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A New Constitutively Active Brain PAK3 Isoform Displays Modified Specificities toward Rac and Cdc42 GTPases*

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Véronique Rousseau‡, Olivier Goupille‡§, Nathalie Morin¶, and Jean-Vianney Barnier‡||

From the ‡Laboratoire de Neurobiologie Cellulaire et Moléculaire, CNRS, 1 Avenue de la Terrasse, 91198 Gif sur Yvette, France and ¶Centre de Recherche de Biochimie Macromoléculaire, CNRS, 34033 Montpellier, France

p21-activated kinases (PAK) are involved in the control of cytoskeleton dynamics and cell cycle progression. Here we report the characterization of a new mammalian PAK3 mRNA that contains a 45-bp alternatively spliced exon. This exon encodes for 15 amino acids that are inserted in the regulatory domain, inside the autoinhibitory domain but outside the Cdc42 and Rac interactive binding domain. The transcript of the 68-kDa new isoform named PAK3b is expressed in various areas of the adult mouse brain. In contrast to PAK3 without the exon b (PAK3a), whose basal kinase activity is weak in resting cells, PAK3b displays a high kinase activity in starved cells that is not further stimulated by active GTPases. Indeed, we demonstrate that the autoinhibitory domain of PAK3b no longer inhibits the kinase activity of PAK3. Moreover, we show that the 15-amino acid insertion within the autoinhibitory domain impedes the ability of PAK3b to bind to the GTPases Rac and Cdc42 and changes its specificity toward the GTPases. Altogether, our results show that the new PAK3b isoform has unique properties and would signal differently from PAK3a in neurons.

p21-activated kinases (PAKs)¹ are serine/threonine kinases that bind to and in some cases are catalytically activated by Rac and Cdc42 GTPases (1, 2). They were shown to actively regulate the actin cytoskeleton dynamics through a number of different targets. They control cell fate commitment, by acting as upstream regulators during cell cycle progression and apoptosis (3–7). Their function in the control of neurite outgrowth and growth cone collapse (8–11) may explain the discovery that mutations in the human PAK3 gene are responsible for X-linked mental retardation (12, 13).

Mammalian PAK1 to -6 as well as *Drosophila*, *Caenorhabditis elegans*, and yeast PAKs (2, 14) all share a highly conserved CRIB domain inside the GTPase-binding domain/p21-binding domain (PBD). That domain is also conserved among most other Cdc42 and Rac effectors (15). Mammalian PAKs of

subgroup I (PAK1/ α , PAK2/ γ , and PAK3/ β) share another feature, which is an autoinhibitory domain (AID) partially overlapping the CRIB domain (2, 16, 17). The AID is composed of a dimerization segment, an inhibitory switch segment (IS), and a kinase-inhibitory segment (18). Intermolecular interactions were shown to occur between the conserved AID and the catalytic domain of two different kinase monomers, resulting in inactive PAK dimers under resting conditions (18, 19).

The main mechanism of PAK catalytic activation results from their interaction with the GTP-bound form of the GTPases Rac and Cdc42 (1). GTPase binding to the PAK CRIB domain induces a conformational change of the inhibitory switch in the AID, which interrupts the interaction between the AID and the catalytic domain and leads to monomeric PAK in an opened conformation (18). Autophosphorylation at several sites in both the regulatory domain and in the kinase domain then occurs and maintains the open conformation, allowing kinase activation (20). However, membrane recruitment of PAK is also required for its activation (21), and to date several potential mechanisms have been described. Indeed, under resting conditions, PAK1 to -3 interact with the Nck adapter that upon activation links tyrosine kinase receptors and brings PAK to the membrane (for a review, see Ref. 22). However, PAKs also form stable complexes with the guanine exchange factors for Rac and Cdc42 of the PIX/Cool family, and these interactions are not only closely involved in targeting PAK to the membrane but also in regulating its kinase activity (23–25).

Despite sharing high sequence identity, PAK1, -2, and -3 differ in pattern of expression. Indeed, PAK2 is ubiquitously expressed and is activated by proteolytic cleavage during apoptosis and plays a cytostatic role (26). On the other hand, PAK1 and PAK3 are restrained to the nervous system (27) and are regulated at the time of the growth cone guidance (10). But to date, no specific functions were attributed to either neuronal PAK1 or PAK3.

Here we report the first characterization of a splice variant of the mouse PAK3 gene we isolated from brain mRNA. This new cDNA contains an alternatively spliced exon (exon b) of 45 bp that is totally conserved in humans. The 15 amino acids encoded by this exon are located in the regulatory domain, inside the inhibitory switch. This PAK3b isoform is abundant in the brain and possesses high basal kinase activity that is not further activated by active Cdc42 proteins. Moreover, the alternatively spliced exon b inhibits interactions with active GTPases Rac and Cdc42. Our results strongly indicate that PAK3b may act through original pathways designed for specific functions in neuronal signaling.

EXPERIMENTAL PROCEDURES

cDNA Cloning of PAK3 Isoforms—An RT-PCR-based approach was used to isolate cDNAs encoding PAK3 isoforms from adult mouse brain

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|| To whom correspondence should be addressed: Laboratoire de Neurobiologie Cellulaire et Moléculaire, CNRS, 1 Avenue de la Terrasse, 91198 Gif sur Yvette, France. Tel.: 33-1-69-82-34-18; Fax: 33-1-69-82-94-66; E-mail: barnier@nbcn.cnrs-gif.fr.

¹ The abbreviations used are: PAK, p21-activated kinase; CRIB, Cdc42/Rac-interactive binding; PBD, p21 binding domain; AID, autoinhibitory domain; IS, inhibitory switch segment; RT, reverse transcription; HA, hemagglutinin; GFP, green fluorescent protein; GST, glutathione S-transferase; MBP, myelin basic protein.

mRNA. Based on published mouse PAK3 coding sequence (15, 28), a set of two primers containing engineered *Bam*HI and *Xba*I restriction sites (underlined) was designed to amplify the open reading frames of mouse PAK3 (primer set 1: 5'-CCGGATCCAGGATCTGACAGCTTTGGATAAC-G-3' and 5'-GGCTCTAGACTAACGGCTACTGTTCTTAATTGC-3'). A high fidelity *Pfu* polymerase (Promega) was used for the amplification, and the PCR products were cloned into TOPO 2.1 vector (Invitrogen) following the manufacturer's instructions and sequenced. The isoform without insertion was named PAK3a, and the isoform containing the insert was named PAK3b. The accession numbers for the mouse PAK3a and PAK3b are AJ496262 and AJ496263, respectively. The amino acid numbering of PAK3 used in this article corresponds to the coding sequence of mammalian PAK3 protein.

Plasmid Construction—The *Bam*HI/*Xba*I fragments of the PAK3a and PAK3b cDNAs were cloned into the pcDNA3 vector in order to obtain HA-tagged PAK3 expression plasmids named pHA-PAK3a and pHA-PAK3b, respectively. Mutants and constructs were prepared from pHA-PAK3a and pHA-PAK3b plasmids with *Pfu* polymerase, using procedures based upon the QuickChange protocol (Stratagene) and confirmed by sequencing. In order to obtain a constitutive activation of the kinase, the oligonucleotide set (5'-CCTGAGCAAAGTAACGAAG-CGAGATGGTGGAACTCCCTAT-3' and 5'-CCAATAGGGAGTTCC-ACCATCTCGCTTCTGTTACTTTGCTC-3') was used for site-directed mutagenesis of the threonine residue 421 to a glutamate residue, as initially described (29, 30). In order to obtain dead kinase mutants, the oligonucleotide set (5'-CTGACAAGAGGTGGCCATCTGCAGATGACCTTCAACAGCAGCC-3' and 5'-GGCTGCTGTTGAAGGTTTCATCTGCAGTATGGCCACCTCTTGTCCAG-3') was used to change the lysine residue 297 to leucine, located in the putative ATP binding site of the kinase domain of PAK3. The constitutively active mutant was named pHA-PAK3a-ca, and kinase-dead mutants were named pHA-PAK3a-kd and pHA-PAK3b-kd.

The PBD domains of PAK3 (amino acids Lys⁶⁵–Lys¹³⁶) (28) were amplified with the oligonucleotide set (5'-CCGGATCCAAAGAGCGCC-CAGAGATCTC-3' and 5'-GACTCGAGTCATTTCTGGTTGTTGACCG-TTTC-3'). The *Bam*HI/*Xho*I fragments were digested and subcloned into pGEX-6P vector (Amersham Biosciences), leading to pGST-PBD-PAK3a and pGST-PBD-PAK3b constructs. The AIDs of PAK3 isoforms (amino acids His⁷⁸–Ser¹⁴⁶) (17) were amplified with the oligonucleotide set (5'-GCGGATCCATACGATTCATGTGGGT-3' and 5'-GCCTCGAG-TTAACCTTTATCTCCTGACGT-3'). Fragments were *Bam*HI/*Xho*I subcloned into pGEX-6P (Amersham Biosciences), leading to pGST-AID-PAK3a and pGST-AID-PAK3b constructs.

The vector pEGFP-C3 was purchased from Clontech. Plasmids expressing GFP-fused active GTPases (RhoAG14V, RhoQ77L, RhoGG12V, Rac1G12V, Cdc42G12V, TC10Q75L, TCLQ79L, and ChpQ89L) were a generous gift from P. Fort (CNRS, CRBM, Montpellier). The GST-Rac1wt, GST-Cdc42wt, and GST-Cdc42V12 prokaryotic expression plasmids were previously described (3).

Expression Analysis by RT-PCR—RNAs from human brain or from adult mouse tissues were prepared and reverse transcribed as previously described (31). Expression of the new PAK3b isoform was characterized from mouse or human brain cDNA using an exon b-specific sense primer and a species-specific reverse primer, leading to a mouse set (primer set 2: 5'-CCAGATCTCTATGGCTCACAG-3' and 5'-GGAG-GAGCCAAAGGAGGTTTC-3') or a human set (primer set 3: 5'-CCAGATCTCTATGGCTCACAG-3' and 5'-GGAGGGCCAATGGAGGC-3'), giving rise to a 275-bp fragment. A mouse multiple tissue cDNA panel was used to analyze the expression of PAK3 isoforms by using the exon b-specific primer set 2 as mentioned above and the mouse PAK3 isoform primer set 4 (5'-TGAGCAATGGGCACGACTAC-3' and 5'-CTTGGTG-CAATGACAGCGG-3'), giving rise to a 297-bp fragment. To confirm successful reverse transcription and to normalize the samples, a 500-bp fragment of β -actin was amplified in parallel using primer set 5 (5'-CAACCGTAAAAGATGACC-3' and 5'-AATTGAATGTAGTTTCATG-GATG-3'). The PCR products were separated on agarose gel and analyzed after ethidium bromide staining.

Purification of Recombinant Proteins—GST-PBD-PAK3a, GST-PBD-PAK3b, GST-AID-PAK3a, GST-AID-PAK3b, GST-Rac1wt, GST-Cdc42wt, and GST-Cdc42V12 recombinant proteins were expressed in *Escherichia coli* and purified on glutathione-agarose beads as described by the manufacturer (Amersham Biosciences).

Antibodies—Antibodies to HA (12CA5) and to GFP were purchased from Roche Molecular Biochemicals. The anti-PAK3-N19 antibody (N19) directed against the 19 N-terminal amino acids was purchased from Santa Cruz Biotechnology, and the anti-phospho-PAK1 (Thr⁴²³), which also recognizes the phospho-Thr⁴²¹ of PAK3, was purchased from Cell Signaling Technology.

A rabbit polyclonal antiserum was raised against the exon b-encoded synthetic peptide PDLYGSQMCPGKLP conjugated to keyhole limpet hemocyanin (Sigma). Antibodies were affinity-purified by Ultralink column chromatography (Pierce) after covalent attachment of the corresponding peptide, following the manufacturer's instructions. For Western blot analysis, protein samples were separated by 10% SDS-PAGE and transferred to polyvinylidene difluoride membrane (Millipore). Immunodetection was performed using the SuperSignal chemiluminescent reagent (Pierce). Quantification of chemiluminescence was performed after acquisition with a CDD camera (SynGene) and quantification software (GeneSnap and GeneTools; SynGene).

Cell Culture and Transfections—COS-7 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 units/ml penicillin, and 100 μ g/ml streptomycin (Invitrogen). Plasmid DNA (10 μ g) was transfected into 3×10^6 COS-7 cells using the electroporation method with an electroporator (Bio-Rad).

Immunoprecipitation and in Vitro Kinase Assay—Transfected cells were lysed as previously described (31). In some experiments, in order to activate the kinase, cleared cell extracts from PAK3-transfected cells were incubated with 5 μ g of recombinant Cdc42V12 in the presence of 25 μ M ATP during 30 min at room temperature. Extracts were then immunoprecipitated by incubating with 4 μ l of 12CA5 anti-HA antibody plus 40 μ l of Pansorbin (Calbiochem) for 3 h at 4 °C. After washing, aliquots of immunocomplexes were subjected to immunoblotting to ensure that PAK proteins were correctly expressed and immunoprecipitated.

For kinase reactions, immunoprecipitates were washed once more time in the kinase buffer (25 mM HEPES, pH 7.4, 25 mM MgCl₂, 25 mM β -glycerophosphate, 2 mM dithiothreitol, 0.1 mM orthovanadate). Immunoprecipitates were then incubated in the kinase buffer containing 20 μ M ATP and 5 μ Ci of [γ -³²P]ATP (Amersham Biosciences) for 20 min at 30 °C, in the absence or in the presence of H2B (3 μ g; Sigma) or MBP (3 μ g; Invitrogen) as a substrate. Boiling in SDS-Laemmli sample buffer stopped the reaction, and the products were resolved by SDS-PAGE. The incorporation of ³²P was quantified using a PhosphorImager (Amersham Biosciences).

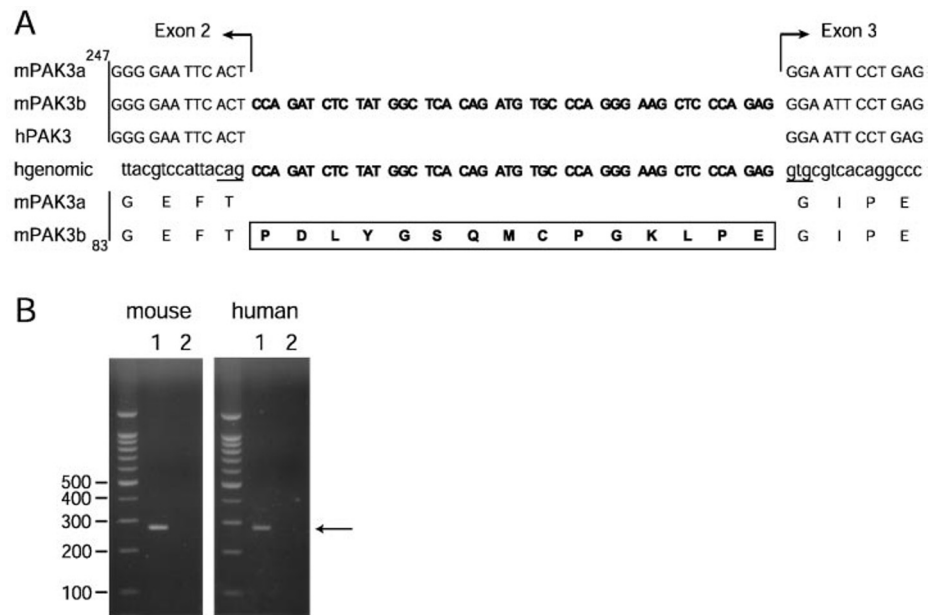
For the kinase activity inhibition by AID, immunoprecipitated HA-PAK3a protein was incubated with increasing concentrations of GST-AID-PAK3 proteins in the presence of Cdc42V12 and ATP for 30 min at 30 °C before performing a kinase assay as previously described (17).

GTPase Overlay, Pull-down Assay, and Co-immunoprecipitation—The overlay assay was done as previously described by Faure *et al.* (3). Briefly, 1 μ g of purified recombinant GST alone or in fusion with PBD-PAK3a or PBD-PAK3b was loaded onto a 12% SDS-polyacrylamide gel and transferred to polyvinylidene difluoride membrane, and the overlay assay was performed using recombinant purified wild-type GST-Cdc42wt or GST-Rac1wt loaded with [γ -³⁵S]GTP (3). For overlay assays with full-length PAK3 proteins, the same procedure was used from HA-PAK3 plasmid-expressing COS-7 cells. Briefly, COS-7 cells were transfected with pHA-PAK3a, pHA-PAK3b, or mutants. The amount of PAK3 protein in the immune complexes was first determined by Western blotting on an aliquot. Samples containing equal amounts of PAK3 proteins were used for the overlay assay as described before. Quantification of the binding of [³⁵S]GTPases was done using a PhosphorImager.

Pull-down assays were performed as described by Vignal *et al.* (32). Briefly, COS-7 cells were transfected with constructs expressing GFP alone or fused to the constitutive active mutants of GTPases of the Rho/Rac/Cdc42 family. 30 h later, cells were washed in cold phosphate-buffered saline and lysed in 50 mM Tris-HCl, pH 7.5, 2 mM MgCl₂, 1% Triton, 100 mM NaCl, 10% glycerol, and protein inhibitor mixture. After 15 min on ice, lysates were cleared by centrifugation at 20,000 \times g for 15 min. Cleared cell extracts were incubated with 20 μ g of GST, GST-PBD-PAK3a, or GST-PBD-PAK3b recombinant proteins, immobilized on glutathione beads, for 30 min at 4 °C. The beads were washed with the lysis buffer, and precipitated proteins were analyzed by Western blotting, using the anti-GFP antibody.

Co-immunoprecipitation was performed as described by Reeder *et al.* (33). Briefly, COS-7 cells were transfected with 5 μ g of active GFP-tagged Rac or Cdc42 along with 5 μ g of HA-tagged PAK3a and PAK3b constructs. Cell lysates in Robert's lysis buffer were immunoprecipitated overnight with anti-HA antibodies (anti-HA affinity matrix; Roche Molecular Biochemicals). Immune complexes were separated by electrophoresis and transferred to polyvinylidene difluoride membranes before Western blotting analysis with anti-PAK3 N19 antibodies to detect PAK3 isoforms or with anti-GFP antibodies to detect co-precipitated Cdc42 or Rac1 proteins.

FIG. 1. Identification of the mammalian PAK3 alternatively spliced exon b. *A*, partial nucleotide and predicted amino acid sequences of the new mouse PAK3 insert and sequence alignment between mouse and human PAK3 isoforms at the border of exons 2 and 3 (indicated with *arrows*). The *numbering* refers to published sequences and starts at the ATG/initiation codon of the mouse PAK3 gene. Exonic sequences are indicated by *capital letters*, and intronic sequences are indicated by *lowercase letters*. The insert sequence is indicated in *bold-face letters*. *B*, RT-PCR analysis of the expression of the new PAK3b isoform. Amplification reactions that were performed from mouse and human brain RNAs (*lanes 1*) or no RNA as negative control (*lanes 2*) using an exon b-specific primer set were loaded on 2% agarose gels. Size markers are indicated in bp. The lengths of amplified DNA fragments are in good agreement with the expected size.



RESULTS

Identification of a New PAK3 cDNA—In order to clone mouse PAK3 cDNA, we performed RT-PCRs on mouse brain mRNAs and subcloned and sequenced the PCR-amplified products. The sequence of the mouse gene we isolated (accession number AJ496262) is slightly different from published sequences (accession numbers U39738 and AF082297) (15, 28). The main difference resides in Leu to Phe amino acid substitution at position 271. A Phe residue is present as well in the homologous position in human (AF068864) and rat (U33314) (34, 35). But most importantly, we found that some of the clones contained an additional in-frame 45-bp sequence (Fig. 1A). Sequence of several clones generated by independent RT-PCR confirmed the presence of this insert in certain PAK3 mRNA. Moreover, this sequence was identified in several mouse expressed sequence tags (BE952172, AU08080098, BB621877, and BE952177) (36). The isoform without insertion was named PAK3a, and the isoform containing the insert was named PAK3b (accession number AJ496263).

Using the same approach, we then searched for this insert in different mammalian species. To do so, human and mouse brain RNAs were reverse transcribed and amplified using a specific forward primer for the 45-bp insert and reverse species-specific primers, both designed to amplify a 275-bp fragment. The size of the DNA fragment that we amplified in both species is in agreement with a 45-bp insertion as shown in Fig. 1B. An identical product was obtained from rat brain RNA (data not shown). Finally, a BLAST search conducted in human genomic data banks, using the 45-bp inserted sequence, demonstrated that this sequence is strictly identical to a sequence lying on chromosome X in position Xq22.3-q23 within the human PAK3 gene (34). The sequence is located within the 5540-bp intron lying between exon 2 and exon 3 and is flanked by 4342 and 1172 base pairs of intronic sequences at its 5'- and 3'-end, respectively. The flanking sequences of the 45-bp insert are in good agreement with the consensus for donor and acceptor splicing sites (37). Altogether, these results demonstrate that the 45-bp insertion in mouse PAK3 cDNA defines a new alternatively spliced exon, henceforth referred to as exon b. We further show that this alternatively spliced exon of PAK3 is strictly conserved in different mammalian species.

Identification of the 68-kDa PAK3b Isoform—Exon b translation leads to a 15-amino acid in-frame addition that has no

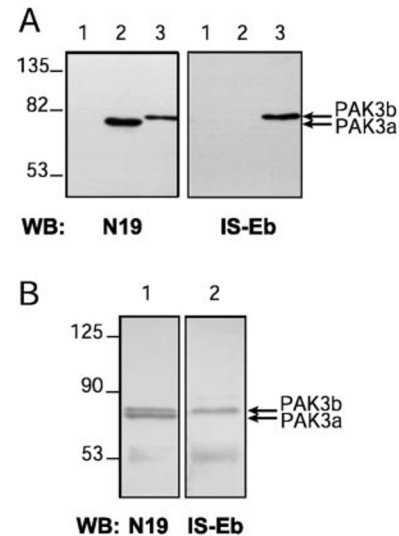


FIG. 2. Immunodetection of the PAK3b isoform. *A*, characterization of the IS-Eb antibodies. Control vector (*lanes 1*), PAK3a (*lanes 2*), and PAK3b (*lanes 3*) encoding plasmids were transfected in COS-7 cells. Lysates were run on SDS-PAGE and analyzed after Western blotting (WB) with antibodies against the PAK3-N terminus region (N19) or the 15-amino acid PAK3 insert (IS-Eb). Molecular masses are indicated in kDa on the left. As shown, IS-Eb antibodies recognize specifically the PAK3b isoform. *B*, PAK3a and -b isoforms were expressed in mouse brain. Mouse brain extracts were immunoprecipitated with the N19-PAK3 antibodies and resolved by Western blotting with the N19 antibodies (*lane 1*) or IS-Eb-specific antibodies (*lane 2*). The *arrows* indicate specific bands. The size difference between recombinant proteins in COS cells and endogenous proteins in brain is due to the presence of a HA tag at the amino-terminal extremity of the recombinant proteins.

significant identity with any sequence in the currently available databases. Alignment of PAK3a, the previously characterized PAK3 isoform without an alternatively spliced exon, with the different PAK3b sequences in the region surrounding exon b is shown in Fig. 1A.

In order to specifically identify PAK3 isoforms, we developed antibodies (IS-Eb) directed against the peptide encoded by the alternatively spliced exon b. To ensure the specificity of IS-Eb antibodies, COS-7 cells were transiently transfected with pHA-PAK3a (Fig. 2A, *lane 2*) and pHA-PAK3b (Fig. 2A, *lane 3*) constructs. Transfected cells were lysed 24 h later and further

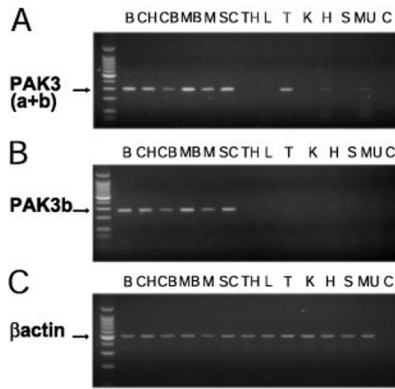


FIG. 3. Semiquantitative RT-PCR analysis of mouse PAK3 and PAK3b isoform expression on different tissues. RT-PCR was performed from RNAs isolated from total brain (B), cerebral hemisphere (CH), cerebellum (CB), midbrain (MB), medulla (M), spinal cord (SC), thymus (TH), liver (L), testis (T), kidney (K), heart (H), spleen (S), skeletal muscle (MU), and negative control (C). Amplification was performed using specific primer sets for PAK3a and -b (primer set 4, upper panel), PAK3b (primer set 2, middle panel), and β -actin for normalization (primer set 5, lower panel).

analyzed in Western blots. In pHA-PAK3b transfected cells, affinity-purified IS-Eb recognized a single 70-kDa protein but failed to detect any band in pHA-PAK3a-transfected COS cells (Fig. 2A). In contrast, the N19-PAK3 polyclonal serum (as well as anti-HA monoclonal antibodies; data not shown) recognized 67- and 70-kDa proteins in pHA-PAK3a- and pHA-PAK3b-transfected cells, respectively (Fig. 2A). Indeed, these controls demonstrate that IS-Eb antibodies are specific of the PAK3b isoform. Next, to detect and identify PAK3 endogenous isoforms in adult mouse brain, we performed immunoprecipitation of PAK3 proteins from brain lysate using the N19-PAK3 serum that reacts with both isoforms. Immunoprecipitates were further analyzed by Western blot and probed with both N19 and IS-Eb sera. Two 65- and 68-kDa protein species were recognized with N19 antibodies, whereas the IS-Eb serum detected a unique 68-kDa species (Fig. 2B). Thus, the latter 68-kDa/PAK3b species is expressed in adult mouse brain at a significant and slightly lower level than PAK3a. In summary, our results show that two different PAK3 isoforms, namely PAK3a and PAK3b, are expressed in adult mouse brain.

Expression—These results raised the question of whether the two PAK3 gene products PAK3a and PAK3b may display a narrow and distinct tissue specificity. To address this question, we studied the expression profile of mouse PAK3b by semiquantitative RT-PCR in various adult tissues. Because PAK3 expression is known to be restricted to brain and, at a lower level, testis (35), we investigated PAK3 RNA expression in different adult mouse tissues as well as in different regions of the brain. First, equal amounts of first strand cDNA were analyzed after normalization using β -actin (Fig. 3C). Then the tissue distribution of both PAK3 mRNAs was investigated by RT-PCR amplification of the carboxyl-terminal part of the regulatory domain, since this domain is not subject to alternative splicing. Our results demonstrate that PAK3 (a and b isoforms) RNAs are highly expressed in the different parts of the adult mouse brain, with a slightly higher expression in the spinal cord and in the midbrain. PAK3 is highly expressed in testis and to a lower extent in heart and muscles. No expression was detected in other tissues (Fig. 3A). In parallel, the analysis of expression of the PAK3b isoform was performed using an exon b-specific forward primer as shown in Fig. 3B. PAK3b expression was detected in the different parts of the brain and in the spinal cord, and, like PAK3a, PAK3b displays a slightly higher expression in midbrain and spinal cord. Nonetheless, PAK3b

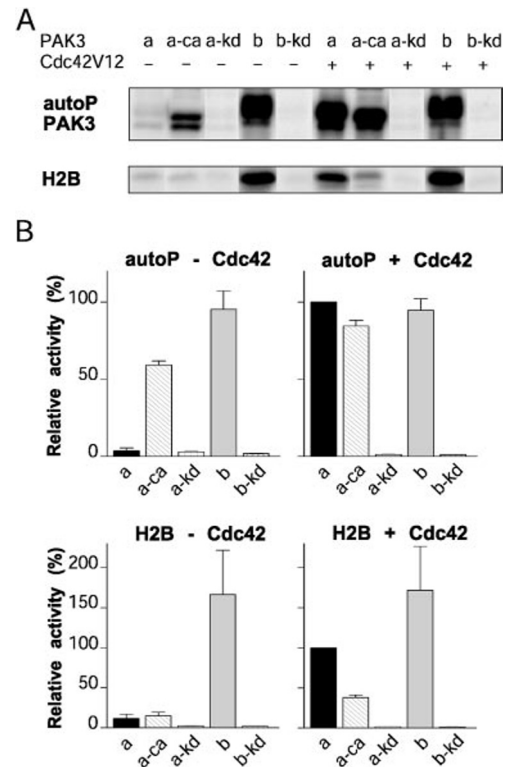


FIG. 4. Kinase activity of PAK3 isoforms and PAK3 mutants. COS-7 cells were transfected with plasmids encoding for wild type PAK3a (a) and PAK3b (b) or for constitutively active kinase mutant PAK3a-ca (a-ca) or kinase-dead mutants PAK3a-kd (a-kd) and PAK3b-kd (b-kd). Cell lysates were incubated in the absence or presence of the active protein Cdc42V12 and immunoprecipitated with anti-HA serum. The immunoprecipitates were subjected to an *in vitro* kinase assay in the absence or presence of H2B as a substrate. Phosphorylated proteins were resolved by SDS-PAGE, and the gel was autoradiographed. Western blotting with aliquots confirmed that similar amounts of recombinant protein were present in each sample (data not shown). A, a representative autoradiography from autophosphorylation of PAK3 proteins in the absence of exogenous substrate (upper panel) or from the phosphorylation of H2B as a substrate (lower panel). B, the histograms shown represent an average of three independent experiments. Gels were scanned and analyzed with an Amersham Biosciences PhosphorImager using ImageQuant software. Results are expressed as percentage of the Cdc42-activated PAK3a kinase activity in each experimental condition (*i.e.* for autophosphorylation (upper histograms) and for H2B phosphorylation (lower histograms)).

transcript distribution is narrower than PAK3a, since, unlike PAK3a, it is not detected in testis. Whereas PAK3b tissue distribution is not specific to any brain territory, it appears to be restricted to the brain in the adult mouse.

Kinase Activity—Since the alternatively spliced exon b is located within the N-terminal regulatory domain of PAK3, we investigated whether its expression might interfere with the intrinsic kinase activity in an *in vitro* kinase assay (Fig. 4). To do so, COS-7 cells were transfected with plasmids coding for either wild type PAK3a (a) and PAK3b (b) isoforms or their corresponding kinase-dead mutants that are mutated on their ATP binding site (a-kd and b-kd) or for a constitutively active mutant for PAK3a (a-ca) (see “Experimental Procedures”). 48 h after transfection, cell lysates were incubated with or without recombinant active Cdc42 protein, and the kinase activity was tested on the purified immune complexes in two ways. First, PAK3 autophosphorylation was estimated in the absence of substrate (*autoP*), and second, kinase activity was tested by measuring the phosphorylation of the histone H2B as a substrate (*H2B*) in independent assays (Fig. 4, A and B). Nevertheless, the addition of the H2B substrate in the kinase assay

did not significantly affect the capacity of PAK3 to autophosphorylate (data not shown). The kinase activity was calculated with reference to the Cdc42-activated PAK3a activity in each condition.

In agreement with previously published results (28, 35), we found that, in the absence of GTPases, wild type PAK3a activity (*a*) measured by autophosphorylation or toward H2B substrate was low and weakly detectable and corresponds to the basal activity. As expected, the kinase-dead mutants of both PAK3a (*a-kd*) and PAK3b (*b-kd*) had no detectable kinase activity. The constitutively active (*a-ca*) mutant displayed a 16-fold increase in autophosphorylation activity in the absence of GTPases. Surprisingly, we reproducibly found no significant increase of the kinase activity of this mutant toward H2B. Very interestingly, we found that wild type PAK3b activity is high in resting cells with an average 25-fold increase in activity compared with wild type PAK3a activity, as measured by autophosphorylation. Note that the PAK3b isoform phosphorylated H2B with a very high efficiency in resting cells. In fact, as seen in Fig. 4, *A* and *B*, PAK3b basal kinase activity is higher than the constitutive PAK3a-ca mutant (compare *a-ca* with *b*).

We then analyzed the potential activation of PAK3a and PAK3b following incubation with a recombinant constitutively active GST-Cdc42V12 protein. As expected, Cdc42 incubation strongly increased PAK3a kinase activity (27-fold in autophosphorylation), whereas no activation was detected for PAK3a-kd protein. The PAK3a-ca mutant is also activated upon Cdc42V12 interaction, although its activity toward the exogenous H2B substrate was repeatedly lower than the activity developed by the activated wild type kinase (PAK3a). Interestingly, the PAK3b isoform was not further activated by active Cdc42. The level of the PAK3b activity was identical to that of Cdc42-activated PAK3a activity.

The GTPase-mediated activation of PAK is accompanied by several autophosphorylation events (29), one of them being the phosphorylation of the threonine 421 that is implicated in activation (38). Thus, to confirm that PAK3b is constitutively active in cells, we looked for its autophosphorylation. Using a specific anti-phosphopeptide Thr⁴²³ (homologous to the Thr⁴²¹ of PAK3), we indeed detected the PAK3b but not the PAK3a isoform by Western blot after immunoprecipitation of PAK3 from transfected COS-7 cells (data not shown). This suggests that PAK3b is constitutively phosphorylated on threonine 421 in resting cells. Altogether, these results show that the PAK3b isoform possesses a high basal kinase activity, which is not stimulated by active Cdc42 protein.

The AID of PAK3b Did Not Inhibit Kinase Activity—The main mechanism of PAK autoinhibition is the inhibitory interaction of the N-terminal part of the protein with the catalytic domain. This interaction is inhibited by phosphorylation of the Thr⁴²¹ residue (17, 18, 39). As previously reported, the peptide, which encompassed residues 78–146 in the AID, is sufficient to inhibit the kinase activity of PAK (17). We thus investigated the respective ability of the PAK3a AID and the modified PAK3b AID to negatively regulate kinase activity of the wild type PAK3a (Fig. 5). AID-PAK3a (AID-3a) and AID-PAK3b (AID-3b) (amino acids 78–146) were purified as recombinant GST fusion proteins. The wild type PAK3a protein was immunoprecipitated from transfected COS-7 cells and then incubated with increasing amounts of recombinant GST-AID and active Cdc42 (Fig. 5). The AID-3a inhibited PAK3a kinase activity with a curve of concentration dependence with an IC₅₀ of about 50 nM (0.1 μg). However, when the same experiment was done with the AID-3b, no inhibition of the kinase activity was detected even at a concentration of 500 nM (1 μg). Thus, the

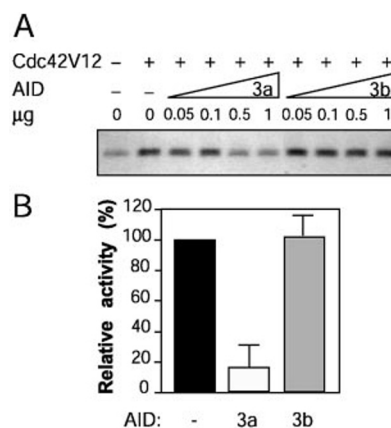


FIG. 5. The autoinhibitory domain of PAK3b does not inhibit kinase activity. Increasing amounts (0.05–1 μg) of recombinant GST-AID proteins of either PAK3a or PAK3b isoforms were incubated with immunoprecipitated HA-PAK3a protein in the presence of active Cdc42V12 and ATP. The reaction was then subjected to an *in vitro* kinase assay in the absence or presence of MBP as a substrate. Phosphorylated proteins were resolved by SDS-PAGE, and the gel was autoradiographed. GST alone did not inhibit the kinase activity of PAK3a (data not shown). *A*, a representative autoradiography from autophosphorylation. Similar results were obtained with MBP substrate (data not shown). *B*, the histogram shows the average of three independent experiments on MBP phosphorylation by PAK3a kinase in the presence of 1 μg of recombinant GST-AID-PAK3a (*3a*) or GST-AID-PAK3b (*3b*). The basal activity value (in the absence of Cdc42V12) was first subtracted from each value. Results are expressed as kinase activity, relative to the Cdc42-activated PAK3a kinase activity (–).

presence of the 15 amino acid insert of PAK3b greatly impairs the inhibitory properties of the autoinhibitory domain.

Interaction with GTPases—We showed that PAK3b kinase activity is not stimulated by active Cdc42. To test whether the 15-amino acid PAK3b insert might impede PAK3b interaction with Rac and Cdc42, we analyzed PAK3 isoforms binding to active GTPases (Fig. 6). GST fusions of PBD-PAK3a (*PBD-3a*), PBD-PAK3b (*PBD-3b*), and GST alone were purified and tested for their ability to bind in overlay assays with [γ -³⁵S]GTP-loaded Cdc42 and Rac1. As expected, Fig. 6*A* shows that the PBD-PAK3a binds both active Rac and Cdc42. In contrast, the binding of PBD-PAK3b to the GTPases is dramatically reduced. Indeed, we quantified PBD-3b binding from several independent experiments and found that it was reduced to 9 and 0% with Cdc42 and Rac1, respectively (Fig. 6*A*, lower panel). We observed the same level of binding inhibition using the entire regulatory domain (fragment 2–272) of PAK3 proteins (data not shown). Thus, our results strongly suggest that the exon b impairs the binding of the PAK3b isoform with Rac and Cdc42 GTPases.

To confirm this result, we analyzed the interaction of wild type PAK3a and PAK3b and the mutant PAK3a-ca, PAK3a-kd, and PAK3b-kd proteins expressed in transfected COS-7 cells with [γ -³⁵S]GTP-loaded Cdc42 and Rac1 GTPases (Fig. 6*B*). We verified by Western blot against PAK3 antibodies that all PAK3 proteins were expressed and immunoprecipitated at similar levels in the transient assay (Fig. 6*B*, upper panel). As shown in Fig. 6*B*, lower panel, GTP-Rac1 bound to PAK3a, PAK3a-ca, and PAK3a-kd proteins with a similar efficiency, suggesting that the GTPase/PAK3a interaction does not depend upon PAK3a kinase activity. In contrast, the PAK3b isoform has a reduced capacity to bind to Rac. Similar results were obtained with GTP-Cdc42. PAK3b binding to Cdc42 and Rac corresponds to 52 and 39%, respectively, of PAK3a binding (Fig. 6*B*, histogram). We observed in several independent experiments that PAK3b-kd always bound to a lesser extent to Cdc42 and Rac than PAK3b. This indicates that the impaired

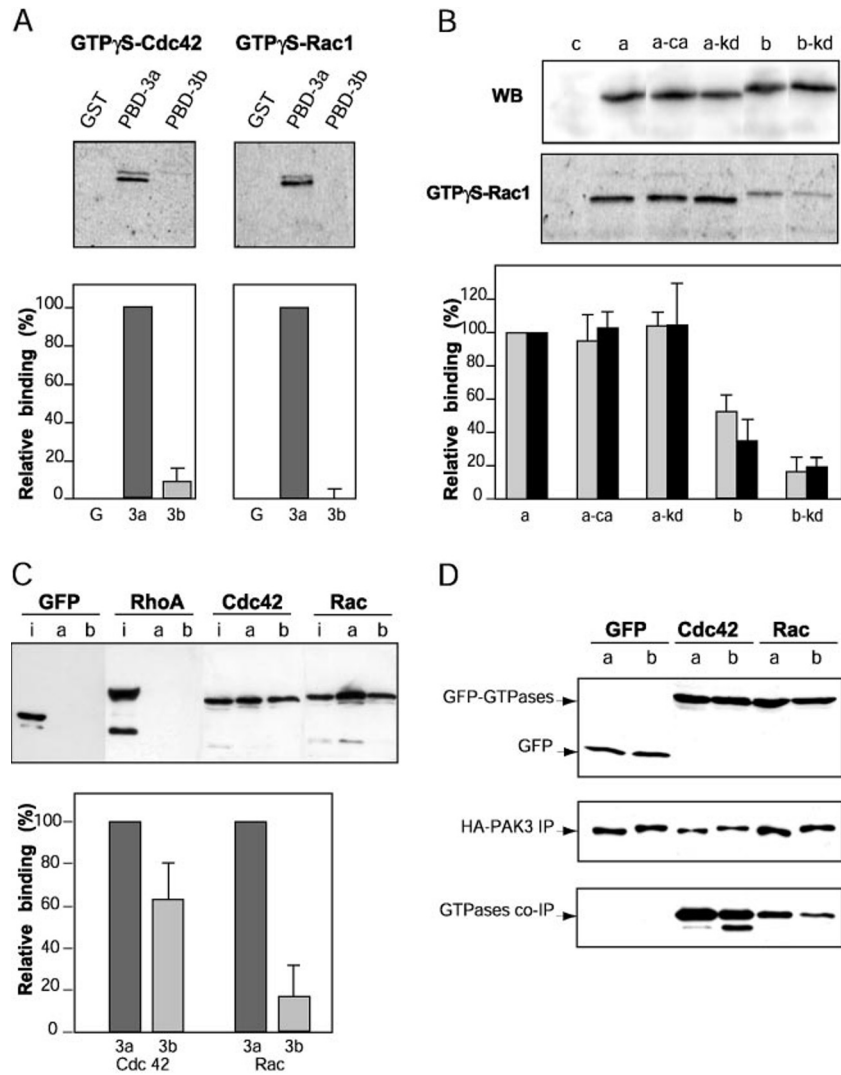


FIG. 6. GTPase binding to PAK3 isoforms. *A*, upper panels, GTPase binding of PBD-PAK3a (PBD-3a) or PBD-PAK3b (PBD-3b) proteins. 1 μ g of purified recombinant protein GST alone or in fusion with PBD-PAK3a or PBD-PAK3b was separated by SDS-PAGE, transferred to polyvinylidene difluoride membranes, and overlaid with [γ - 35 S]GTP-loaded Cdc42 or Rac1. Lower panel, quantification of the relative binding to the active GTPases of PBD-3b compared with PBD-3a binding. These data were averaged from three independent experiments. *B*, expression and GTPase binding of wild type and mutant HA-PAK3 isoforms. COS-7 cells were transfected with plasmids encoding for PAK3a (a) or PAK3b (b), for constitutively active kinase PAK3a mutant (a-ca), and for kinase-dead mutants (a-kd) and (b-kd). PAK proteins were immunoprecipitated, and the amount of PAK proteins in the immune complexes was determined by Western blotting (WB) on an aliquot (upper panel). Samples containing equal amounts of PAK3 proteins were separated by electrophoresis, transferred to polyvinylidene difluoride membrane, and overlaid with [γ - 35 S]GTP-loaded Cdc42 or Rac1. The typical profile of an autoradiography is shown (middle panel). Quantification of interactions with Cdc42 (gray) and Rac (black) from three independent experiments was averaged (histogram, lower panel). *C*, pull-down of active GTPase by PAK3 isoforms. COS-7 cells were transfected with plasmids encoding GFP-tagged active GTPases. Cell lysates were directly loaded (input i) or were incubated with 20 μ g of GST-PAK3-PBD3a (a) or GST-PAK3-PBD3b (b). Retained proteins were run on SDS-PAGE and were analyzed by Western blotting with antibodies against GFP protein. A typical profile is shown. Lower panel, quantification from three independent experiments was averaged after acquisition of the chemiluminescence with a CDD camera. *D*, in vivo interaction of active GTPases with PAK3 isoforms. HA-tagged PAK3 isoforms were co-expressed into COS-7 cells with either GFP as control or active GFP-tagged Cdc42 or Rac. Upper panel, the presence of GFP-GTPases in the cell lysates was controlled by anti-GFP immunoblotting. Middle panel, PAK3 proteins were immunoprecipitated (IP) using anti-HA antibodies, and immune complexes were separated by electrophoresis and transferred to polyvinylidene difluoride membranes. The amount of immunoprecipitated PAK3 proteins was controlled using anti-PAK3 N19 immunoblotting. Lower panel, the presence of the GFP-tagged Cdc42 or Rac protein in the immune complexes was revealed by anti-GFP Western blotting. Data shown are representative of a typical experiment.

PAK3b binding to GTPases we observed is not a consequence of the high kinase activity of PAK3b. These data confirm the results obtained with the recombinant PBD-(65–136) or Nter-PAK3-(2–272) proteins, but the inhibition of binding of PAK3b isoform was lower with the full-length proteins than with recombinant shorter proteins.

To further demonstrate that the expression of exon b interferes with the ability of PAK3 to interact with members of the Rho/Rac/Cdc42 family, we developed an approach based on pull-down assays of the active GTPases with GST alone or with GST-PBD-PAK3a and GST-PBD-PAK3b proteins. COS-7 cells

were transfected with either GFP alone or constitutively active (V12/V14) GFP-tagged GTPase mutants. Transfected cell lysates were incubated with the GST fusion proteins before pull-down (Fig. 6C). As expected, control GST protein alone did not precipitate any GTPase (data not shown), and expressed GFP protein alone, as control, did not interact with any GST-PBD bait. RhoA binds neither to GST-PBD-PAK3a, as initially reported (28, 35), nor to GST-PBD-PAK3b. GST-PBD-PAK3a, indeed, interacts with Rac and Cdc42 (Fig. 6C). GST-PBD-PAK3b did not pull down RhoA either and pulled down Rac and Cdc42 GTPases with a lower efficiency than GST-PBD-PAK3a.

GST-PBD-PAK3b consistently pulled down less Rac than Cdc42 (Fig. 6C, histogram).

Finally, to assess the binding of the PAK3 isoforms to Cdc42 and Rac in an *in vivo* environment, we performed co-immunoprecipitation assays. HA-tagged PAK3 isoforms were co-expressed into COS-7 cells with either GFP as control or active GFP-tagged Cdc42 or Rac. PAK3 proteins were then immunoprecipitated using anti-HA antibodies, and the presence of the associated GTPase in the immune complexes was revealed by anti-GFP immunoblot (Fig. 6D). We ensured that similar levels of active Cdc42 and Rac were present in the cell lysates using anti-GFP immunoblotting (*upper panel*) and verified that PAK3a and PAK3b proteins were immunoprecipitated at similar levels (*middle panel*). Co-immunoprecipitation of active GTPases with HA-tagged PAK3 isoforms is presented in the *bottom panel*. As expected, the two PAK3 isoforms did not interact with control GFP protein, and PAK3a isoform bound active Cdc42 and Rac, although with a lower efficiency for the latter. In contrast, relative to PAK3a binding, the efficiency of PAK3b interaction with Cdc42 and Rac was reduced to 55 and 32%, respectively. We always noticed that two species of Cdc42 were resolved in Western blot, after co-immunoprecipitation with PAK3. This probably results from the cleavage occurring during the immunoprecipitation procedure, since no cleavage was observed in the cell lysate (*upper panel*). For binding quantification, both bands were considered.

Taken together, our results demonstrate that the 15-amino acid insert in PAK3b PBD impairs PAK3b ability to interact correctly with both Rac and Cdc42.

DISCUSSION

In the present study, we have shown that the mouse PAK3 gene, as well as the human gene, encodes two isoforms of 544 and 559 amino acids. The two isoforms are generated by exon skipping of a short sequence of 45 bp. The sequence of the alternatively spliced exon is strictly conserved between mice and humans. Thus, we report here the first characterization of an isoform for a member of the p21-activated kinase family. The alternatively spliced exon codes for 15 amino acids that do not possess any identity with known sequences and appear to be unique for 3b. Thus, the PAK3b mRNA codes for a new isoform identified as a 68-kDa protein that is only slightly less abundant than the PAK3a isoform in adult mouse brain extract.

The PAK3b insert is located between Thr⁹² and Gly⁹³ of the sequence FT*GIP, which is highly conserved in the PAK family and is present in the six mammalian PAK1–6 proteins and in other orthologs of PAK, *i.e.* in *Xenopus*, *Drosophila*, *C. elegans*, *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*, and *Candida albicans* (17, 40). This sequence FT*GIP is located immediately after the CRIB domain, in the overlapping region of the GTPase-binding domain/PBD and of the AID. It adopts different conformations depending on the interaction with the GTPases (18, 41, 42). Indeed, in absence of GTPase, this segment forms a β strand at the extremity of a β hairpin that interacts with the α -helices of the IS domain, packing it in a compact structure (18, 41). The active GTPase interacts with the CRIB domain and with residues of the IS domain, producing a modification of the structure, which allows the G protein to contact the β hairpin and the α -helices of the IS domain and which disrupts the inhibitory conformation. Previous analysis of mutations in PAK sequences, which interfere with GTPase binding and the interaction between the regulatory domain and the catalytic domain, have demonstrated that the region where the PAK3b 15-amino acid insert lies is very critical for both GTPase interaction and regulation of the kinase activity. In-

deed, we found that PAK3b possesses unique GTPase binding properties and kinase activity regulation.

Kinase Activity—The PAK3b isoform clearly differs from the constitutively active mutant T421E. Whereas the mutant PAK1-T423E has only a weak constitutive kinase activity toward the substrate H2B, its ectopic expression is sufficient to deregulate the cell cytoskeleton network in fibroblasts (30, 43). Indeed, PAK3b basal kinase activity is more elevated than the constitutively engineered T421E PAK3-ca mutant and is not, unlike this latter, further activated upon Cdc42 interaction (30, 43). Since the kinase activity of PAK3a-ca and PAK3b are not similarly regulated, it is likely that their biological roles may differ as well. Interestingly, it was reported that the double mutation of Ser⁴²² and Thr⁴²³ stimulates PAK1 activity more than the Thr⁴²³ mutation (29). This highlights the multistep mechanism of PAK activation and the difference between autophosphorylation and phosphorylation of exogenous substrates.

How could the presence of the 15-amino acid insert activate the kinase? Mutations inside the autoinhibitory domain are known to suppress its inhibitory properties (16, 17). It was previously reported that mutations of the conserved residues Phe⁹¹, Gly⁹³, and Pro⁹⁵, which are located near the insertion site, lead to a high basal kinase activity that was not further activated by active Cdc42 (44). Similar results were obtained by a genetic approach in the *S. pombe* PAK1 protein, where the mutation of the homologous residues Phe⁸⁴, Gly⁸⁹, Glu⁹⁰, Phe⁹¹, Thr⁹², Trp⁹⁵, and Leu¹⁰² leads to the suppression of intramolecular interaction and activation of the kinase (45). We have shown that the AID of the PAK3b isoform cannot inhibit the PAK3 kinase activity. Thus, the 15-amino acid insert probably modifies the inhibitory conformation of the IS and disrupts this functional domain. Another hypothesis is that the alternatively spliced exon inhibits the formation of dimers that is necessary to allow transinhibition (19). The dimerization segment, as well as some other regions including the IS segment, contributes to the stabilization of dimers (19). Thus, it is possible that the insert of PAK3b suppresses dimer formation and, by consequence, the associated inactive state of the kinase. Another hypothesis is that the deformation of the closed conformation of the regulatory domain allows the direct phosphorylation of the crucial Thr⁴²¹, which in turn leads to the kinase activation (20, 39). Finally, another possibility is that the insertion could promote the activation of the kinase by one of these previously described mechanisms, which in turn induces the autophosphorylation of two residues, Ser¹³⁹ and Thr⁴²¹, leading to an inhibition of the closed conformation (39, 46). We are currently investigating which of these mechanisms is implicated.

GTPase Binding—We observed a drastic decrease of GTPase binding for the PAK3b isoform. This result was obtained by overlay, pull-down, and co-immunoprecipitation assays. The overlay test was done with three different constructs, corresponding to the PBD domains (fragment 65–136), to the amino-terminal regulatory domain (fragment 2–272), and to full-length proteins from PAK3a and PAK3b isoforms. Pull-down experiments of constitutively active mutants of Cdc42 and Rac were performed with the GST-PBD proteins. Finally, the *in vivo* GTPase interaction was measured after immunoprecipitation of PAK3 proteins from co-transfected cells. The main result we obtained is that the PAK3b isoform interacts significantly less with GTPases than PAK3a. The binding of the PBD or the N-terminal region of PAK3b is between 0 and 10% compared with that of PAK3a. On the other hand, the interaction of the full-length PAK3b protein relative to PAK3a was around 50% for Cdc42 and only 15% for Rac. In the same way,

the co-immunoprecipitation of Cdc42 with PAK3b was diminished to 45%, whereas the co-immunoprecipitation of Rac was reduced to 32%. Thus, we conclude that the presence of the insert decreases GTPase interaction.

The CRIB domain was identified as a conserved motif present in the different target proteins for both Rac and Cdc42 (15). This domain is necessary but not sufficient for a high affinity binding (47, 48). The smallest fragment of PAK that binds small G proteins with high affinity consists of residues Ile⁷⁰–Lys¹⁷³ (47). Some residues, (PAK1 Met⁹⁹, homologous to the PAK3 Ile⁹⁴, and also the conserved residues Trp⁹⁸, Leu¹⁰¹, and Leu¹⁰²), located in the proximity of the insertion, are involved in interaction with the GTPase (41). Particularly, the mutation of PAK1 Gly⁹⁸/PAK3 Gly⁹³ decreases the affinity of PAK for GTPases (17). Thus, it may be possible that the insertion of the exon b changes the IS structure and impedes GTPase interactions in a similar manner.

It was also reported that following activation, the full-length PAK3 protein does not bind active GTPases (1, 35). But the fact that neither the kinase-dead mutant of PAK3b nor its amino-terminal domain devoid of kinase activity was able to bind the GTPases strongly suggests that the inability of PAK3b to bind GTPases is not a consequence of its high basal kinase activity. On the contrary, the fact that the PAK3b-kd mutant binds the active GTPases to a lesser extent than the wild type PAK3b suggests that some autophosphorylation events may increase the binding of PAK3b to Cdc42 by an as yet unknown mechanism.

Moreover, this region of GTPase binding that encompasses the CRIB domain is also implicated in the selectivity of the interaction with Rac versus Cdc42 (33, 49). Interestingly, the mutation of the residue Phe⁹¹ of PAK3, homologous to the Phe⁹⁶ of PAK1, abolished Rac binding more strongly than Cdc42 binding (33). Consistent with this, the binding of the PAK3b isoform is more impeded for Rac than for Cdc42. One hypothesis could be that the presence of the exon b inside the regulatory domain can modify the interaction with some GTPases of the Rac/Cdc42 family. We tested this by pull-down experiments with different GTPases of the Rho/Rac/Cdc42 family. We did not find any interaction with Rif and RhoG, which are paralogs of RhoA (data not shown) (50, 51). However, we found that three other members of the Rac/Cdc42 family, TC10, TCL, and Chp (32, 52, 53), bind PAK3a more strongly than PAK3b with a ratio identical to that of Cdc42 (data not shown). It is interesting to note that whereas active Rac binds PAK3a with the same affinity as Cdc42, Rac is only a weak activator of PAK3a (see Refs. 28 and 35; our results). Thus, we can hypothesize that the different GTPases function either to recruit and activate PAK3, as does Cdc42, or to only recruit PAK3 as does Rac. On the other hand, PAK3b brings in a new mechanism whereby Cdc42 could act only to recruit it, since it is already active. These mechanisms are particularly interesting with regard to the biological function of Rac and Cdc42 in neuritegenesis (54).

Another interesting question is whether the PAK3b isoform acts independently of the Rac and Cdc42 GTPases. It was proposed that PAK activation proceeds by several successive steps, the first one being membrane recruitment by the Nck adaptor or the guanine exchange factor of the PIX family and the second step being the activation by GTPase or lipids (for a review, see Ref. 23). It may be possible that PAK3b that possesses high basal kinase activity is activated in a signaling pathway only by membrane recruitment, by a mechanism independent from GTPases, and this remains to be tested. However, it was shown that following activation, PAK autophosphorylation prevents further binding to both Nck and Pix (55).

Thus, we can hypothesize that PAK3b may not interact with these two partners and cannot be recruited by these pathways. Finally, PAK mutants that have high kinase activity disrupt actin filaments and focal adhesions (for a review, see Ref. 23) and cooperate with Ras, Rac, and Rho GTPases in transformation or tumor cell line invasiveness (56–58). This further indicates that PAK3b is unlikely to be constitutively active in the nervous system and argues that a novel unknown PAK regulatory mechanism independent of GTPases exists and remains to be identified.

Finally, we report here the first characterization of an isoform for the PAK3 kinase. This isoform is detected at similar levels in the different parts of adult mouse brain. Future investigations of the biological properties of each PAK3 isoform, in particular in neuronal cells, may be of help to understand the function of PAK3 in the brain. The PAK3 gene was found to be implicated in mental retardation twice (12, 13). In one case, it is a missense mutation of the residue Arg⁶⁷, which probably disrupts GTPase binding, and in the second case, the mutation is a nonsense mutation inside the kinase domain generating a truncated protein. In both cases, these mutations could affect the functions of the two isoforms. Whether the PAK3a or PAK3b isoforms are implicated in mental retardation or in synaptic plasticity is a crucial question. Our results indicate a complex role for PAK3 isoforms in neuronal signaling.

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