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Human Immunodeficiency Virus Type 1 Nef Selectively Associates with a Catalytically Active Subpopulation of p21-Activated Kinase 2 (PAK2) Independently of PAK2 Binding to Nck or β-PIX

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We have recently identified the Nef-associated serine-threonine kinase (NAK) as the p21-activated kinase 2 (PAK2). Here we have taken advantage of the possibility to manipulate the functional properties of NAK by transfecting PAK2 cDNA or its mutant derivatives in order to further characterize the Nef-NAK complex. To exclude the possibility that some Nef variants might interact with PAK1 instead of PAK2, we also examined the identity of NAK complexed with divergent human immunodeficiency virus type 1 HIV-1 Nef proteins. All tested Nef proteins, including SF2, NL4-3, BH10, and HAN-2, associated with PAK2 but not with PAK1. By exchanging different regions between these two PAK proteins, the selective ability of PAK2 to associate with Nef could be mapped to the carboxy-terminal part of its regulatory domain. Binding of PAK2 with the adapter protein Nck or β-PIX was found to be dispensable for the assembly of the Nef-PAK2 complex, whereas an intact Cdc42-Rac1 interactive binding motif was required. Most importantly, we found that NAK represented a distinct subpopulation of the total cellular PAK2 characterized by a high specific kinase activity. Thus, although only a small fraction of cellular PAK2 could be found in complex with Nef, NAK represented a major part of cellular PAK2 activity.

The Nef gene of primate immunodeficiency viruses increases viral replication and is critically important for the clinical outcome of infected humans and macaques (7, 9, 10). At the cellular level, different effects of this 27- to 34-kDa myristoylated protein have been identified and studied (reviewed in references 16, 18, and 20). These include downregulation of CD4 and major histocompatibility complex class I cell surface expression and an increased infectivity phenotype of virus particles produced in Nef-expressing cells. Furthermore, Nef has been found to modulate cellular signaling events. Several host cell proteins that are implicated in mediating these effects of Nef have been identified (16, 18, 20).

The interaction with the Nef-associated serine-threonine kinase (NAK) has been reported to correlate with the ability of Nef to enhance viral infectivity (23, 30). Although the protein was already suspected for some time to be a member of the p21-activated kinase (PAK) family, the identity of NAK remained elusive until recently, when we showed that NAK is PAK2 (19). This conclusion was based on several independent lines of evidence. NAK that was eluted from anti-Nef immunoprecipitations could be reimmunoprecipitated with specific anti-PAK2 antibodies but not with antibodies with unique reactivity to PAK1 or PAK3. Also, partial proteolysis mapping of NAK and PAK2 yielded identical maps, and finally, NAK, like PAK2 (but unlike PAK1 and PAK3), was sensitive to cleavage by caspase 3 (19).

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The mammalian PAK family consists of the three highly homologous members PAK1 to -3 and the less related PAK4 (reviewed in references 1, 11, and 25). The cellular functions described for PAKs are numerous and include morphogenetic regulation (reviewed in reference 6), modulation of signaling cascades leading to transcriptional regulation (reviewed in reference 1), and regulation of apoptotic pathways (21, 24, 26).

The carboxy-terminal kinase domains of all PAKs are almost identical, and also, the aminoterminal regulatory domains contain regions of high sequence conservation. PAK1 to -3 contain an amino-terminal PXXP-motif that has been shown to function in PAK1 as a target for the second SH3 domain of the adapter protein Nck (2, 13, 32). Activation of any of the PAKs by the Rho family p21-GTPases Cdc42 and Racl is mediated by the Cdc42-Racl interactive binding (CRIB) motif (5, 12, 17, 27). The CRIB motif is part of a bigger region that is conserved in PAK1 to -3 (the PAN domain, or autoregulatory [AR] region). Studies using mutational analysis and yeast two-hybrid techniques have shown that this region negatively regulates kinase activity by interacting with the kinase domain (28, 31). A recent crystal structure of the kinase domain of PAK1 together with its AR region not only confirms this interaction but also shows how binding of the Rho GTPases will trigger several conformational changes that result in a catalytically active state of the kinase (12). The PAK-interacting exchange protein (β-PIX; also called COOL-1), which binds via its SH3 domain to a proline-rich region of the PAKs that is different from the Nck binding site, has been found to be involved in targeting PAK1 to focal complexes (3, 15). A distinguishing feature of PAK2 is the presence of a recognition site for DEVD-sensitive caspases, which is located between its regulatory and kinase domains (21).

In this paper, we show that selection of PAK2 (rather than PAK1) as NAK is a common feature of divergent HIV type 1 (HIV-1) Nef proteins and demonstrate that this specificity lies in a region of the amino terminus of PAK2 that is relatively poorly conserved in PAK1. We also show that this interaction is independent of the PAK2 PXXP-motif, the caspase cleavage site, and the PIX-binding domain. Finally, we demonstrate that Nef interacts with a highly active subpopulation of PAK2, which, although composing only a small fraction of the total pool of PAK2, represents the majority of cellular PAK2 activity.

MATERIALS AND METHODS

Cell lines and transfections. 293T human embryonic kidney fibroblast-derived cells were maintained as described previously (14). Transfections were done using the Lipofectamine transfection agent (Gibco BRL) according to the manufacturer's instructions.

Antibodies. The anti-Nef antibodies used were a polyclonal sheep serum raised against glutathione *S*-transferase-Nef (kindly provided by M. Harris) and a monoclonal antibody (2F2) that was raised against a peptide (amino acids 151 to 170) of Nef of the HIV-1 BRU isolate (kindly provided by Vladimir Ovod, Institute of Medical Technology, University of Tampere, Tampere, Finland). PAK1- and PAK2-specific antisera (anti-PAK1-R1 and anti-PAK2-R2) were raised in rabbits against a cocktail of selected sequences that are divergent in these proteins, as described previously (19). Anti-hemagglutinin (HA) antibodies were purchased from BAbCO (Richmond, Calif.).

Plasmids and construction of PAK2 mutants. Generation of the pEBB-PAK2-HA plasmid was described before (19). pEBB-PAK1-HA and an expression plasmid for dominant-active Cdc42 were obtained from B. Mayer. The expression vector for pEF-BOS-SF2-Nef was kindly provided by Andreas Baur. HAN-2 Nef cDNA (obtained from Kai Krohn, Institute of Medical Technology) was amplified by PCR and cloned into the pEBB expression vector. pEBB-NL4-3-R71 Nef has been described before (22). The Nef allele NL4-3-R71 was used in all experiments unless otherwise indicated.

All PAK2 mutagenesis was done by PCR in the pUC18 vector. PAK1N2C, PAK1/2N2C, and PAK2/1N2C were made by PCR amplification of parts of PAK1 that were used to replace the corresponding parts of PAK2. The PAK2 mutants PIX^- (P185G; R186A), $caspase^-$ (D212N), $CRIB^-$ (H82/85L), and the AXXA mutant (P12/15A) were made by overlap PCR using a specific mutant primer pair and common outer primers containing restriction sites used for cloning. All constructs were sequenced and recloned as *Bam*HI-*Kpn*I fragments into pEBB-HA.

To facilitate the discrimination of the transfected PAK2 proteins from the endogenous PAK2, a multiepitope (ME) tag was generated by cloning an anti-Myc epitope and a 6-histidine stretch between the PAK cDNA and the HA tag.

IVKA. Transfected cells were lysed in in vitro kinase assay (IVKA) lysis buffer (50 mM HEPES [pH 7.4], 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1 mM EGTA, 1.5 mM $MgCl_2$, 1 mM Na-orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 10 μg of aprotinin/ml), and the lysates were cleared by centrifugation, adjusted to identical total protein concentrations and subjected to immunoprecipitations using protein G-Sepharose beads. After immunoprecipitation, the beads were washed with IVKA lysis buffer (three times) and then with IVKA buffer (50 mM HEPES, [pH 7.4], 5 mM $MgCl_2$) (two times). The kinase assay was performed in 80 μl of IVKA buffer containing 2.5 μCi of [γ-^{32}P] ATP for 20 min at 30°C. The assay was stopped by the addition of ice-cold phosphate-buffered saline (PBS). The supernatant was removed, and the Sepharose beads were boiled in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer for analysis of autophosphorylation activity. Proteins were separated on 13-cm-long SDS-8% PAGE gels.

Elution and reimmunoprecipitation of NAK. After IVKA, radiolabeled NAK was eluted from the sheep anti-Nef immunoprecipitates by incubation for 1 h at 37°C in PBS with 0.1% SDS. The eluate was diluted 10-fold with PBS and cleared with protein G-Sepharose before reimmunoprecipitation with the specific rabbit anti-PAK1 or rabbit anti-PAK2 antiserum.

RESULTS

Selection of PAK2 as NAK is common to diverse alleles of HIV-1 Nef. We have recently reported the identification of NAK as PAK2 (19). In these experiments, two HIV-1 LAI-derived Nef alleles, NL4-3-R71 and BH10, were used. Recently, Fackler et al. raised the possibility that the HIV-1 SF2 Nef allele might have a different specificity and interact instead with PAK1 (8). This prompted us to characterize NAK recovered from complexes obtained with a panel of divergent HIV-1 Nef proteins.

Anti-Nef immunoprecipitations and subsequent in vitro kinase assays were performed using extracts from 293T cells transfected with different Nef proteins. As shown in Fig. 1A, in the absence of transfected Nef, no NAK signal was observed even from cells that expressed high levels of active PAK2 (lane 2), thus confirming the specificity of this assay. To increase cellular PAK activity a dominant active form of Cdc42 (Cdc42V12) was included in most transfections. The effects of Cdc42V12 on total and Nef-associated PAK2 activity are shown in Fig. 1A.

As shown in Fig. 1B, NAK that coprecipitated with NL4-3-R71, SF2, or HAN-2 Nef had identical apparent molecular weights. PAK1 and PAK2, which have calculated sizes of 61 and 58 kDa, respectively, can be easily separated under the electrophoretic conditions used here (Fig. 2B), suggesting that NAK associated with these different Nef proteins represents only one PAK species. More importantly, after being released from the anti-Nef immunoprecipitations, radiolabeled NAK could in all cases be reimmunoprecipitated with an anti-PAK2 antiserum but not with an anti-PAK1 antiserum (Fig. 1B). We have previously established that these antibodies are monospecific for their respective target PAK proteins (19). Based on these experiments, we concluded that the NAK associated with all three different Nef proteins was PAK2.

Divergence in the regulatory domains of PAK1 and PAK2 is responsible for their different abilities to associate with Nef. Since PAK1 and PAK2 are highly homologous proteins, we were interested in investigating what part of the PAK2 protein was responsible for its specific association with Nef. Of note for the design of these experiments is the fact that, although transfected PAK2 protein has been shown to efficiently replace endogenous PAK2 in the complex with Nef, overexpression of PAK2 does not increase the overall NAK signal (19). Therefore, when transfecting mutant forms of PAK2, it would have been difficult to determine whether these proteins could still interact with Nef or whether NAK activity coprecipitating with Nef was due to endogenous PAK2. To overcome this problem, we increased the molecular weight of PAK2 by adding a 38-amino-acid-long ME tag to the carboxy terminus in order to discriminate between endogenous and exogenous PAK2. The ME-tagged PAK2 retained the capacity to associate with Nef and thereby efficiently (albeit not completely) replaced the endogenous PAK2 in the coprecipitating complex (Fig 2B, lane 2, and results not shown).

We constructed several PAK1/PAK2 chimeric proteins, as shown on the right in Fig. 2B. Because the autoregulation of PAKs involves coordinated intramolecular interactions between part of the amino-terminal regulatory domain and the carboxy-terminal kinase domain, we engineered the fusion borders (Fig. 2A) to be within the highly homologous regions in order to increase the likelihood of properly folded, functional kinases. The chimeric, ME-tagged proteins were expressed in 293T cells and were found to be active kinases that could be

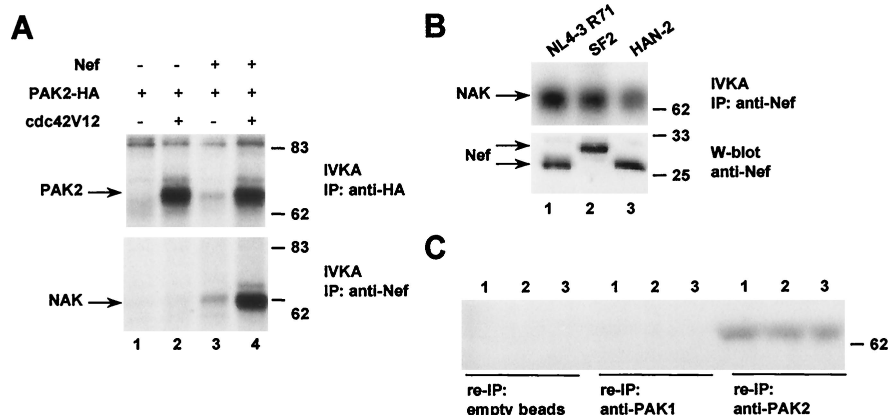


FIG. 1. Selective association with PAK2 is common to diverse alleles of HIV-1 Nef. (A) 293T cells were transfected with expression plasmids for HA-tagged PAK2 (all lanes), NL4-3-R71 Nef (lanes 3 and 4), and Cdc42V12 (lanes 2 and 4). Cell lysates were split in two and immunoprecipitated (IP) with anti-HA or anti-Nef antibodies. The immunoprecipitates were subjected to IVKAs and separated by SDS-PAGE. +, present; −, absent. (B and C) 293T cells were transfected with expression plasmids for NL4-3-R71 Nef (lanes 1), SF2 Nef (lanes 2), or HAN-2 Nef (lanes 3) together with Cdc42V12. The cell lysates were subjected to immunoprecipitation with a polyclonal sheep anti-Nef antiserum and subsequent IVKA. A fraction of these samples was directly examined by SDS-PAGE (B, top), whereas the rest was eluted from the Sepharose beads and reimmunoprecipitated with either anti-PAK1 or anti-PAK2 antiserum or empty beads (control) before SDS-PAGE (C). Panel B, bottom, shows an anti-Nef Western blot analysis of the lysates before any immunoprecipitation. Molecular mass markers are indicated in kilodaltons.

normally regulated by Cdc42V12 (Fig. 2B and data not shown), thus validating this approach. Although not all chimeras showed the same specific activity, they could be readily detected after immunoprecipitation and IVKA. The different molecular weights of the chimeric proteins are due to the longer carboxy-terminal part of the regulatory domain of PAK1.

As shown before (19), overexpressed PAK1-ME did not coimmunoprecipitate with Nef (Fig. 2B, lanes 1). When PAK1-ME was overexpressed together with SF2 or HAN2 Nef instead of NL4-3-R71 Nef, the results were equally negative (data not shown). The results with the PAK1/PAK2 chimeric proteins indicated that the PAK1 N terminus in the chimeric protein PAK1N2C failed to substitute for the corresponding region of PAK2 in mediating an interaction with Nef (lanes 3). Experiments with the chimeric proteins containing smaller pieces of PAK1 (PAK1/2N2C and PAK2/1N2C [lanes 4 and 5]) indicated that the carboxy-terminal part of the PAK2 regulatory domain (amino acids 101 to 259) was required for the specific interaction of Nef with PAK2.

Role of functional motifs of PAK2 in the interaction with Nef. Several defined sequence motifs in PAK are known to mediate its interactions with other cellular proteins. The amino-terminal PXXP motif has been shown to be a ligand for Nck (2, 13, 32), the interaction with the guanidine exchange factor β-PIX is mediated by the PIX-binding motif (3, 15), the CRIB motif is required for interaction with the Rho GTPases Rac1 and Cdc42 (5, 12, 17, 27), and finally, the amino acid sequence HVDGA between the regulatory domain and the kinase domain renders PAK2 a substrate for caspases (21). To determine whether these motifs might also be important for the PAK2-Nef interaction, we introduced missense mutations in PAK2 that disrupted these sites. As described above for the PAK1/PAK2 chimeras, these mutant proteins were also equipped with a C-terminal ME tag to allow their distinction from the endogenous PAK2.

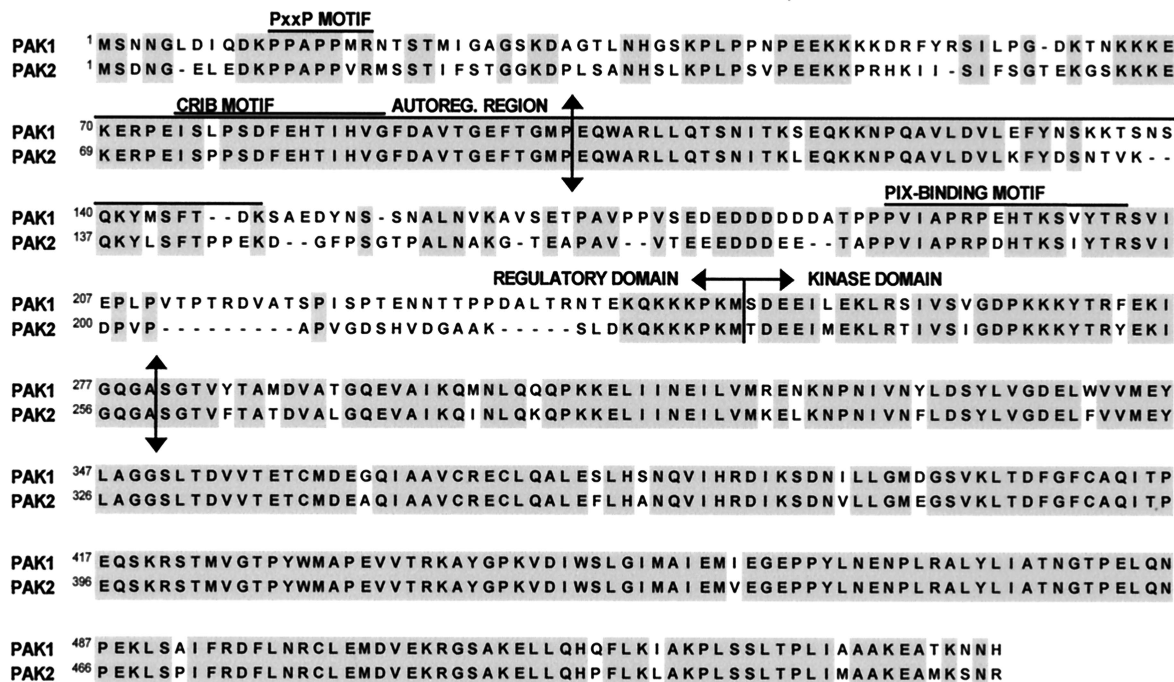
The mutant PAK2 proteins were transfected into 293T cells together with an expression vector for Nef with or without cotransfection of Cdc42V12. Lysates of these cells were split in two and immunoprecipitated with anti-HA or anti-Nef, followed by IVKA. As shown in the upper gel of Fig. 3, the mutations in the PXXP motif (AXXA-PAK2), the caspase target sequence (caspase$^-$-PAK2) site, or the PIX-binding motif (PIX$^-$-PAK2) did not affect the ability of PAK2 to become activated by cotransfected Cdc42V12. As expected, the CRIB$^-$ mutant PAK2 did not become activated upon cotransfection of Cdc42V12. However, it could be visualized by IVKA because of its considerable constitutive autophosphorylation. Besides increasing their catalytic activities, cotransfection of Cdc42V12 caused the appearance of additional slower-migrating forms in the case of the wild-type and all mutant PAK2 proteins, except for CRIB$^-$-PAK2.

Analysis of the labeled proteins that coprecipitated with PIX$^-$-PAK2 revealed the absence of the high-molecular-weight phosphoproteins (p85 and p88) that were found associated with all PAK2 proteins that had a functional PIX-binding motif. These PIX-binding-site-associated substrates of PAK2 may correspond to the NAK-associated phosphoproteins observed in a recent study by Brown et al. (4).

When the presence of the ME-tagged PAK2 mutants in anti-Nef immunoprecipitations was examined, it was found that the PXXP mutant, the caspase mutant, and the PIX mutant were all able to interact with Nef (Fig. 3, middle gel). By contrast, CRIB$^-$-PAK2 had lost its ability to interact with Nef, as evidenced by the absence of the ME-tagged PAK2 among the proteins of the anti-Nef immunocomplex labeled by IVKA (Fig. 3, middle gel, lanes 11 and 12). It is unlikely that this was due to misfolding of the mutant protein, since CRIB$^-$-PAK2 still interacted with the p85 and p88 phosphoproteins.

We therefore concluded that the formation of the Nef-PAK2 complex is not dependent on the interaction of PAK2 with caspases, the adapter protein Nck, the exchange factor

A



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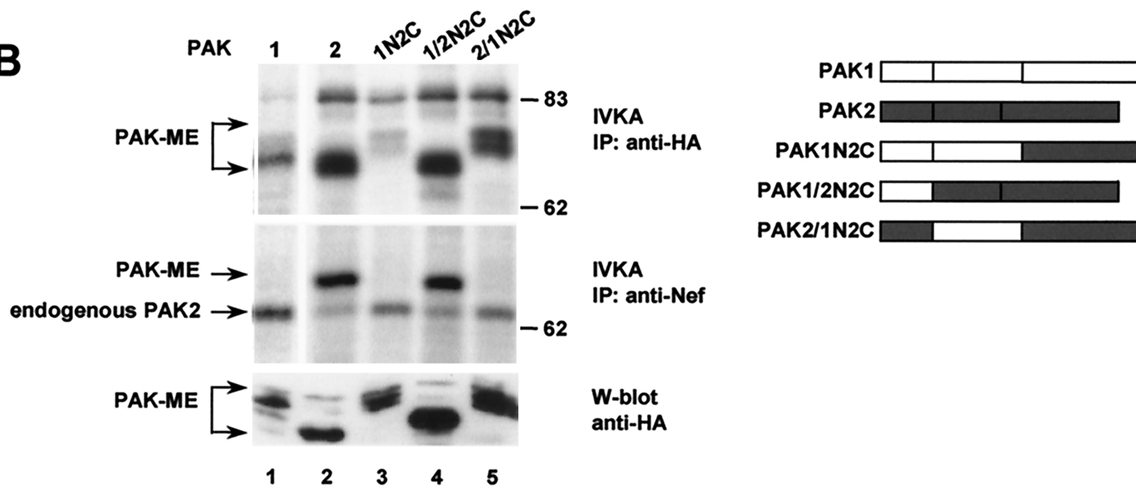

FIG. 2. Specificity of Nef for PAK2 is determined by a region in the regulatory domain. (A) Alignment of PAK1 and PAK2. The proteins consist of a carboxy-terminal kinase domain and an amino-terminal regulatory domain that contains regions known to be involved in protein-protein interactions (indicated with lines over the sequences). The autoregulatory region is involved in autoinhibition of the kinase activity and contains the CRIB motif required for Cdc42 binding. Identical amino acids are shaded. The vertical arrows indicate the borders of the PAK1/PAK2 chimeras. (B) 293T cells were transfected with Nef, Cdc42V12, and different ME-tagged PAK1/PAK2 chimeras. The cell lysates were split in two and immunoprecipitated (IP) with anti-HA or anti-Nef antibodies. The immunoprecipitates were subjected to IVKAs and separated by SDS-PAGE. On the right is a schematic representation of the hybrid proteins. Molecular mass markers are indicated in kilodaltons.

β-PIX, or, consequently, other factors that interact with PAK2 via these proteins. Instead, either the CRIB motif itself or CRIB-mediated activation of PAK2 by Cdc42 (or Rac1 [results not shown]), which was associated with the appearance of slower-migrating species in the IVKAs, was essential for the interaction with Nef.

NAK represents a distinct subpopulation of PAK2 with high kinase activity. Various observations made during the course of these and previous studies (19), in particular the difficulty of detecting Nef-associated PAK2 by Western blotting, suggested that NAK might be a small but highly active subpopulation of PAK2. To directly test this hypothesis, radioactive IVKA samples were transferred to a blotting membrane and subjected to Western blot analysis, which allowed simultaneous detection of the amounts of PAK2 and its catalytic activity from the same membrane. In this way it was also possible to precisely compare the electrophoretic mobility of the radioactively labeled PAK2 to that of the bulk of the PAK2 protein detected by

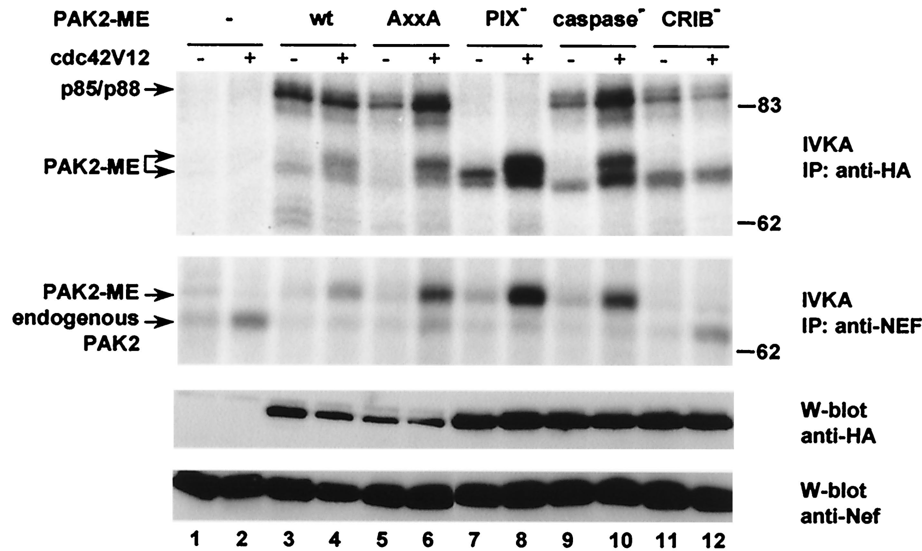

FIG. 3. Mutational analysis of PAK2 residues potentially involved in association with Nef. 293T cells were transfected with Nef, Cdc42V12 (where indicated), and different PAK2 mutant constructs, all carrying the ME tag. The cell lysates were split in two and immunoprecipitated (IP) with anti-HA or anti-Nef antibodies. The immunoprecipitates were subjected to IVKAs and separated by SDS-PAGE (upper two gels). Part of the cell lysates was removed before the immunoprecipitations, separated by SDS-PAGE, and analyzed by Western blotting (W-blot) for PAK and Nef expression (lower two blots). +, present; −, absent. Molecular mass markers are indicated in kilodaltons.

Western blotting by overlaying the X-ray film with the Western blot film. The upper panel in Fig. 4, showing the IVKA results, demonstrated that NAK comigrated with the higher-molecular-weight form of PAK2 that appeared upon cotransfection with Cdc42V12 (compare lanes 2 and 4). Using an identical experimental setup, we have previously shown that this protein can be reimmunoprecipitated with anti-HA (19), thus confirming that it is indeed PAK2-HA. Western blot analysis of the anti-HA immunoprecipitation of total transfected PAK2 (lower panel, lanes 1 and 2) indicated that the higher-molecular-weight species of PAK2 detected by IVKA was present in amounts too small for immunodetection, showing that it was only a minute fraction of the total pool of PAK2. Likewise, Nef-associated PAK2, which was readily detected by IVKA, could not be detected by Western blotting (lower panel, lanes 3 and 4). This experiment indicated that activation of PAK2 by Cdc42V12 correlated with the appearance of a PAK2 species which, besides its identical electrophoretic mobility, resembled NAK by being highly catalytically active but present in amounts too small to be readily detected by Western blotting. We concluded that this subspecies of PAK2 is NAK and that, unlike the majority of PAK2, which is not associated with Nef, a significant fraction of this highly active subpopulation of cellular PAK2 is in complex with Nef.

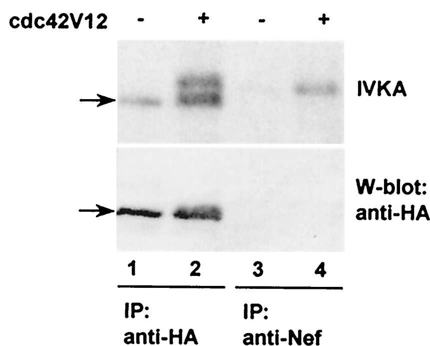

FIG. 4. NAK is a catalytically active subpopulation of total cellular PAK2. 293T cells were transfected with expression vectors for PAK2-HA and Nef with or without Cdc42V12. The cell lysates were split in two and immunoprecipitated (IP) with anti-HA or anti-Nef antibodies. The immunoprecipitates were subjected to IVKAs and separated by SDS-PAGE. After electrophoresis, the proteins were blotted to nitrocellulose for Western blot (W-blot) analysis using anti-HA. Top, exposure of the radiolabeled proteins; bottom, immunostaining of the same blot. The arrows indicate the exact points of alignment when the autoradiogram of the IVKA and the filter of the Western blot were superimposed.

DISCUSSION

In this paper we have reported a series of experiments to characterize the molecular composition of a protein complex containing PAK2 and HIV-1 Nef. In addition, we have demonstrated that the selective association of Nef with PAK2 (rather than PAK1) is common to divergent alleles of Nef. In disagreement with this conclusion, a recent report by Fackler et al. suggested that some HIV-1 Nef alleles, such as SF2, might instead select PAK1 as their binding partner (8). This discrepancy, however, can be fully explained by the incomplete specificity of the reagents used by these authors. The commercial anti-PAK1 antiserum used in their study has been shown to recognize PAK2 almost as well PAK1 (19), whereas the PAK1-derived peptide used by Fackler et al. to inhibit Nef-associated kinase activity in transfected cells overlapped with the conserved PAK AR domain and hence would be expected to interfere with CRIB-mediated activation of all PAKs (12, 28, 31).

Interestingly, Fackler et al. also reported an in vitro inter-

action between recombinant PAK1 and Nef proteins. Although this observation does not directly address the identity of the NAK in cells, it could be relevant to the assembly of this complex. Because the assembly of the NAK complex is strictly dependent on the SH3-binding capacity of Nef (despite the fact that none of the PAKs contain an SH3 domain) (14), it appears evident that if a direct contact between Nef and PAK2 indeed takes place in cells, it is not sufficient to hold this complex together. Therefore, it is possible that the PAK2 residues that mediate a possible direct contact with Nef are also conserved in PAK1, and a specific ability of PAK2 to participate in other molecular contacts required to stabilize the NAK complex would instead account for selective recruitment of PAK2 to this complex.

Nonetheless, the results reported here together with our previous study (19) clearly establish that, regardless of the HIV-1 Nef alleles used, only PAK2 and not PAK1 can serve as NAK. Moreover, in this study, the different abilities of these two homologous kinases to associate with Nef could be mapped to residues 101 to 259 in PAK2. The replacement of this region with the corresponding fragment of PAK1 resulted in a functional PAK that retained the capacity for catalytic autoinhibition through intramolecular interactions and could be normally activated by Cdc42 but failed to associate with Nef.

An intriguing aspect of the Nef-NAK interaction, which initially complicated the identification of NAK as PAK2, is that the amount of PAK2 associated with Nef cannot be significantly increased by overexpression of either of these proteins. One explanation for this observation could be that the cellular factor(s) required for stabilizing the NAK complex is present in limiting amounts. The critical role of the SH3-binding surface of Nef suggests that an SH3 domain-containing adapter protein could be involved. One obvious candidate for this role would be Nck, which contains three SH3 domains, including one that is known to bind PAKs via their PXXP-motifs (2, 13, 32). However, disruption of the PXXP motif in PAK2 did not affect its ability to associate with Nef, thus excluding a role for Nck or other PAK PXXP-motif binding adapter proteins in assembly of the NAK complex.

Another promising candidate for this role would be β-PIX, which binds PAKs with high affinity via the proline-rich PIX-binding motif and plays an important role in coordination of the recruitment of PAKs into macromolecular assemblies in cells, such as the focal adhesion complexes (15). Moreover, β-PIX has recently also been directly implicated as a component of the NAK complex by Brown et al. (14), who suggested that the β-PIX-associated protein p95PK, the 95-kDa paxillin kinase linker that is known to bind to β-PIX (29), is one of the cellular proteins that coprecipitate with Nef and become phosphorylated by NAK in vitro. While the identities of these phosphoproteins remain to be confirmed, their presence in the anti-PAK2 immunoprecipitates was clearly dependent on the PIX-binding motif of PAK2 (Fig 3, lanes 7 and 8). Nevertheless, disruption of the PIX-binding motif had no effect on the ability of PAK2 to associate with Nef, thus excluding β-PIX and its associated factors as essential adapters in the assembly of the NAK complex.

In contrast to disruption of the protein interaction motifs discussed above, mutations that affected the CRIB motif of PAK2 completely abolished Nef-PAK2 complex formation. Because these mutations also slightly interfered with the negative autoregulation of the kinase activity of PAK2, the presence of this mutant protein could be easily detected in anti-PAK2 immunocomplexes by IVKA despite its failure to interact with and become activated by Cdc42. An interesting question, which needs to be addressed in future investigations, is whether the CRIB motif per se was important for association with Nef or whether changes in PAK2 conformation and/or phosphorylation status caused by its CRIB-mediated activation were more relevant. Our observation that NAK represented a highly active subpopulation of PAK2 that was characterized by an altered electrophoretic mobility would seem to favor the latter possibility. Interestingly, even in cells that were provided with a robust PAK-activating stimulus (transfection of Cdc42V12), only a minute fraction of cellular PAK2 molecules were converted to this NAK-like species. Thus, in addition to the hypothetical SH3 protein involved in Nef-PAK2 interaction, the cellular levels of such activated PAK2 molecules could also be a major limiting factor in assembly of the NAK complex.

So far, the pathophysiological significance of the Nef-NAK interaction has remained unclear. A major reason for the slow progress in this area has been, and still is, the lack of suitable experimental models which would be known to faithfully reflect the pathogenic potential of HIV-1 Nef in infected individuals. A more trivial but yet equally material obstacle has been the lack of understanding of the molecular composition of the Nef-NAK complex, which has prevented the design of experiments involving rational manipulation of this interaction. While the present results shed new light on this question, much remains to be done in order to fully characterize the components, organization, and cellular functions of the multiprotein complex involving Nef and PAK2.

Perhaps the most important new insight into the Nef-NAK complex provided by this study is that NAK represents a small but highly active subpopulation of cellular PAK2. The low stoichiometry of the Nef-NAK complex, as reflected by the absence of sufficient amounts of PAK2 in anti-Nef immunoprecipitates to be readily detected by Western blotting, has seemed incompatible with an important role of this interaction in the cell biology of HIV-1 infection. In the light of the present finding showing that a major part, if not most, of the catalytically active fraction of cellular PAK2 is in complex with Nef, this apparent disagreement could be resolved. Besides explaining past controversies in studies aimed at the identification of NAK, this notion should also help to guide future efforts to uncover the pathophysiological role of this conserved cellular function of Nef.

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REFERENCES

1. **Bagrodia, S., and R. A. Cerione.** 1999. PAK to the future. Trends Cell Biol. **9:**350–355.
2. **Bagrodia, S., S. J. Taylor, C. L. Creasy, J. Chernoff, and R. A. Cerione.** 1995. Identification of a mouse p21Cdc42/Rac activated kinase. J. Biol. Chem. **270:**22731–22737.
3. **Bagrodia, S., S. J. Taylor, K. A. Jordon, L. Van Aelst, and R. A. Cerione.** 1998. A novel regulator of p21-activated kinases. J. Biol. Chem. **273:**23633–23636.
4. **Brown, A., X. Wang, E. Sawai, and C. Cheng-Mayer.** 1999. Activation of the PAK-related kinase by human immunodeficiency virus type 1 Nef in primary human peripheral blood lymphocytes and macrophages leads to phosphorylation of a PIX-p95 complex. J. Virol. **73:**9899–9907.
5. **Burbelo, P. D., D. Drechsel, and A. Hall.** 1995. A conserved binding motif defines numerous candidate target proteins for both Cdc42 and Rac GTPases. J. Biol. Chem. **270:**29071–29074.
6. **Daniels, R. H., and G. M. Bokoch.** 1999. p21-activated protein kinase: a crucial component of morphological signaling? Trends. Biochem. Sci. **24:** 350–355.
7. **Deacon, N. J., A. Tsykin, A. Solomon, K. Smith, M. Ludford-Menting, D. J. Hooker, D. A. McPhee, A. L. Greenway, A. Ellett, and C. Chatfield.** 1995. Genomic structure of an attenuated quasi species of HIV-1 from a blood transfusion donor and recipients. Science **270:**988–991.
8. **Fackler, O. T., X. Lu, J. A. Frost, M. Geyer, B. Jiang, W. Luo, A. Abo, A. S. Alberts, and B. M. Peterlin.** 2000. p21-activated kinase 1 plays a critical role in cellular activation by Nef. Mol. Cell. Biol. **20:**2619–2627.
9. **Kestler, H. W., III, D. J. Ringler, K. Mori, D. L. Panicali, P. K. Sehgal, M. D. Daniel, and R. C. Desrosiers.** 1991. Importance of the nef gene for maintenance of high virus loads and for development of AIDS. Cell **65:**651–662.
10. **Kirchhoff, F., T. C. Greenough, D. B. Brettler, J. L. Sullivan, and R. C. Desrosiers.** 1995. Brief report: absence of intact nef sequences in a long-term survivor with nonprogressive HIV-1 infection. N. Engl. J. Med. **332:**228–232.
11. **Knaus, U. G., and G. M. Bokoch.** 1998. The p21Rac/Cdc42-activated kinases (PAKs). Int. J. Biochem. Cell Biol. **30:**857–862.
12. **Lei, M., W. Lu, W. Meng, M. C. Parrini, M. J. Eck, B. J. Mayer, and S. C. Harrison.** 2000. Structure of PAK1 in an autoinhibited conformation reveals a multistage activation switch. Cell **102:**387–397.
13. **Lu, W., S. Katz, R. Gupta, and B. J. Mayer.** 1997. Activation of Pak by membrane localization mediated by an SH3 domain from the adaptor protein Nck. Curr. Biol. **7:**85–94.
14. **Manninen, A., M. Hiipakka, M. Vihinen, W. Lu, B. J. Mayer, and K. Saksela.** 1998. SH3-domain binding function of HIV-1 Nef is required for association with a PAK-related kinase. Virology **250:**273–282.
15. **Manser, E., T. H. Loo, C. G. Koh, Z. S. Zhao, X. Q. Chen, L. Tan, I. Tan, T. Leung, and L. Lim.** 1998. PAK kinases are directly coupled to the PIX family of nucleotide exchange factors. Mol. Cell. **1:**183–192.
16. **Marsh, J. W.** 1999. The numerous effector functions of Nef. Arch. Biochem. Biophys. **365:**192–198.
17. **Morreale, A., M. Venkatesan, H. R. Mott, D. Owen, D. Nietlispach, P. N. Lowe, and E. D. Laue.** 2000. Structure of cdc42 bound to the GTPase binding domain of PAK. Nat. Struct. Biol. **7:**384–388.
18. **Peter, F.** 1998. HIV nef: the mother of all evil? Immunity **9:**433–437.
19. **Renkema, G. H., A. Manninen, D. A. Mann, M. Harris, and K. Saksela.** 1999. Identification of the Nef-associated kinase as p21-activated kinase 2. Curr. Biol. **9:**1407–1410.
20. **Renkema, G. H., and K. Saksela.** 2000. Interactions of HIV-1 NEF with cellular signal transducing proteins. Front. Biosci. **5:**D268–D283.
21. **Rudel, T., and G. M. Bokoch.** 1997. Membrane and morphological changes in apoptotic cells regulated by caspase-mediated activation of PAK2. Science **276:**1571–1574.
22. **Saksela, K., G. Cheng, and D. Baltimore.** 1995. Proline-rich (PxxP) motifs in HIV-1 Nef bind to SH3 domains of a subset of Src kinases and are required for the enhanced growth of Nef+ viruses but not for down-regulation of CD4. EMBO J. **14:**484–491.
23. **Sawai, E. T., I. H. Khan, P. M. Montbriand, B. M. Peterlin, C. Cheng-Mayer, and P. A. Luciw.** 1996. Activation of PAK by HIV and SIV Nef: importance for AIDS in rhesus macaques. Curr. Biol. **6:**1519–1527.
24. **Schurmann, A., A. F. Mooney, L. C. Sanders, M. A. Sells, H. G. Wang, J. C. Reed, and G. M. Bokoch.** 2000. p21-activated kinase 1 phosphorylates the death agonist Bad and protects cells from apoptosis. Mol. Cell. Biol. **20:**453–461.
25. **Sells, M. A., and J. Chernoff.** 1997. Emerging from the pak: the p21 activated protein kinase family. Trends Cell Biol. **7:**162–167.
26. **Tang, Y., H. Zhou, A. Chen, R. N. Pittman, and J. Field.** 2000. The Akt proto-oncogene links Ras to Pak and cell survival signals. J. Biol. Chem. **275:**9106–9109.
27. **Thompson, G., D. Owen, P. A. Chalk, and P. N. Lowe.** 1998. Delineation of the Cdc42/Rac-binding domain of p21-activated kinase. Biochemistry **37:** 7885–7891.
28. **Tu, H., and M. Wigler.** 1999. Genetic evidence for Pak1 autoinhibition and its release by Cdc42. Mol. Cell. Biol. **19:**602–611.
29. **Turner, C. E., M. C. Brown, J. A. Perrotta, M. C. Riedy, S. N. Nikolopoulos, A. R. McDonald, S. Bagrodia, S. Thomas, and P. S. Leventhal.** 1999. Paxillin LD4 motif binds PAK and PIX through a novel 95-kD ankyrin repeat, ARF-GAP protein: a role in cytoskeletal remodeling. J. Cell Biol. **145:**851–863.
30. **Wiskerchen, M., and C. Cheng-Mayer.** 1996. HIV-1 Nef association with cellular serine kinase correlates with enhanced virion infectivity and efficient proviral DNA synthesis. Virology **224:**292–301.
31. **Zhao, Z. S., E. Manser, X. Q. Chen, C. Chong, T. Leung, and L. Lim.** 1998. A conserved negative regulatory region in alphaPAK: inhibition of PAK kinases reveals their morphological roles downstream of Cdc42 and Racl. Mol. Cell. Biol. **18:**2153–2163.
32. **Zhao, Z.-S., E. Manser, and L. Lim.** 2000. Interaction between PAK and Nck: a template for Nck targets and role of PAK autophosphorylation. Mol. Cell. Biol. **20:**3906–3917.