Identification of the Nef-associated kinase as p21-activated kinase 2

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The Nef protein of primate immunodeficiency viruses plays an important role in the pathogenesis of acquired immunodeficiency syndrome (AIDS) [1,2]. The interaction of Nef with the Nef-associated kinase (NAK) is one of the most conserved properties of different human and simian immunodeficiency virus (HIV and SIV) Nef alleles. The role of NAK association is currently not known but it has been implicated in enhanced viral infectivity in cell culture and in disease progression in SIV-infected macaques [3]. Previous studies have indicated that NAK shares many features with the p21-activated kinases (PAKs) [3], but the molecular identity of NAK has remained unknown. We have generated specific antisera against PAKs 1–3, and expressed these kinases individually as epitope-tagged proteins. By using these reagents in experiments involving partial proteolytic mapping, and exploiting the unique ability of PAK2 to serve as a caspase substrate, we have positively identified NAK as PAK2. Interestingly, although ectopic PAK2 overexpression efficiently replaced endogenous PAK2 from the complex with Nef, the total Nef-associated PAK2 activity was not increased, indicating the abundance of another cellular factor(s) as the limiting factor in Nef–PAK2 complex formation. Identification of NAK as PAK2 should now facilitate elucidation of its role as a mediator of the pathogenic effects of Nef.

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Results and discussion

Detailed studies of the role of NAK in mediating the actions of Nef have been hampered by the failure to identify this kinase. Although PAK1 has been considered a probable candidate [4], the prevailing hypothesis has been that NAK represents a new member of the PAK family [5,6]. PAKs are a subfamily of STE20 kinases and, at present, there are four PAKs: PAK1 (αPAK or PAK65 in rat), PAK2 (hPAK65, rat γ-PAK, rabbit PAK-I), PAK3 (rat β-PAK) and PAK4. In contrast to the other PAKs, PAK4 lacks an amino-terminal proline-rich region that has been shown to target PAK1 to the adapter protein Nck [7,8]. As NAK also has the ability to associate with Nck [5], we ruled out PAK4 as a NAK candidate.

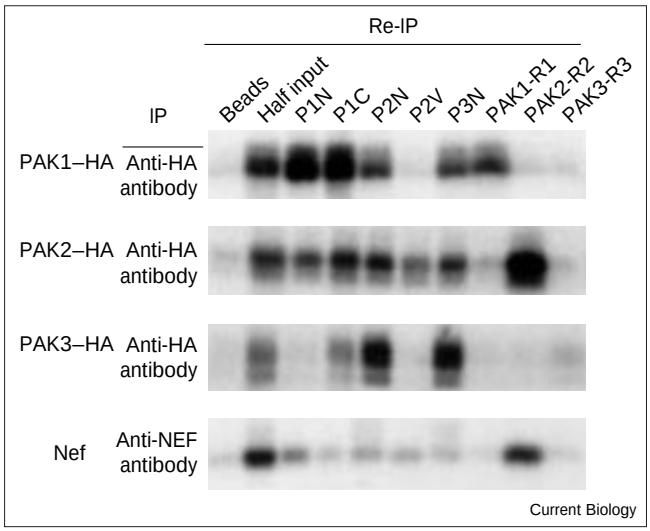
Serological characterisation of NAK

Previous studies have shown that autophosphorylated NAK that has been radiolabelled in an *in vitro* kinase assay and eluted from anti-Nef immunocomplexes can be re-immunoprecipitated by antisera raised against peptides corresponding to the amino- and carboxy-terminal sequences of PAK1 [4,9]. Notably, however, the amino and carboxyl termini of PAK2 and PAK3 share extensive sequence homology with PAK1. Although we could readily confirm the ability of anti-PAK antibodies to re-immunoprecipitate NAK (see later), we found that the amount of anti-PAK reactive material in anti-Nef immunoprecipitates was too low to be detected by western blot using the same antibodies. Therefore, in our further serological studies on the identity of NAK, we chose to use the sensitive *in vitro* kinase assay/re-immunoprecipitation approach.

To produce more specific anti-PAK antibodies, we immunised rabbits with PAK1, PAK2 and PAK3 peptides corresponding to the few divergent regions in these proteins. To test the specificity of our own as well as the commercial anti-PAK antisera, we separately expressed PAK1, 2 or 3 as haemagglutinin (HA)-tagged proteins. These were immunoprecipitated with anti-HA antiserum, eluted from the immunocomplexes with the HA peptide, and subsequently subjected to re-immunoprecipitation using the different anti-PAK antibodies (Figure 1, upper three panels). In parallel experiments, we eluted NAK from anti-Nef antibody immunocomplexes from Nef-transfected cells, and compared the ability of the different anti-PAK antisera to recognise NAK (Figure 1, bottom panel) with their relative specificities in re-immunoprecipitation of HA- tagged PAK 1, 2 and 3.

As shown in Figure 1, our studies confirmed that the widely used amino- and carboxy-terminal anti-PAK1 antisera (P1N and P1C) were able to re-immunoprecipitate NAK that was released from an anti-Nef immunoprecipitation. Nevertheless, from the parallel re-immunoprecipitation of PAKs 1–3 it can be seen that P1N

Figure 1



Re-immunoprecipitation of PAKs 1–3 and NAK using different antibodies. Human embryonic kidney 293T cells were transfected with HA-tagged PAK1, PAK2 or PAK3, or with Nef. All were cotransfected with the dominant active version of Cdc42 (Cdc42V12). Immunoprecipitation (IP) was performed using anti-HA antibody (for PAKs 1–3) or with the 2F2 anti-Nef monoclonal antibody. After *in vitro* kinase assay, the radiolabelled proteins were eluted with the HA epitope peptide (for PAKs 1–3) or with the 2F2 epitope peptide (for Nef). Eluted proteins were re-immunoprecipitated (re-IP) with the different antibodies and run on SDS–polyacrylamide gels. The commercial anti-PAK antibodies used are: P1N, raised against the amino terminus of PAK1; P1C, raised against the carboxyl terminus of PAK1; P2N, raised against the amino terminus of PAK2; P2V, raised against a sequence at the amino terminus of PAK2; and P3N, raised against the amino terminus of PAK3. Antibodies PAK1-R1, PAK2-R2 and PAK3-R3 were raised in rabbits against sequences that are divergent in the different PAK proteins.

and P1C, which efficiently precipitated PAK1 as expected, were also able to precipitate PAK2. Among the commercial antibodies, the only relatively specific antiserum was the P2V antibody, raised against a region in the PAK2 amino terminus. By contrast, the antisera we raised against peptides corresponding to divergent regions of the PAKs were remarkably specific (Figure 1, compare the signals of the lanes PAK1-R1 and PAK2-R2 in the upper three panels). The most notable finding using this set of antibodies was that both the P2V antibody and our anti-PAK2-R2 antibody could precipitate both PAK2 and NAK and not PAK1 and PAK3. From these experiments, we concluded that NAK is immunologically identical to PAK2.

Characterisation of NAK by proteolytic digestion

To further substantiate this conclusion, we performed limited in-gel digestions for the three PAKs and NAK using two different proteases. As seen in Figure 2, PAK2 and NAK had very similar digestion patterns for both chymotrypsin and the endoproteinase Glu-C, whereas the digestion patterns of PAKs 1 and 3 were clearly different from NAK. Minor differences in the relative intensities of fragments in the NAK and PAK2 digestions could well be explained by small differences in the phosphorylation of these proteins.

Although the PAKs are highly homologous proteins, only PAK2 is known to be a substrate of caspase 3 (also known as CPP32 or apopain), one of the downstream effector proteases of the proteolytic cascades occurring during apoptosis [10]. Only PAK2 contains the consensus cleavage site for DEVD-sensitive caspases located between Asp212 and Gly213 in the linker region between the amino-terminal regulatory and the carboxy-terminal kinase domain [11]. We have exploited this remarkable difference between the PAKs to obtain further proof that NAK is indeed PAK2. After immunoprecipitation and subsequent *in vitro* kinase assay, the radiolabelled proteins were incubated with caspase 3 for the indicated time periods. From this experiment (Figure 2b), it can be clearly seen that the only proteins that were cleaved by caspase 3 were PAK2 and NAK. As most (6 out of 7) of the potential PAK2 phosphorylation sites are located in the amino-terminal domain of the protein [12], only this cleavage product was visible in this experimental set-up. From the comparable chymotrypsin and Glu-C digestion patterns and the sensitivity of NAK to caspase 3 we conclude that, in 293T cells, NAK is identical to PAK2

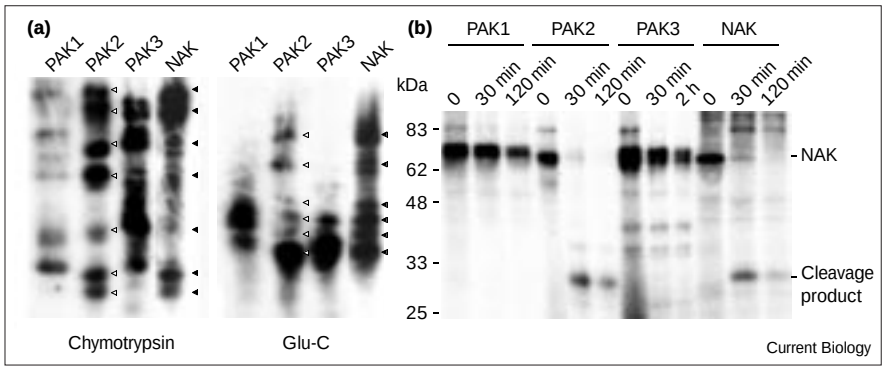
As it was formally possible that NAK represents different PAKs in different cell types, we analysed NAK from Nef-transfected HeLa and Jurkat cells. We found that the chymotrypsin pattern of NAK from 293T cells was identical to the patterns from HeLa and Jurkat cells. Also, NAK from these three different cell types was similarly sensitive to caspase 3 digestion *in vitro* (see Supplementary material).

Except for exploiting the ability of caspase 3 to specifically cleave PAK2, our experimental approaches were not very different from those used in previous studies, which did not lead to the identification of NAK as PAK2 [4,6,9]. Nevertheless, it is easy to see how crossreactivity of the anti-PAK antibodies used for re-immunoprecipitation of NAK from anti-Nef immunocomplexes, as well as the uncertain identity of the kinases precipitated from cells with anti-PAK reagents for comparison of their proteolytic patterns with NAK, could have contributed to the inconclusive results of these studies. In addition to serological characterisation, previous studies aimed at identifying NAK used dominant-negative forms of different PAKs. Because of the extensive homology of the PAKs, however, such results are equally prone to misinterpretation, as different dominant-negative PAKs are expected to be cross-inhibitory.

Transfected PAK2 can substitute for endogenous NAK

Having identified NAK as PAK2, we were puzzled by our initial findings indicating that overexpression of PAK2 did

Figure 2



Proteolytic comparison of NAK with PAKs 1–3. Cells (293T) were transfected with HA-tagged PAK1, PAK2, PAK3, or Nef. All were cotransfected with Cdc42V12. Immunoprecipitations were performed with anti-HA antibody (for PAK-transfected cells) or with sheep anti-Nef antibody (for Nef-transfected cells). (a) After an *in vitro* kinase assay the radiolabeled PAK/NAK proteins were excised from a preparative gel and subjected to a subsequent in-gel digestion electrophoresis with chymotrypsin and Glu-C. The open and filled arrowheads indicate the corresponding proteolytic fragments in the PAK2 and NAK lanes in this audiograph. (b) After the *in vitro* kinase assay, these reaction products were subjected to caspase 3 digestion for the indicated time periods and analysed by electrophoresis.

not increase the NAK signal. Indeed, when we repeated these experiments by transfecting PAK2–HA along with Nef, we did not see an increase in NAK signal compared with transfection of Nef alone (Figure 3, uppermost lanes). We did, however, observe a small upward shift in the mobility of the NAK when HA-tagged PAK2 was coexpressed, suggesting that the overexpressed PAK2–HA, which has a slightly slower mobility because of the tag, might be able to replace the endogenous PAK2 in the complex with Nef. We addressed this by eluting the NAK band from anti-Nef immunoprecipitates from cells transfected with either only Nef or with Nef and PAK2–HA. As a control, cells cotransfected with Nef and PAK1–HA were used. The eluted NAK was then re-immunoprecipitated with the PAK2-R2 antiserum or with anti-HA antibody. NAK from cells transfected with Nef alone could only be re-precipitated with the PAK2 antiserum but not with the anti-HA antibody, and therefore represented endogenous PAK2. NAK from cells cotransfected with PAK2 could be precipitated with both antisera, however, proving that overexpressed PAK2–HA could replace the endogenous PAK2 in the Nef–NAK complex. By contrast, PAK1–HA could not replace the endogenous PAK2 as the NAK band from cells cotransfected with PAK1–HA could only be precipitated with the PAK2 antiserum, but not with anti-HA antibody. Similar, high-level expression of PAK1–HA and PAK2–HA in these experiments was confirmed by western blot analysis (data not shown). Thus, although overexpression of PAK2–HA did not increase the NAK signal, it could replace the endogenous PAK2 in the complex with Nef, suggesting that another component(s) in the complex is limiting.

PAKs have been implicated in many different functions [13], such as cytoskeletal regulation, activation of the Jun N-terminal kinase (JNK)/p38 mitogen-activated protein (MAP) kinase signalling pathway and apoptotic signalling. Many of these functions could be involved in regulation of the replicative cycle of HIV, and thus represent potential targets of Nef action. It is possible that Nef could modulate the effects of PAK2 in mediating signalling from the cytoplasm into the nucleus and/or in inducing re-organisation of the actin cytoskeleton, both of which could facilitate some step(s) in the HIV life cycle, such as transcription, reverse transcription, or budding. Our current observation that Nef specifically interacts with PAK2, the only PAK family member that is cleaved and activated by pro-apoptotic caspases, adds even more possibilities to this list. For example, the Nef–PAK2 interaction might modulate the sensitivity of HIV-infected cells to apoptosis or, alternatively, induce some apoptosis-like changes in the cellular physiology, which could favour HIV replication without causing an immediate destruction of the infected cells.

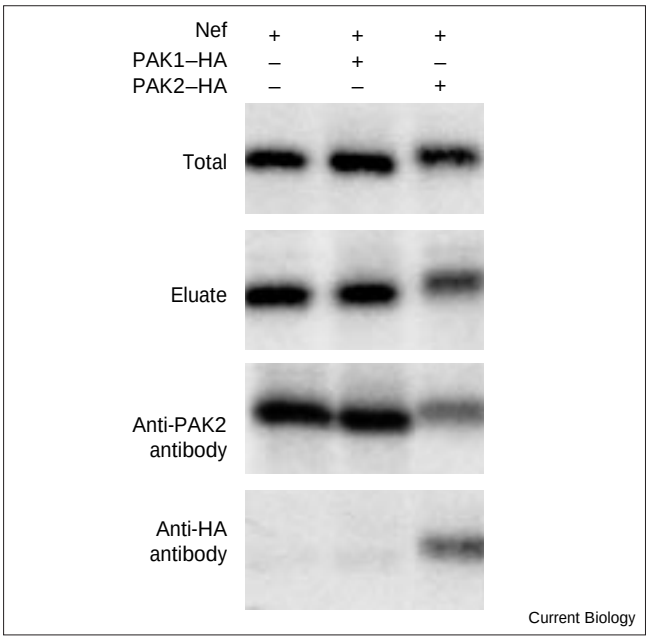
Identification of NAK as PAK2 will now enable more sophisticated experiments to address these and other outstanding questions regarding the role of PAK2 as a mediator of the pathogenic effects of Nef, as well as to examine its value as a potential therapeutic target in AIDS.

Materials and methods

Plasmids and transfections

Transfections to 293T human embryonic kidney fibroblast-derived cells were done using the lipofectamine transfection agent (Gibco BRL) according to the manufacturer's instructions. Plasmid pEBB-PAK1HA [8] was kindly provided by B. Mayer (Harvard); pEBB-PAK2HA was generated by replacing the *Bam*H1–*Kpn*1 fragment of pEBB-PAK1HA containing the PAK1 coding sequence by a PCR product containing the PAK2 sequence derived from human cDNA. Similarly, pEBB-PAK3HA was made by cloning a rat PAK3-containing fragment generated by PCR from pXJ40-HA-PAKβ (kindly provided by E. Manser). The NL4-3 Nef allele R71 [14] was cloned into the pEBB expression vector, without the HA epitope tag. B. Mayer kindly provided pEBG-cdc42V12, which expresses a fusion of glutathione-S-transferase (GST) with dominant-active Cdc42.

Figure 3



Cotransfection of Nef and PAK2. Cells (293T) were transfected with the plasmids encoding the indicated proteins. All cells were also transfected with the Cdc42V12 plasmid. Cell extracts were immunoprecipitated with the 2F2 anti-Nef antibody and subjected to *in vitro* kinase assay. Part of each sample was subjected to electrophoresis (total); the rest was eluted with the 2F2 epitope peptide. Part of the eluate was analysed directly on gel (eluate); the rest was split into two fractions, one of which was re-immunoprecipitated with the PAK2-R2 antibody, the other with the anti-HA antibody before they were subjected to electrophoresis.

Antibodies

The Anti-Nef antibodies used were a polyclonal sheep serum raised against GST–Nef (M. Harris) and a monoclonal antibody (2F2 [15], kindly provided by Vladimir Ovod, IMT, Tampere) that was raised against a peptide (amino acids 151–170) of the HIVnef BRU isolate. Anti-PAK antibodies P1N, P1C, P2N, PAK2V and P3N were purchased from Santa Cruz Biotechnology. Antibodies against PAK1-R1, PAK2-R2 and PAK3-R3 were raised in rabbits against cocktails of selected sequences that are divergent in the different PAK proteins. Peptides were made as multiple-antigen peptides, $(peptide)_8–K_4–K_2–K–A$. Anti-HA antibodies were derived from BAbCO.

Re-immunoprecipitation

After immunoprecipitations and subsequent *in vitro* kinase assays (performed as described before [5]), beads were washed with PBS and incubated with 1 mg/ml of the peptide corresponding to the epitope used for immunoprecipitation. Eluted proteins were subjected to beads pre-bound with antibodies and incubated overnight at 4°C. Beads were washed with PBS and boiled in SDS sample buffer. Proteins were run on SDS–PAGE and visualised by autoradiography.

Limited in-gel protease digestion

Radiolabelled bands were cut from SDS–polyacrylamide gels and allowed to rehydrate in 1:10 diluted SDS–PAGE sample buffer. Gel pieces were transferred to the wells of a 13% SDS–polyacrylamide gel and minced with a needle. Chymotrypsin (500 ng per well, Sigma) or endoproteinase Glu-C from *Staphylococcus aureus* V8 (2 μg per well, Sigma) were added in 1:10 sample buffer. After stacking, electrophoreses was stopped for 30 min and then continued overnight. After electrophoreses, the gel was dried and exposed to Biomax MR film (Kodak).

In vitro *caspase 3 digestion*

After the *in vitro* kinase assay, as described above, beads were washed with PBS, equilibrated with digestion buffer (DB, 25 mM HEPES pH 7.4, 1 mM DTT), and incubated with 40 ng caspase 3 in DB (Calbiochem) at 37°C for the indicated time points. The reactions were stopped by the addition of SDS–PAGE sample buffer.

Supplementary material

Supplementary material including additional methodological detail and a figure demonstrating that NAK from 293T cells is identical to NAK from Jurkat T cells and HeLa cells is available at http://current-biology.com/supmat/supmatin.htm

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Supplementary material

Identification of the Nef-associated kinase as p21-activated kinase 2

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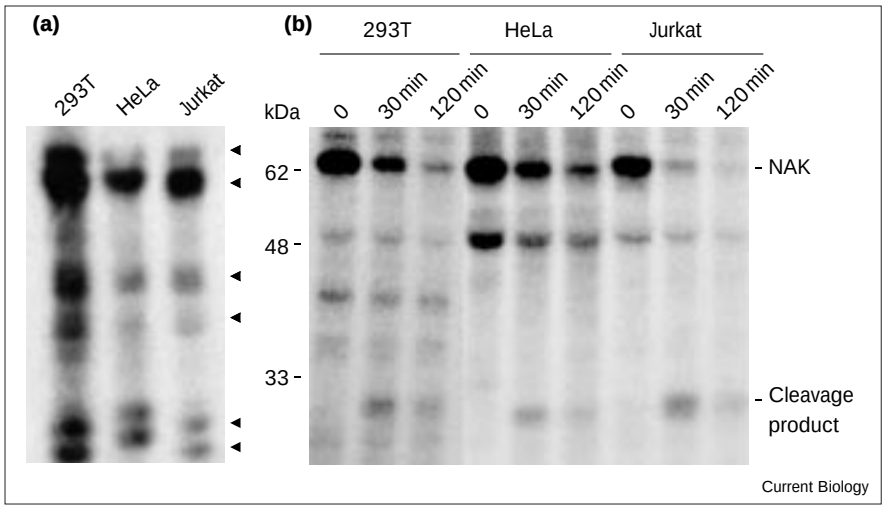
Figure S1

In-gel digestion and caspase 3 sensitivity of NAK from different cell types. Extracts of 293T, HeLa and Jurkat cells expressing Nef were immunoprecipitated with the sheep anti-Nef antiserum. (a) *In vitro* kinase assays were subjected to electrophoresis and the radiolabelled protein bands (arrowheads) were cut out and subjected to in-gel digestion with chymotrypsin. (b) After *in vitro* kinase assay, caspase 3 digestion was performed for the indicated time periods.



Supplementary materials and methods

Generation of cell lines containing stably transfected inducible Nef constructs, HeLa (BH10) and Jurkat (51–31), and the control Jurkat line (MT-11) have been described [S1]. The low basal level of Nef expression from these regulatable but leaky constructs was sufficient for optimal NAK signal in these cell lines.

Supplementary reference

S1. Cooke SJ, Coates K, Barton CH, Biggs TE, Barrett SJ, Cochrane A, *et al.*: Regulated expression vectors demonstrate cell-type-specific sensitivity to human immunodeficiency virus type 1 Nef-induced cytostasis. *J Gen Virol* 1997, 78:381-392.