y.), which had been preadsorbed for 30 min at 4°C with unlabeled VB cell extract. Beads were collected by centrifugation and washed three times with lysis buffer, and the immunoprecipitated proteins were analyzed by reducing sodium dodecyl sulfate–10% polyacrylamide gel electrophoresis (SDS-PAGE). Radiolabeled proteins were detected by autoradiography with preflashed Kodak XAR-5 film. Quantitative analysis of Nef-FLAG-associated proteins was performed with a Molecular Dynamics Phosphorimager (Sunnyvale, Calif.).

Kinase assays. Nef-FLAG and associated proteins, immunoprecipitated from 1 ml of unlabeled cell extracts (50 million cells) and washed as described above, were incubated in 100 μ l of kinase buffer (1% Triton X-100, 0.1 M NaCl, 5 mM MgCl₂, 10 mM PIPES [pH 7.2]) containing 2 μ Ci of [γ - 32 P]ATP (3,010 Ci/mmol) for 5 min at room temperature and, in the absence of added substrates, subjected to an additional wash in 1 ml of lysis buffer prior to analysis by SDS-PAGE (10% polyacrylamide). Assays including added substrate (0.1 mg/ml), either myelin basic protein (Sigma, St. Louis, Mo.), or histones (Boehringer Mannheim, Indianapolis, Ind.), were performed in a reaction volume of 20 μ l with 5 μ Ci of [γ - 32 P]ATP and 10 μ M ATP for 10 min at room temperature after further washing the immunoprecipitated proteins once with 0.5 M LiCl, 10 mM Tris (pH 7.4), and once with kinase buffer prior to assay. Phosphorylated substrates were also analyzed by SDS-PAGE (15% polyacrylamide).

Analysis of kinase inhibitors was performed on the Nef-FLAG and associated proteins isolated from 1 ml of Nef-FLAG expressing VB cell lysate. Washed beads were distributed into four separate aliquots, and kinase reactions were carried out with the addition of either 5% dimethyl sulfoxide in kinase buffer (control) or staurosporine or genistein diluted 20-fold from dimethyl sulfoxide stocks. Preincubation of beads with the ATP analog 5'-[μ -(fluorosulfon-

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yl)benzoyl]adenosine (FSBA) (11) diluted in kinase buffer was performed for 5 min at 37°C, followed by a single wash. Similar treatment of beads in the absence of FSBA had no effect on the activity of kinase (not shown).

Nef-FLAG and associated proteins isolated by immunoprecipitation from 5 ml of cell lysate were labeled with 5 μ Ci of [γ - 32 P]ATP in a kinase assay, and the products were released into lysis buffer with the addition of 100 μ g of FLAG peptide (DYKDDDDK, Eastman Kodak) per ml for 10 min at room temperature. After the M2-agarose beads were removed, proteins were reprecipitated by incubation with antibodies (2 μ g) in 0.5 ml lysis buffer for 2 h at 4°C and collected on GammaBind G Sepharose (Pharmacia, Piscataway, N.J.), with two subsequent washes in lysis buffer prior to analysis by SDS-PAGE.

ATP-binding assay. Immunoprecipitated proteins isolated from 5 ml of VB cell lysate were assayed for ATP binding by immunoblot by using reagents and protocols available from Boehringer Mannheim, Indianapolis, Ind. Briefly, washed immunoprecipitates were incubated with either 500 μ M FSBA or 500 μ M FSBA plus 1 mM ATP for 5 min at 37°C and subjected to SDS-PAGE (7% polyacrylamide) followed by electrophoretic transfer to a nitrocellulose filter. After blocking of the filter and incubation with polyclonal anti-FSBA antisera, bound antibodies were detected with alkaline phosphatase-conjugated goat anti-rabbit antibody and BCIP/NBT (5-bromo-4-chloro-3-indolylphosphate toluidinium/nitroblue tetrazolium) substrate (Kirkegaard-Perry, Gaithersburg, Md.).

Expression of activated GTPases. Nef-FLAG-expressing or nontransduced VB cells were electroporated with 5 μ g of pcDNA3 plasmid DNAs encoding mutated forms of the small p21 GTPases Rac1, Cdc42Hs, or RhoA (12) by using a BTX Technologies apparatus, according to a protocol provided by the manufacturer. Briefly, 7.5×10^6 cells, washed with medium, were mixed with DNA in 0.25 ml RPMI 1640 and subjected to a pulse of 230 V over 65 ms in a chamber of 0.4 mm width. Cells were incubated in 30 ml of RPMI 1640 supplemented with 10% fetal bovine serum for 20 h prior to lysis and analysis of the Nef-FLAG-associated proteins by kinase assay. Electroporation of cells with a similar expression plasmid encoding green fluorescent protein (Clontech, Palo Alto, Calif.) indicates that more than 40% of viable cells express protein under these conditions.

RESULTS

In order to identify T-cell proteins that interact directly with Nef, a variant form of the *nef* gene has been synthesized in vitro to incorporate an octapeptide epitope (FLAG) at the Nef carboxy terminus. This modified gene has been cloned into a retroviral expression vector, and recombinant viruses have been produced to allow the direct transduction of human cells. Expression of the modified *nef* gene in the human T-cell line VB results in the same decrease of cell surface CD4 shown previously with the wild-type *nef* (not shown). A 31-kDa Nef-FLAG protein can be precipitated from metabolically labeled VB cells with antibody directed against the octapeptide (Fig. 1, lane 2) as well as polyclonal anti-Nef antisera (not shown). In addition, several candidate Nef-associated cellular proteins of 65, 79, and 97 kDa coprecipitate with Nef (Fig. 1, lane 2), and were present at levels, compared to the control, of approximately 3, 3, and 8%, respectively, of precipitated Nef by mass, assuming even distribution of labeled residues incorporated at equilibrium into protein.

A species of 65 kDa is also labeled in an in vitro kinase assay (Fig. 1, lane 5) and represents the major phosphate acceptor for a Nef-associated protein kinase. A similar phosphoprotein is seen in labeled immunoprecipitates from monocytes (U937) and murine fibroblasts (PA317, data not shown). These results are similar to those reported previously, which identified a 62-kDa phosphoprotein in kinase assays of an immunoprecipitated Nef fusion protein (49).

Studies with protein kinase inhibitors (Fig. 2A) indicate that the Nef-associated kinase activity is sensitive to the inhibitors staurosporine and FSBA (11) but not the tyrosine kinase inhibitor genistein. Additional studies with specific inhibitors of PKA, PKC, PKG, PKR, and myosin light chain kinase (KT5720, chelerythrine, KT5823, 2-aminopurine, and KT5926, respectively) also show little effect on the Nef-associated kinase activity (not shown). The 65-kDa protein p65 binds and can be labeled by the ATP analog FSBA in an ATP-dependent manner (Fig. 2B). P65 is the only protein that specifically binds

FIG. 1. Isolation of Nef-FLAG and in vitro phosphorylation of associated proteins. Proteins were immunoprecipitated from extracts of VB cells (lanes 1 and 4) or VB cells expressing Nef-FLAG (lanes 2 and 5). In immunoprecipitates from in vivo 35 S-labeled extracts (lanes 1 and 2) Nef-FLAG is apparent along with several candidate Nef-associated proteins (indicated by dots, lane 2). Addition of 32 P-ATP to proteins immunoprecipitated from unlabeled extracts in an in vitro kinase assay (lanes 4 and 5) results in phosphorylation of proteins associated with Nef-FLAG (lane 5), most notably species at 65 and 97 kDa. Molecular weight standards are shown in lane 3, with sizes (10^3) indicated (10% polyacrylamide gel).

FSBA in this assay, indicating that it itself is the Nef-associated protein kinase and is subject to autophosphorylation.

Myelin basic protein and histones can be phosphorylated by the p65 kinase as exogenous substrates (Fig. 3A). Comparison with control immunoprecipitates indicates that histone H4 is the most specific substrate (Fig. 3B), while histone H1 was not recognized by the kinase. Limited HCl hydrolysis of phosphorylated histone H4 and thin-layer chromatographic analysis (41) identified incorporation of label into both *o*-phosphoserine and *o*-phosphothreonine (not shown).

The preferential recognition of histone H4 as substrate by a 65-kDa serine/threonine kinase and the capacity for autophosphorylation are reminiscent of the previously described pla-

FIG. 2. P65 is a Nef-associated kinase. (A) Phosphorylation of Nef-associated p65 in the in vitro kinase assay (lane 1) is inhibited in the presence of 10 μ M staurosporine (lane 4) but not by 100 μ M genistein (lane 3). The Nef-associated kinase is also inhibited by pretreatment of the immunoprecipitate with 100 μ M FSBA (lane 2). (B) P65 is an ATP-binding protein. P65 can be labeled by FSBA, as detected by Western blot (lane 1), which is inhibited by the addition of ATP during the labeling reaction (lane 2).

FIG. 3. Histone H4 is a preferred substrate for p65 kinase. (A) Kinase substrates were assayed together with immunoprecipitates from control (odd-numbered lanes) or Nef-FLAG expressing VB cells (even lanes). Phosphorylated bovine myelin basic protein (MBP, lanes 1 and 2), or the histones H4 (lanes 3 and 4), H3 (5 and 6), H2b (7 and 8), H2a (9 and 10), and H1 (11 and 12) were analyzed by SDS-PAGE (15% polyacrylamide). (B) Phosphorimage analysis of phosphorylated substrates. Counts incorporated into protein in the presence of Nef are presented as a multiple of the counts incorporated in control reactions. The data shown are representative of three independent experiments.

cental-derived S6/H4 kinase (15) and the fMet-Leu-Phe-activated histone H4 protein kinase of neutrophils (26). Both of these serine/threonine kinases have recently been shown to be members of the PAK family (6, 30). The PAKs, which have a molecular size range of 62 to 68 kDa, undergo autophosphorylation-dependent activation upon binding to the GTP forms of Cdc42 and Rac and utilize myelin basic protein and histone H4 as substrates (4, 30, 35–37, 55).

In order to determine whether there is a relationship between p65 and the PAK family of enzymes, Nef-associated p65 was immunoprecipitated with antibodies to several PAKs. Active p65 kinase, labeled by autophosphorylation in a complex with the modified Nef, was released into solution by elution with the FLAG peptide. Figure 4 shows the immunoprecipitation of labeled p65 by either amino-terminal (lane 1) or carboxy-terminal (lane 2) polyclonal anti-peptide antisera to rat α PAK (36) but not by several antisera directed against other serine/threonine kinases (lanes 3 to 7). In a similar experiment, p65 can also be immunoprecipitated with antisera directed against *Escherichia coli* recombinant rat PAK1 or PAK2 (30) (not shown). Phosphorimager analysis in several similar experiments indicates that 1 to 4% of input p65 is precipitated by anti-rat PAK antisera. Inefficiencies of precipitation are presumably due to low cross-reactivity of the anti-rat protein antisera with the human kinase, since partial digestion of 32 P-labeled p65 and p65 immunoprecipitated by anti-rat α PAK with endoproteinase Glu-C generated identical peptide maps (data not shown). These experiments indicate that the Nef-associated p65 kinase is serologically related to PAKs.

The PAK enzymes are defined by the ability of GTP-bound Rac and Cdc42 to positively regulate kinase activity (36). In order to test the Nef-associated kinase for similar activation, Nef-FLAG-expressing cells were electroporated with plasmid DNAs encoding mutated (QL) forms of Rac, Cdc42, and, as a control, Rho, which are constitutively activated for the binding of GTP (31, 56, 57). PAKs demonstrate a preference for the GTP-bound form of Cdc42 over Rac in activation and are not

activated or are suppressed by Rho (36, 37). In agreement with the PAK G-protein preferences, the Nef-associated kinase activity is elevated in the presence of Cdc42 and to a lesser extent with Rac1 but is suppressed by RhoA (Fig. 5). Phosphorimage analysis of histone H4 phosphorylation in three similar experiments indicates that the kinase activity associated with Nef is increased by Cdc42-QL 2.5 ± 0.3 (mean $\pm$ standard deviation)-fold. These results define the Nef-associated kinase as a

FIG. 4. P65 is related to the rat brain PAK. Purified 32 P-labeled p65 was immunoprecipitated with antisera to several protein kinases: rabbit polyclonal anti-rat brain PAK65 N-terminal peptide (Santa Cruz Biotechnology) (lane 1), rabbit polyclonal anti-rat brain PAK65 C-terminal peptide (Santa Cruz Biotechnology) (lane 2), rabbit polyclonal anti-human PKR peptide (Santa Cruz Biotechnology) (lane 3), mouse monoclonal anti-RAF (Ulf Rapp) (lane 4), rabbit polyclonal anti-cdc2 kinase (PSTAIR) (Upstate Biotechnology) (lane 5), sheep polyclonal anti-human ERK3 (Upstate Biotechnology) (lane 6), and rabbit polyclonal anti-mouse ERK2 (Upstate Biotechnology) (lane 7).

FIG. 5. Nef-associated kinase is preferentially activated by Cdc42 and Rac. VB (lane 1) or Nef-FLAG (lanes 2 to 5)-expressing cells were transfected with pcDNA3 vector (lane 2), or with plasmids encoding RhoA-QL (lane 3), Rac1-QL (lane 4), or Cdc42-QL (lanes 1 and 5) by electroporation. After 20 h in culture, Nef-FLAG-associated proteins were isolated and kinase assays were performed in the presence of histone H4. Phosphoproteins were analyzed by SDS-PAGE (15% polyacrylamide) and autoradiography.

p21-activated kinase and provide evidence that Nef can associate with an activated form of p65 PAK.

DISCUSSION

Our characterizations of the Nef-associated kinase identify it as a member of the PAK family. PAKs play integral roles in cellular responses to environmental stimuli and may be part of the costimulus pathway in T cells. From sequence data there appear to be a minimum of three mammalian PAK members: (i) rat p65PAK or rat α PAK (36) and human PAK1 (10a) (GenBank accession number U24152), predominantly expressed in brain; (ii) human PAK65 (37), human PAK2 (10a) (GenBank accession number U24153), and rat γ PAK (55), which are expressed ubiquitously; and (iii) murine PAK3 (4) and rat β PAK (35), another brain isoform. Available sequence data on placental S6/H4 kinase identify it as belonging to the second PAK (PAK65/PAK2/ γ PAK) subfamily (6). Additionally, 62- and 65-kDa kinases, isolated from chemoattractant receptor-activated human neutrophils, have been shown to be serologically related to human PAK2 and PAK1, respectively (30). Initial comparison of the Nef-associated PAK with PAKs isolated directly from the VB cells has been attempted. In experiments not shown here, the Nef-associated kinase comigrates with the 65-kDa PAK precipitated by rabbit antisera (30) directed against recombinant rat PAK1 (α PAK) protein but not PAK2. Partial endoproteinase Glu-C digestion of the in vitro autophosphorylated VB cell PAK1 gives a digestion pattern similar to that of an in vitro phosphorylated purified recombinant rat PAK1 protein, suggesting PAK1 is expressed in this T-cell line. However, the GluC protease-digested Nef-associated PAK does not share the PAK1 pattern. This may indicate that the Nef-associated kinase is subject to autophosphorylation at sites different from those of purified PAK1 or that it is an as-yet-unidentified T-cell PAK. Further characterization of the PAK isoform associated with Nef is ongoing.

Although the PAK enzymes are expressed in a variety of tissues, none have previously been identified directly in T cells, and a role for PAKs in T-cell functions has not been demonstrated. It has been suggested that activated PAK initiates the kinase cascade leading to the activation of c-Jun N-terminal kinase, JNK (12, 40), and stimulation of CD28, a T-cell activation surface receptor, has been shown to activate JNK (53).

This signal transduction pathway could be affected by Nef-mediated membrane association of PAK. Like PAK, the serine kinase Raf is activated by association with a small G protein. The GTP form of Ras becomes part of a membrane complex that activates and translocates the Raf kinase to the plasma membrane in response to mitogenic stimuli (32, 52). The experimental targeting of Raf to the membrane by the fusion of a membrane localization peptide to the kinase eliminates the normal requirement for activated (GTP-bound) Ras (32). A GTP-inducible membrane association of Cdc42/Rac has also

been described (7), although subsequent recruitment of PAK has not yet been shown. The recruitment of PAK by membrane-associated Nef might facilitate the PAK activation pathways. The recruitment of a signal pathway molecule to inappropriate cellular compartments may also induce novel and unanticipated activities. This latter concept is supported by the findings that different cellular locations of Nef result in different T-cell activation state phenotypes (5).

The Nef-mediated alterations in T-cell function may be secondary to an evolved role for Nef in HIV replication. As noted above, when expressed in the virus-generating cell, Nef increases the infectivity of viral particles. This enhanced infectivity may in part be due to kinase-mediated phosphorylation of the HIV matrix protein. The matrix protein is myristoylated, like Nef, and becomes localized to the plasma membrane, facilitating viral assembly at this site (8, 21). In addition, matrix protein (10), like the *vpr* gene product (25), affects nuclear targeting of the HIV preintegration complex in newly infected cells. Alteration of nuclear localization sequences in matrix protein and Vpr eliminates viral replication in nondividing macrophages (25). The matrix-mediated nuclear import and subsequent viral replication in nondividing cells, however, is dependent on phosphorylation of the matrix protein on both tyrosine and serine residues (9, 18, 19). The partially purified Nef-associated serine/threonine kinase can phosphorylate matrix protein at yet unidentified sites (data not shown). The relevance of this activity to in vivo function remains to be tested, although preliminary work (54) has shown that matrix serine phosphorylation is dependent upon the expression of Nef.

The association of Nef with PAK as identified in this report implies a role for Nef in the promotion of signal transduction responses in virally infected cells, leading to the Nef-mediated alteration of T-cell activation and enhancement of HIV replication and pathogenesis. Identification of the Nef-associated kinase will now permit studies to resolve its importance in the biological activities of the HIV Nef protein.

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