The p21-activated Kinase PAK3 Forms Heterodimers with PAK1 in Brain Implementing Trans-regulation of PAK3 Activity

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Background: Regulation of group I p21-activated kinases is based on dimeric conformation.

Results: PAK3a forms regulatory heterodimers with PAK1 in vitro and in vivo.

Conclusion: Heterodimerization of PAK3a and PAK1 links PAK pathways in brain.

Significance: Analyzing the molecular mechanisms of PAKs regulation could help to understand the pathophysiology of some cancers and mental disabilities.

The p21-activated kinase 1 (PAK1) and PAK3 belong to group I of the PAK family and control cell movement and division. They also regulate dendritic spine formation and maturation in the brain, and play a role in synaptic transmission and synaptic plasticity. PAK3, in particular, is known for its implication in X-linked intellectual disability. The pak3 gene is expressed in neurons as a GTPase-regulated PAK3a protein and also as three splice variants which display constitutive kinase activity. PAK1 regulation is based on its homodimerization, forming an inactive complex. Here, we analyze the PAK3 capacity to dimerize and show that although PAK3a is able to homodimerize, it is more likely to form heterodimeric complexes with PAK1. We further show that two intellectual disability mutations impair dimerization with PAK1. The b and c inserts present in the regulatory domain of PAK3 splice variants decrease the dimerization but retain the capacity to form heterodimers with PAK1. PAK1 and PAK3 are co-expressed in neurons, are colocalized within dendritic spines, co-purify with post-synaptic densities, and co-immunoprecipitate in brain lysates. Using kinase assays, we demonstrate that PAK1 inhibits the activity of PAK3a but not of the splice variant PAK3b in a trans-regulatory manner. Altogether, these results show that PAK3 and PAK1 signaling may be coordinated by heterodimerization.

The p21-activated kinases (PAK) are effectors of Rac and Cdc42 GTPases and control cell proliferation and movement...
Unlike PAK3a, these splice variants are constitutively active and their interaction with active GTPases of the Rac/Cdc42 family are profoundly diminished compared with PAK3a. Moreover, their AID cannot inhibit the kinase activity of a PAK3a kinase domain (19). The molecular basis of the specific functions of PAK3 and its splice variants are largely unknown (20), and their mechanisms of regulation deserve to be extensively studied. Mechanisms of regulation of PAK1 were largely studied in regard to its role in cancer (21, 22) and the basis of PAK1 regulation is dependent upon its capacity to form dimeric complexes. In an attempt to characterize the mechanisms of regulation of PAK3a and of the three PAK3 splice variants, we analyzed, in vitro, the ability of PAK3 proteins to form dimers and to interact with the other neuronal PAK protein, PAK1. In transfected cells, we have demonstrated the existence of PAK3 homodimers and also heterodimeric complexes between PAK1 and the four PAK3 proteins. However, we observed a preference for heterodimeric complexes with PAK1 rather than PAK3 homodimers. In vivo, we showed that the different pAK3 mRNAs are expressed together with pAK1 in single pyramidal neurons and that PAK1 and PAK3 proteins colocalized in dendritic spines and are both present in the postsynaptic density (PSD) fraction. Moreover, we report that PAK1 and PAK3 co-immunoprecipitate with each other in mouse brain lysates. Finally, we demonstrated that PAK1/PAK3 heterodimers allow a trans-inhibition of the catalytic activity of PAK3a but not of the PAK3b splice variant. Altogether, our data show that PAK3 proteins form heterodimers with PAK1 and suggest that PAKs heterodimerization can coordinate PAK signaling and help to synchronize actin polymerization and spine stabilization.

**EXPERIMENTAL PROCEDURES**

**Antibodies**—Immunoblot and immunchemistry analyses were performed using anti-HA (rabbit: Santa Cruz Biotechnologies and rat: Roche), anti-FLAG (rabbit and mouse:Sigma-Aldrich), anti-PSD95 K28/43 (NeuroMab), anti-paxillin (Upstate-Millipore), HRP-conjugated secondary antibodies (Bio-Rad), Rabbit TrueBlot antibody (eBioscience), anti-rabbit-FITC-, anti-rat-TRITC-, anti-mouse-Alexa-568-, and anti-mouse-Alexa-633-conjugated secondary antibodies (Jackson ImmunoResearch, Sigma, and Invitrogen). The anti-synapsin rabbit serum was previously described (23). For PAK analysis, several commercial or homemade sera were used and their characteristics were described in supplemental Fig. S1. N19-PAK3 directed against the 19-N-terminal amino acids of PAK3 and the N20-PAK1 directed against the 20 N-terminal amino acids of PAK1 were from Santa Cruz Biotechnologies, and the monoclonal antibody, named 3A12-PAK3 was from Sigma-Aldrich. A new polyclonal antiserum was generated in rabbit (Sigma-Aldrich), a new polyclonal antiserum directed against the 19-N-terminal amino acids of PAK1 was provided by M. C. Parrini (7). Mutagenesis was performed using procedures based upon the QuickChange protocol (Stratagene) with the oligonucleotides listed in supplemental Table S2. PCR on pcDNA3-HA-PAK3a-K297L with the oligonucleotides set 1 gave rise to the pcDNA3-HA-PAK3a-L102F-K297L plasmid. The KpnI/XbaI digested fragment from the pcDNA3-HA-PAK3a-L102F-K297L plasmid was introduced in the pFLAG3S2X vector (Sigma), to obtain the pFLAG-PAK3a-L102F-K297L plasmid. Mutagenesis on the pFLAG-PAK3a-WT and the pFLAG-PAK3a-K297L plasmids using the oligonucleotides set 2 led to the pFLAG-PAK3a-R436E-K437D and the pFLAG-PAK3a-R436E-K437D-K297L plasmids, respectively. A fragment obtained after Pfnll digestion of the pFLAG-PAK3a-R436E-K437D-K297L construct, was introduced in the Pfnll digested pFLAG-PAK3a-L102F-K297L plasmid, giving rise to the pFLAG-PAK3a-L102F-R436E-K437D-K297L plasmid. The pFLAG-PAK3a-R436E-K437D-K297L was digested by KpnI/XbaI and the fragment obtained was cloned in the pcDNA3-HA vector, leading to the pcDNA3-HA-PAK3a-R436E-K437D-K297L plasmid. PCR amplifications were performed from the pFLAG-PAK3a-K297L and the pFLAG-PAK3a-R436E-K437D using the oligonucleotides set 3. pLex and pGAD plasmids previously were previously described (5). PCR products were digested by BamHI and the fragments obtained were inserted in the pLex vector to obtain the pLex-PAK3-(232–254) plasmid (named pLex-PAK3-Cter) and the pLex-PAK3-R436E-K437D-(232–254) plasmid (named pLex-PAK3-Cter-R436E-K437D), respectively. PCR amplification was also performed on pcDNA3-HA-PAK1-K299R with the oligonucleotide set 4. PCR products were digested by BamHI and the fragments obtained were inserted in the pLex vector, giving rise to the pLex-PAK3a-(2–270) plasmid. PCR products were digested by BamHI and the fragments obtained were inserted in the pLex vector to obtain the pLex-PAK3-(232–254) plasmid (named pLex-PAK3-Cter) and the pLex-PAK3-R436E-K437D-(232–254) plasmid (named pLex-PAK3-Cter-R436E-K437D), respectively. PCR amplification was also performed on pcDNA3-HA-PAK1-K299R with the oligonucleotide set 4. PCR products were digested by BamHI and the fragments obtained were inserted in the pLex vector, giving rise to the pLex-PAK3a-(2–270) plasmid, named pLex-PAK3a-Nter. To obtain the pGAD-PAK3-(232–254) plasmid, named pGAD-PAK3-Cter, a PCR was performed on pcDNA3-HA-PAK3a-K297L with the oligonucleotide set 5. PC products were digested by BamHI/XbaI and the insert obtained was cloned
into the GAD vector. PCR amplifications were performed from the plasmids pcDNA3-HA-PAK3a-L102F-K297L, pcDNA3-HA-PAK3b, pcDNA3-HA-PAK3c, and pcDNA3-HA-PAK3cb with the oligonucleotide set 7. PCR products digested by BamHI/XbaI were cloned into the pGAD vector to obtain pGAD-PAK3a(2–270)-LI02F, pGAD-PAK3b(2–270), pGAD-PAK3c(2–270), and pGAD-PAK3cb(2–270) constructs (all named Nter in place of 2–270). The BamHI/XbaI insert obtained after digestion of the pFLAG-PAK1-K299R plasmid (5) was inserted in the pcDNA3-HA vector (Invitrogen) in order to obtain the pcDNA3-HA-PAK1-K299R plasmid. The pGAD-PAK1(2–270) plasmid, named pGAD-PAK1-Nter was obtained by amplifying pcDNA3-HA-PAK1 with set 6. The insert was then cloned in the GAD vector. The GST-Cdc42-V12 prokaryotic expression plasmid was previously described (19). All plasmid coding sequences obtained were confirmed by sequencing.

Cell Line and Neuron Culture, Immunocytochemistry, and Imaging—HeLa cell cultures, primary cultures of dissociated hippocampal neurons and transfactions were previously described (5). For HeLa cells, PAK-transfected cells were fixed after 24 h and triple immunolabeled using FLAG, HA, and paxillin antibodies, followed by anti-rabbit-FITC-, anti-rat-TRITC- and anti-mouse-Alexa-633-conjugated secondary antibodies, respectively. At 21 days-in vitro (DIV), neurons were fixed and labeled for native PAK proteins, using specific PAK1 and PAK3 antibodies, followed by anti-rabbit-FITC- and anti-mouse-Alexa-568-conjugated secondary antibodies, respectively, in the presence of Alexa-633-phalloidin to label actin cytoskeleton. Images were acquired in a sequential mode, as previously described (5), using a Zeiss LSM 700 confocal microscope, equipped with 488, 555, and 639 nm lasers. Bleed-through was checked by imaging cells labeled with a single fluorophore and by acquiring dual channel images with the same setup used for the triple-labeled cells, indicating the total absence of overlap between the different fluorophores used. Analysis of the PAK1/PAK3 co-localized pixels was performed from the same focal plane acquired sequentially in the two channels, using the RG2B co-localization plug-in of NIH ImageJ software.

Immunoprecipitation and Western Blot—24–30 h after transfection, HeLa cells were lysed in KLB buffer as described by Ref. 7. Cell lysates were immunoprecipitated overnight with HA-agarose conjugate or with protein G-agarose (Sigma-Aldrich) in the presence of the FLAG antibody. For brain immunoprecipitation, tissues were cut in small pieces and resuspended in 5% (weight/volume) of resuspension solution A (5 mM Hepes, 320 mM sucrose, 150 mM NaCl, 2 mM EDTA, pH 7.4, and protease and phosphatase inhibitors), as described in Ref. 27 (supplemental Fig. S3). Immunoprecipitation was then performed over-night at 4 °C by adding protein G-agarose and appropriate PAK antibodies. Samples were resolved by electrophoresis and Western blotting as previously described (5). Expression of transfected proteins was checked in each experiment by Western blotting of Total Cell Lysate (TCL) samples.

Kinase Assay—The immunoprecipitates obtained from transfected cells were resuspended in the kinase buffer (25 mM HEPES, pH 7.4, 25 mM MgCl₂, 25 mM-glycerophosphate, 2 mM dithiothreitol, 0.1 mM orthovanadate) and incubated in the absence or the presence of the active GTPase GST-Cdc42V12, to activate the kinase activity, as previously described in Thévenot et al., (5). Samples were then incubated in the presence of 5 μCi of [γ-32P]ATP (Amersham Biosciences) for 20 min at 30 °C under agitation, in the presence of 3 μg of Myelin Basic Protein (Invitrogen) as a substrate. Reaction was stopped by adding SDS-Laemmlí sample buffer. Boiled samples were resolved by SDS-PAGE and incorporation of 32P was quantified using a PhosphorImager (Amersham Biosciences).

Yeast Two-hybrid Assay, Histidine Test, and β-Galactosidase Activity—The two-hybrid assay was applied according to standard procedures. L40 yeasts strain (L40 Mata, Trp-1, Leu-2, His-3, lys::(lexAop)4-HIS3, ura3::(LexAop)8-LacZ), were co-transfected with pLex and pGAD constructs by the lithium acetate method and transformants were grown on appropriate selective medium. Following overnight growth on YPDA medium, patches were replicated on plates lacking His, Trp, and Leu for selection of diploids. Liquid β-galactosidase assay was performed following the method of Crouin et al. (25). In each experiment, the quantification of the interaction between the PAK3a-Nterminal domain and the PAK3-C-terminal domain constitutes the 100% reference for all other tested interactions.

Single Cell RT-PCR on Cortical Neurons—Experiments were performed as previously described (26). Briefly, layer V visual cortex neurons of 3-week-old rats were patched and following whole cell recording to identify neurons, as much cytoplasm as possible was aspirated into the patch pipette. The cytoplasm was added to a reverse-transcription mix and processed for first strand elongation for three hours at 48 °C. The RT products were then amplified by two successive rounds of PCR. First, a multiplex PCR was performed directly in the same tubes by adding 90 μL of the PCR mix. This mix contained pairs of primers that hybridize with similar Tm. A second specific amplification was then performed in several parallel PCRs, using 1 μL of the PCR1 products (25 μL final PCR volume) and one primer set. PCR products were analyzed after electrophoresis in ethidium bromide containing agarose gels. Primer sequences are described in supplemental Table S2 and in Ref. 17.

Synaptosome Extraction and PSD Purification—Mice brains were cut in small pieces and resuspended in 5% (weight/volume) of resuspension solution A (5 mM Hepes, 320 mM sucrose, 150 mM NaCl, 2 mM EDTA, pH 7.4, and protease and phosphatase inhibitors), as described in Ref. 27 (supplemental Fig. S3). Homogenization of tissues was performed by a Precellys tissue homogenizer. The TBL was centrifuged to give the P2 fraction, according to the protocol of Ref. 28. P2 was then resuspended in a small volume of solution B (320 mM sucrose, 1 mM NaHCO₃ with protease and phosphatase inhibitors), which was then mixed with 15 mL of 14% Ficoll (GE Healthcare). This preparation was loaded below a 7.5% Ficoll solution. Both Ficoll solutions were diluted in solution A. Gradients were centrifuged a few hours in the presence of protein G-agarose as a clearing step. Immunoprecipitation was then performed over-night at 4 °C by adding protein G-agarose and appropriate PAK antibodies. Samples were resolved by electrophoresis and Western blotting as previously described (5). Expression of transfected proteins was checked in each experiment by Western blotting of Total Cell Lysate (TCL) samples.
in ice then centrifuged 20 min at 32,800 × g. The pellet containing the PSD fraction was resuspended in a small volume of Kreb’s buffer (20 mM Hepes, 10 mM glucose, 1.2 mM NaH2PO4, 1.3 mM MgCl2, 1.2 mM CaCl2, 5 mM KCl, 145 mM NaCl, 2 mM EDTA, with protease, and phosphatase inhibitors). All the collected fractions were then analyzed by Western blotting using appropriate antibodies.

**Animal Experimentation**—All experiments were conducted under appropriate biological containment in accordance with European Communities Council Directive (CEE 86/609) for animal care and experimentation, and were conducted following the guidelines of the animal facility in Orsay (France) approved by the national direction of veterinary services (Direction des Services Vétérinaires, France, agreement no. B91-471-104).

**RESULTS**

**PAK3a Forms Homodimers**—Crystallographic analysis and co-immunoprecipitation assays showed that PAK1 forms homodimers (6, 7). Since PAK1 and PAK3 possess a high sequence identity and share an overall similar structure (Fig. 1A), we first asked whether PAK3 may form homodimers like PAK1 does. The ability of PAK3a to form dimers was tested by a co-immunoprecipitation assay. HeLa cells were co-transfected with HA- and FLAG-tagged PAK3 constructs. As a control, cells were transfected by HA- or FLAG- PAK3 plasmids alone or with empty vectors. To bypass the problem of dissociation of the dimers following PAK activation, we chose to work with kinase-dead (KD) constructs (PAK3-K297L and PAK1-K299R) as previously performed for PAK1 (7). The mutation of the PAK3 lysine 297 residue that is homolog to the lysine 299 in the PAK1 protein, totally inactivates the kinase activity of PAK3 (19). Co-immunoprecipitated proteins were revealed by Western blot analysis with anti-FLAG antibodies on HA-precipitated fractions. The presence of the mutation R436-E-K437D in both partners strengthened the effect of a single mutation (25.36 ± 5.88% of PAK3a homodimer). Moreover, the presence of the mutations L102F and R436-E-K437D in one PAK3 protein totally abrogates dimer formation (1.39 ± 0.8% of PAK3a homodimer). These results show that L102 and R436-K437 residues are strongly involved in PAK3 dimer formation. To define the precise domains involved in the complex formation, a two-hybrid assay was performed with the N-terminal moiety and the C-terminal moiety of PAK3 fused to the Lex binding domain or the GAD transactivation domain. Protein interactions were observed on selective medium devoid of histidine (histidine test) and quantified by measure of β-galactosidase activity. Double transfected yeasts that co-expressed N-terminal and C-terminal moieties displayed a significant growth on selective media and a high β-galactosidase activity, indicating a strong interaction between the N-terminal and the C-terminal domains of PAK3a (Fig. 1E). In contrast, interactions between two N-terminal moieties of PAK3a and between two C-terminal moieties of PAK3 were very weak referred to the interaction between the N-terminal and C-terminal parts of the protein: 4.19 ± 1.39% for PAK3a-Nter/PAK3a-Nter interaction and 3.45 ± 1.15% for PAK3-Cter/PAK3-Cter interaction. The L102F or the R436E-K437D mutations both strongly decrease the N-terminal/C-terminal interaction (L102F: 17.06 ± 5.68%; R436E-K437D: 1.22 ± 0.40%). Taken together, these data show that PAK3a forms homodimers via a strong N-terminal/C-terminal interaction, leucine 102, arginine 436, and lysine 437 being critical residues for this complex.

**PAK3a Forms Heterodimers with PAK1**—Given that PAK1 and PAK3 share a very high level of sequence identity, we wondered whether PAK3a can form heterodimers with PAK1. Because PAK1 and PAK3 proteins can form heterodimers only if they localize in the same subcellular regions, we analyzed their co-localization, in particular in focal adhesions whose structure and reorganization are highly regulated by PAKs (4). HeLa cells were co-transfected with FLAG-tagged PAK3a-KD and HA-tagged PAK1-KD expressing plasmids (Fig. 2A). Immunolabeling was performed using FLAG and HA antibodies to detect PAK3 and PAK1 proteins, respectively, and paxillin antibody to detect focal adhesions. We observed that PAK3a and PAK1 proteins colocalize (Fig. 2A, right panel), and colocalize also with paxillin in the focal adhesions at the edge of the cell.

Thus, since these proteins were localized in the same cellular structures, we examined whether they could form heterodimers by a co-immunoprecipitation assay. As mentioned above, since PAK activation may interfere with the capacity to form dimers (7), we tested interaction with kinase-dead proteins. HeLa cells were co-transfected with plasmids encoding differentially tagged PAK3a-KD or PAK1-KD (Fig. 2B) and a co-immunoprecipitation assay revealed that PAK3a could form homodimers. Interestingly, significantly higher levels of heterodimers were formed with PAK1 (Fig. 2B). Comparison of the ratio co-immunoprecipitated/precipitate intensity reveals that PAK3a interacts about 7-fold more with PAK1 than with itself (674.71% of...
PAK3a homodimer \( \pm 224.90 \) (Fig. 2C). In contrast, PAK1 forms similar amounts of homodimers (431.22% of PAK3a homodimer \( \pm 143.74 \)) than heterodimers.

We then compared co-immunoprecipitation between wild-type and kinase-dead proteins (Fig. 2D). Controls with empty vectors verified the specificity of the assay (first two lanes).
observed that the interaction between PAK1 and PAK3a is maintained for wild-type partners, demonstrating that this interaction is not abrogated by phosphorylation. On the contrary, we observed that PAK3a-WT co-immunoprecipitates more with PAK1-KD than the PAK3a-KD does. This suggests that, in contrast to what previously reported for PAK1 (7), the wild-type PAK3a protein more easily forms heterodimers than the inactive protein, and that the regulation of dimer stability by autophosphorylation may differ for the different PAK isoforms.

Using a two-hybrid assay, we observed that the PAK3 C-terminal domain strongly interacts with the PAK1 N-terminal domain of its partner, and reciprocally, contributing to heterodimer formation (Fig. 2E). The L102F and R436E-K437D mutations also disrupted heteromeric interaction between PAK3a and PAK1, as analyzed by the two-hybrid assay. Note that the quantitative difference observed between PAK3a/PAK3a homodimers and PAK3a/PAK1 heterodimers in the co-immunoprecipitation assay was not observed in the two-hybrid assay, suggesting that preferential heteromeric conformation is supported by properties dependent on full length structure. Altogether, our results indicate that, in transfected cells, the two proteins PAK1 and PAK3 localize and that PAK3a preferentially forms heterodimers with PAK1.

**Intellectual Disability Mutations Differentially Affect PAK3 Dimerization**—To date, five mutations in the **pak3** gene coding sequence have been identified in patients with a non syndromic form of ID (reviewed in Ref. 20). Because we demonstrated that PAK3 preferentially forms heterodimers with PAK1, we wondered if these ID mutations could affect dimerization. We tested the R67C mutation previously described to decrease both the Cdc42 binding and the induced PAK3 activity, and two other mutations located in the C-terminal moiety, one missense mutation (A365E) and one nonsense mutation (R419X), that were both shown to abrogate PAK3 kinase activity (Fig. 3A) (24). HeLa cells were co-transfected with FLAG-PAK1-KD in

![FIGURE 2. PAK3a forms heterodimers with PAK1.](http://www.jbc.org/)

A. PAK1 and PAK3 colocalize at focal adhesions of HeLa cells. Immunolabeling was performed using FLAG and HA antibodies to detect PAK proteins and paxillin antibodies to identify focal adhesions. The PAK1/PAK3 colocalized pixels are shown (right). B. PAK3a co-immunoprecipitates with PAK1. HA- and FLAG-tagged PAK3a-KD and PAK1-KD plasmids were co-transfected in HeLa cells then cell lysates were HA-precipitated (IP HA). The co-immunoprecipitated PAK proteins were revealed by anti-FLAG Western blotting (first panel). HA-precipitated proteins and TCL were controlled using the corresponding antibodies as indicated (second and third panels, respectively). Images shown are representative of three independent experiments. C. amount of FLAG-PAK proteins co-immunoprecipitated compared with the amount of HA-precipitated PAK proteins. Results are expressed relative to the PAK3a/PAK3a interaction. Comparison with Student’s t test: NS, *p* > 0.05; ***, *p* < 0.001, n = 3. D. wild-type PAK3 isoforms interact together. Cells were co-transfected with HA-PAK1-KD and FLAG-PAK3a-KD (third lane), HA-PAK1-KD, and FLAG-PAK3a-WT (fourth lane), and HA-PAK1-WT and FLAG-PAK3a-WT (fifth lane). HA-proteins were immunoprecipitated (second panel) and co-immunoprecipitated FLAG proteins were visualized by anti-FLAG Western blotting (first panel). Co-transfection of PAK1 with empty vector (empty v.) was done as control (first two lanes). Images shown are representative of three independent experiments. E. analysis of the PAK1/PAK3a interaction using the two-hybrid assay. Yeasts growth on histidine minus media indicated a protein-protein interaction (+). β-Galactosidase activity was expressed relative to the PAK3a-Nter/PAK3a-Cter interaction. Comparison with Student’s t test: NS, *p* > 0.05; *, *p* < 0.05; ***, *p* < 0.001; n = 3.

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the presence of either HA-tagged PAK3 plasmids PAK3a-WT, -KD, -A365E, -R419X, and -R67C. Cell lysates were HA-immunoprecipitated and the co-immunoprecipitated PAK1 proteins were revealed using FLAG antibodies (Fig. 3B). Concerning ID mutations, the R67C mutation did not affect the PAK1/PAK3 interaction. In contrast, the A365E and R419X PAK3 mutations dramatically decreased PAK1/PAK3 complex formation. Thus, PAK3 ID mutations of the Ala-365 residue and C-terminal deletion from Arg-419 residue are directly responsible for the loss of dimerization with PAK1.

**PAK3 Splice Variants Still Form Heterodimers with PAK1**

The b and c inserts are located between amino acids 92 and 93 at the N-terminal part of the inhibitory switch domain (Fig. 4A), which is proximal to the dimerization segment and the Leu-102 residue involved in dimer stability (6, 7, 17). We thus wondered whether PAK3 splice variants could also form dimers and tested this using a co-immunoprecipitation assay (Fig. 4B). Cells were transfected with plasmids expressing FLAG-tagged PAK3a-KD or PAK1-KD proteins in the presence of HA-tagged PAK3 splice variants in their kinase-dead forms. Interestingly, we observed that the presence of the b and c inserts, B, presence of inserts decreases dimer formation. All the PAK1 and PAK3 expressed proteins carry the kinase dead mutation (KD). FLAG-tagged PAK3a-KD or PAK1-KD plasmids were transfected in HeLa cells in the presence of HA-tagged PAK3-KD-encoding variants. Cell lysates were HA-precipitated (IP HA). The co-immunoprecipitated PAK proteins were revealed by anti-FLAG Western blotting (first panel). HA-precipitated proteins and TCL were controlled using the corresponding antibodies as indicated (second and third panels, respectively). Images shown are representative of three independent experiments. C, quantification of the amount of FLAG-PAK3a-KD or FLAG-PAK1-KD proteins co-immunoprecipitated in the experiment illustrated in B, relative to the amount of HA-immunoprecipitated PAK3 proteins. Comparison with Student’s t test: **, p < 0.01; ***, p < 0.001, n = 3.
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With itself (Fig. 4, B and C). Two-hybrid analysis confirmed that the presence of the insert in the N-terminal part of PAK3 protein fused to GAD bait decreases the interaction with the C-terminal moieties of PAK1 and PAK3 fused to Lex, suggesting that the inserts strongly modify structures involved in dimer formation (supplemental Fig. S4). In transfected mammalian cells, PAK3 splice variants (PAK3sv) were relatively impaired in their capacity to form dimers with PAK3a, but they retained their capacity to form heterodimers with PAK1. Interestingly, in both cases, relative to PAK3a-PAK3a, the decrease of dimer formation was more pronounced for PAK3sv-PAK3a (more than 10-fold) than for PAK3sv-PAK1 (3–5-fold), suggesting that in vivo, PAK3 splice variants heterodimerize with PAK1.

PAK3 and PAK1 Form Complexes in Brain—One required condition for heterodimer formation is the expression of transcripts coding for the two partners in the same cell. To test this, we investigated whether pak1 mRNA and the different pak3 transcripts are co-expressed in a neuron by single cell RT-PCR. Pyramidal neurons of the visual cortex layer V of juvenile rat were patched and the cytoplasm was aspirated to be processed for RT-PCR with specific primers that amplified pak1 transcripts, all the pak3 transcripts (indicated as PAK3s) or each pak3 splice variant, i.e. a, b, c, cb variants as previously described (17, 26) (Fig. 5A). Actin was used as control, to attest the ability of the cDNA amplification from each sample. Among the actin-positive neurons (n = 37), 26 were positive for pak1 and 11 for pak3. Importantly, all of the pak3 positive cells were also positive for pak1 transcripts. Among pak3 positive neurons, 5 expressed pak3a, 4 expressed pak3b and 2 expressed pak3a and pak3cb. As an illustration, we show here (Fig. 5B) three neurons, positive for at least one pak3 transcript, indicating that a single neuron could express different pak3 transcripts. These results show that in a single neuron, both pak1 and pak3 genes are expressed and also that several pak3 splice variants can be co-expressed.

To go further, we analyzed PAK1 and PAK3 localization by immunofluorescence and biochemical fractionation. PAK1 immunofluorescence was performed on hippocampal neurons after 21 days of in vitro differentiation using specific antibodies as described in supplemental Fig. S1. PAK3 labeling shows an enrichment of PAK3 proteins in dendritic spines which are enriched in actin, as observed by Phalloidin labeling (Fig. 5C, second and third panels), a result previously described (29–33). PAK1 labeling displays a more widespread distribution in the dendritic shaft, but is also detectable in certain dendritic spines. However, we observed clear colocalization of PAK1 and PAK3 in some spines (Fig. 5C, fourth panel, white arrows), indicating that in some functional domains of neurons, PAK1 and PAK3 proteins colocalize.

We then asked whether PAK1 and PAK3 proteins were enriched in the same subcellular domain in particular in the post-synaptic densities where PAK1 and PAK3 were described to localize (32). Synaptosomes were isolated from mouse brain lysates on Ficoll-sucrose gradient and the postsynaptic densities (PSD) fractions were purified by Triton-X100 treatment (supplemental Fig. S3) as described (27). The different fractions obtained were analyzed by Western blot using the PAK antibodies characterized in supplemental Fig. S1, and as controls, the pre-synaptic marker synaptophysin and the post-synaptic marker PSD-95 (Fig. 5B, two lower panels). Results showed that synaptophysin was highly enriched in the synaptosomal fraction and completely absent from the PSD fraction. By contrast and as expected, PSD-95 is almost completely recovered in the PSD fraction. We observed that PAK1 was enriched in the synaptosomal fraction and was also present in the PSD fraction whereas PAK3 is present at a low level in the synaptosomal fraction and is highly enriched in the PSD fraction. These data are in agreement with the immunolabeling results indicating a more restricted localization of PAK3 in dendritic spines. The different PAK3 splice variants segregate differentially in postsynaptic fractions, the PAK3b splice variant being particularly enriched in PSD whereas the splice variants containing exon c-encoded insert are enriched in synaptosomes. Thus PAK1 and the different PAK3 proteins partially co-purified in the PSD fraction from adult mouse brain.

We next investigated whether dimers exist in the mouse brain between the two endogenous proteins by co-immunoprecipitation using specific immunoprecipitating antibodies (supplemental Fig. S1) and pak3 knock-out mouse brains as control. PAK1 protein was equally detected in both models (Fig. 5E, third panel, and 5F, fourth panel) and equal amount of immunoprecipitated PAK1 proteins were detected in brain lysates from wild-type mice and pak3 KO mice (Fig. 5F, second panel). On the other hand, PAK3 protein was only detectable in WT animals (Fig. 5E, fourth panel, and 5F, third panel), as expected (30). We detected PAK1 protein in the PAK3 immunoprecipitated from wild-type mice and not from pak3 KO mice (Fig. 5E, first panel). In the reverse experiment, we detected PAK3 protein in the PAK1 immunoprecipitated from wild-type mice, while no signal was detected from pak3 knock-out mice (Fig. 5F, first panel). Thus, using pak3 knock-down mice as a negative control and specific immunoprecipitata, we demonstrated by co-immunoprecipitation assays that endogenous PAK1 and PAK3 proteins form heterodimers in mouse brain. To resume, PAK3 and PAK1 are co-expressed in single neurons, and to some extent, colocalize in dendritic spines, co-purify in post-synaptic densities and co-immunoprecipitate in mouse brain demonstrating that endogenous PAK1 and PAK3 proteins form heterodimers in neurons.

PAK3a Is Regulated by Heterodimerization—Since PAK3 can dimerize, and PAK1 is regulated by its homodimerization, we wondered whether PAK3 heterodimerization regulates its own kinase activity. We developed a multi-step assay in which we first immunoprecipitated a PAK1/PAK3 complex. The co-immunoprecipitated material was divided into two equal aliquots, the first one being directly tested for its kinase activity while the second being incubated with active recombinant Cdc42 GTPase, before testing for its kinase activity. In both cases only the activity of the co-immunoprecipitated protein was measured since the precipitated protein was a kinase-dead mutant. We verified for the presence of immunoprecipitated and co-immunoprecipitated proteins by Western blot (Fig. 6, A and B) and the dissociation of the dimers following activation by Cdc42 (data not shown), as previously described (7). Kinase activity was analyzed by measuring the incorporation of γ-32P in myelin basic protein (MBP) as a substrate. The comparison...
PAK3 and PAK1 are coexpressed in single neurons, colocalize in dendritic spines, co-purify with the postsynaptic density, and co-immunoprecipitate in brain extracts. A, schematic representation of the location of the different PAK3 oligonucleotides used for single-cell RT-PCR. Forward and reverse oligonucleotides are indicated by arrows. Primers were chosen near or within the exons 2–3 portion to detect all the PAK3 cDNAs (1), and at exon/exon junction to specifically detect PAK3a (2), PAK3b (3), PAK3c (4), and PAK3cb (5) cDNAs. B, PAK3 and PAK1 are co-expressed in single neuron. RNAs from single pyramidal neurons were extracted and RT-PCR was performed using the different primer sets. Actin amplification was used as control (panel 7). Specific primer sets were used for PAK1 (first panel), all PAK3s (second panel), PAK3a, b, c, cb (third to sixth panel). C, PAK3 and PAK1 partially colocalize in dendritic spines of differentiated hippocampal neurons. Endogenous PAK proteins were immunolabeled with rb-211-PAK1 antibodies (first panel) and monoclonal 3A12 PAK3 antibodies (second panel). Dendritic spines were visualized by Alexa-633-phalloidin labeling (third panel) on dissociated hippocampal neurons at DIV21. On the PAK1/PAK3 colocalization image (fourth panel), white arrows indicate some spines where PAK1 and PAK3 proteins are coexpressed. Scale bar, 2 μm. D, PAK3 and PAK1 partially co-purify with some synaptosomal fractions as shown by Western blots of the different fractions purified from brain lysates. TBL from adult mice brain were fractionated to obtain the second pellet (P2) fraction, the synaptosomal fraction (Syn) and the postsynaptic densities (PSD) fraction. The same amount of protein was loaded for each fraction. The different fractions were probed with either the N20-PAK1 antibodies specific for PAK1 (first panel), the rb-211-PAK3 antibodies that recognized the PAK3s proteins (second panel), the rb-svs-PAK3b antibodies specific for PAK3b variant (third panel), the Ec antibodies that recognized the PAK3c and PAK3cb variants (fourth panel). Western blots with the synaptophysin antibodies as a presynaptic marker (fifth panel), and the PSD-95 antibodies as a postsynaptic density marker (sixth panel) confirm quality of tissue fractionation. Images shown are representative of three experiments. E, PAK1 co-immunoprecipitates with PAK3. TBL were prepared from adult wild-type mice (WT) and as a negative control, from pak3−/− mice (KO). In TBL, PAK1, and PAK3 proteins were detected by immunoblotting using the N20-PAK1 antibodies (third panel) and the N19-PAK3 (fourth panel), respectively. PAK3 proteins were immunoprecipitated with the N19-PAK3 sera (second panel) and co-immunoprecipitated PAK1 proteins were detected with ch-209-PAK1 (first panel). F, PAK3 co-immunoprecipitates with PAK1. TBL were prepared as for E, to analyze PAK3 (third panel) and PAK1 (fourth panel) expression. PAK1 proteins were immunoprecipitated using the N20-PAK1 antibodies (second panel) and co-immunoprecipitated PAK3 proteins were then detected with the rb-211-PAK3 sera (first panel). Images shown are representative of two experiments.
between the kinase activities of the two fractions with or without the GTPase reflects the level of inhibition of the co-immunoprecipitated partner in the non-dissociated complex. To do this, HeLa cells were co-transfected with FLAG-PAK1 kinase-dead and HA-PAK3a or -PAK3b wild type plasmids. Immunoprecipitation and Western blot analysis were performed with appropriate tag antibodies as indicated in Fig. 6B. A control was performed to confirm that PAK3a-WT has a catalytic activity, only in presence of Cdc42-V12, whereas PAK3b is constitutively activated as previously published (17, 19) (Fig. 6C, first panel). Catalytic activity of HA-PAK3a-WT; HA-PAK3b-WT and FLAG-PAK1-KD previously obtained by immunoprecipitation was tested in the presence or in the absence of recombinant Cdc42-V12. D and E, catalytic activity of complexed or dissociated PAK3 proteins. HA-PAK3a-WT (D) or HA-PAK3b-WT (E) obtained by co-immunoprecipitation (B, first panel) was analyzed after incubation with or without recombinant Cdc42-V12 protein. Images shown are representative of three experiments.

**DISCUSSION**

*Molecular Mechanisms of Regulation of PAK3— Kinase dimerization is a key regulatory mechanism in the kinome. A large range of scenarios exist in life and the importance of kinase dimerization was recently highlighted in relation to pathophysiology (34). Dimerization is a canonical step in receptor tyrosine kinase activation (35) but plays more diverse roles in serine/threonine kinases, being responsible for inactivation, activation, subcellular compartment retention or localization and substrate specification (36–38). PAK dimerization was first described ten years ago (6) and since then the PAK1 regulation mechanisms have been extensively studied (6, 7, 11, 12). Data demonstrated that, under cellular resting conditions, PAK1 exists in an autoinhibited inactive dimeric conformation (6, 7). The activation process of PAK1 requires a succession of steps: membrane recruitment, modification of domain folding, and phosphorylation of key residues that modify the conformation of the catalytic cleft and other regulatory sites leading to changes in the intermolecular assembly (1, 13, 39). The PAK1 trans-inhibition regulatory mechanism is thought to be valid also for the other kinases of group I since they share high**
sequence identities and functional similarities. We demonstrated here for the first time, that PAK3 is able to form homodimers, in vitro, as PAK1 does. However, another interesting question concerns the potentiality for PAK kinases to form heterodimers. It was previously mentioned (7) that PAK1 can form heterodimers with PAK2. Here we present the first evidence that PAK3 can also form heterodimers with PAK1, through interactions mainly between the N-ter domain and the C-ter domain. The L102F mutation or the R436E-K437D mutation introduced in the IS domain and the catalytic domain respectively, strongly or totally abrogate homomeric interaction of PAK3a and also the heteromeric interaction between PAK3a and PAK1. This suggests that PAK3a may form homodimers as PAK1 does, and also that heteromeric complexes have similar structures compared with the homodimeric ones described for PAK1. Moreover, we demonstrated that PAK1 regulates PAK3a kinase activity within heterodimers, showing that PAK heterodimerization also allows trans-inhibition.

In vivo, we demonstrated that PAK1 and PAK3a are co-expressed in the same cells, they colocalize in the same subcellular area, and they also biochemically partially co-purify in the same structure, three properties necessary for their interaction. Interestingly, we observed a higher level of PAK3a/PAK1 heterodimers than PAK3a/PAK3a homodimers in vitro whereas PAK1 interacts with itself or with PAK3a with the same efficiency. Moreover, the relative amount of endogenous proteins in neurons is much more in favor of PAK3a/PAK1 heterodimer formation than PAK3a homodimer formation. Actually, all these results strongly reinforce the idea that PAK3 forms complexes with PAK1 in vivo, a hypothesis that we demonstrated by detecting PAK1/PAK3 complexes in brain immunoprecipitates.

Moreover, we report here that PAK3 splice variants can form heterodimers with PAK1. Insertions of 15, 21, and 36 amino acids encoded by the two alternatively spliced exons b and c, and present in the splice variants b, c, and cb, respectively, are localized in the β-sheet of the IS domain, and so are predicted to strongly modify the structure of the regulatory region. We previously demonstrated that the AID of PAK3b does not inhibit a PAK3a wild-type kinase domain (19). PAK3 splice variants show a relatively decreased capacity to form complexes with PAK3a and heterodimers with PAK1, but heterodimers are more efficiently formed than dimers with PAK3 suggesting that splice variants exist mainly as complexes with PAK1. In addition, we demonstrated that whereas PAK1 could regulate PAK3a kinase activity by dimerization, dimerization with PAK3 splice variants does not inhibit their kinase activity. This observation suggests that dimer formation is not sufficient to permit a trans-inhibition of one monomer by another one. It is also possible that the residual activity of PAK3b in dimers is sufficient to phosphorylate crucial residues such as Thr-421 or Ser-139 (144 for PAK1) which play important role in kinase regulation (12). Moreover this suggests that the kinase activity of splice variants is regulated by other unknown mechanisms, such as post-transcriptional modulation of splicing or protein degradation. Finally, recent data demonstrated an asymmetry in the PAK dimer complex, suggesting that the two monomers have distinct roles in the molecular mechanisms of kinase inhibition (11). Since we report that PAK dimers contain one PAK1 and one PAK3 monomers, this questions whether the two isoforms have distinct roles in the asymmetric complex, or there is a random repartition of the two distinct roles.

Biological Consequence of Dimerization—Our data suggest that PAK3 and PAK1 signaling are linked together, at least until the moment of their activation. Interestingly, PAK1 protein is also involved in synaptic plasticity via regulation of coflin phosphorylation and actin cytoskeleton modeling (40) whereas PAK3 probably acts through other pathways involving CREB and Nck2 (5, 30). A recent study that characterized the pak1/pak3 double KO mice pointed out new neuronal defects due to an impaired post-natal brain growth (41). Altogether, these data suggest that in addition to unique functions and to shared functions, some PAK functions may depend on a crosstalk between PAK1 and PAK3 during the activation process. As a consequence, PAK1-dependent PAK3 regulation could coordinate their specific signaling pathway and help to synchronize actin polymerization and spine stabilization.

In addition, a consequence of the link between PAK1 and PAK3 signaling concerns the relationship between intelligence disability PAK3 linked ID mutations and the alteration of PAK1 signaling. The five ID mutations which have been shown to modify different properties of PAK3 such as the kinase activity, the binding to the Rac-Cdc42 GTPases (24), or the binding to Nck2/Grb4 (5), and which are associated with moderate to severe cognitive defects (reviewed in (20)), differently affect dimer formation. We observed that the R67C mutated protein does not alter dimerization with PAK1 in contrast with the two kinase-dead ID mutated proteins. Since the PAK3-R67C protein displays a defect in GTPase binding and a consequent defect in the activation of the kinase (24), the PAK3-R67C protein in complex with PAK1 will probably maintain PAK1 inactive. Thus, the more severe phenotype associated with the R67C mutation could be due to a defect of PAK1 activation, in addition to partial PAK3 impairment, supporting the idea that some mutated PAK3 proteins may act as dominant-negative proteins with respect to PAK1. In contrast, PAK3-A365E and PAK3-R419X mutations that alter the PAK3 ability to dimerize with PAK1 could favor PAK1 to form homodimers and thus these mutations would not impact PAK1 regulation. So, some phenotypic characteristics associated with pak3 mutations may be due to anomalies in PAK1 regulation in addition to the PAK3 signaling defect.

To conclude, it appears that PAK regulation is a complex process that involves homodimer and heterodimer formation dependent upon intra and inter molecular interactions, in a face-to-face conformation, allowing a dynamic succession of trans- and cis- phosphorylation events associated with spatial rearrangements of several domains of the protein, also dependent on activators such as active GTPases, or to a lesser extent on the guanine exchange factor of the PIX/Cool family, on membrane interaction, and on lipid association. We report here a novel level of complexity in one of these parameters, demonstrating that a combinatorial of dimer assembly exists, that a hierarchy in complex formation exists, and that these heteromeric interactions may have a functional role. We show that...
PAK isoforms do not have the same propensity to form homodimers versus heterodimers. Thus, in addition to the concept that heterodimers support a signaling network of related kinases (6), we propose that heterodimers may link together PAK1 and PAK3 signaling pathways in physiological as well as in pathophysiological conditions.

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PAK Heterodimers in Brain

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Supplemental Data

The p21-activated Kinase PAK3 Forms Heterodimers with PAK1 in Brain Implementing Trans-regulation of PAK3 Activity

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Supplemental Figure S1: Characterization of PAK specific sera.

Supplemental Table S2: List of primers used for plasmid construction or RT-PCR.

Supplemental Figure S3: Schematic representation of the method used to prepare synaptosomes and postsynaptic fraction from mice brain.

Supplemental Figure S4: Two-hybrid analysis of the PAK3 splice variant interactions.
**Supplemental Fig. S1: Characterization of PAK specific sera.** To analyze co-localization, copurification and co-immunoprecipitation of endogenous PAK proteins, we developed PAK1 and PAK3 isoform-specific antibodies and PAK3 splice variant-specific antibodies (SD 1A) that would be appropriate for immunofluorescence, for Western blotting, and for immunoprecipitation assays. Location of antigens is indicated on PAK structure (SD 1B). We produced them in two host species (chicken and rabbit) to permit simultaneous use with mouse monoclonal or goat polyclonal antibodies. The specificity of each affinity-purified antibody was tested on HeLa cell lysates previously transfected with HA-tagged PAK1, PAK2, or PAK3a plasmids (SD 1C). We produced PAK1 antibodies directed against a unique sequence (209-219 amino-acids) in chicken and named them ch-209-PAK1, and also a rabbit polyclonal antiserum against a 211-227 peptide, named rb-211-PAK1. Both sera are specific to PAK1 in Western blotting, and the rabbit serum is well suited for immunofluorescence assay. The commercial N20-PAK1 is specific to PAK1 protein in immunoprecipitation assay. For PAK3 proteins, the mouse monoclonal 3A12 gave a specific fluorescent labeling on hippocampal neurons and no signal on pak3- neurons (data not shown). For PAK3 immunoprecipitation, we used a rabbit polyclonal serum generated against a unique PAK3 sequence located between amino acids 211-228 shared by all PAK3 splice variants: this new antibody named rb-211-PAK3 was able to specifically immunoprecipitate PAK3a and not PAK1 nor PAK2 (SD 1C). For analyzing
PAK3 splice variants, we also produced rabbit antibodies (SD ID) directed against a peptide restricted to the PAK3b splice variant by designing a 8-mer peptide whose sequence encompasses the last four amino-acids of the coding exon 2 and the first four amino-acids encoded by the exon b. We carefully showed that this serum named rb-svs-PAK3b recognizes the PAK3b protein and does not recognize the PAK3cb splice variant, since the epitope is interrupted by the sequence encoded by the exon c. The rabbit antibody directed against a peptide issued from an internal sequence of the exon c, named Ec was previously described (17). Thus, we developed very specific antibodies, able to discriminate between the different PAK proteins.

A, table summary of the characteristics and properties of the PAK sera used in this study. B, location of antigens in the PAK structures. C, characterization of PAK1 and PAK3 specific antibodies. HA-tagged PAK1, PAK2, PAK3a plasmids were transfected in HeLa cells and their expression was checked by HA immunoblotting (first panel). Western blot specificity of the rabbit rb-211-PAK1 (second panel), ch-209-PAK1 (third panel), and rb-211-PAK3 (fourth) sera were analyzed on TCL of transfected cells. The specificity of the sera N20-PAK1 (fifth panel) and rb-211-PAK3 (sixth panel) in immunoprecipitation assays were tested on transfected cells and immunoprecipitates were analyzed by Western blot using the HA antibody. D, characterization of the PAK3 variant antibodies. Antibodies specific to the PAK3b variant (rb-svs-PAK3b, first panel) or of the insert c (Ec, second panel) were tested on lysates of HeLa cells transfected with HA-PAK3a, b c or cb plasmids by Western blotting using HA antibodies as control (third panel).
Supplemental Table S2: List of primers used for plasmid construction or RT-PCR. Sets 1 to 7 were used to amplify PAK sequences by PCR in order to construct plasmids. PAK1 set and actin set were used to amplify the PAK1 and actin cDNAs, respectively, to detect their expression in cytoplasm of single neurons.
Supplemental Fig. S3: Schematic representation of the method used to prepare synaptosomes and postsynaptic fraction from mice brain. Along the preparation, protein samples of the different fractions were conserved: the Total Lysate (TL) corresponds to the total brain homogenate from adult male mice; the second pellet (P2) corresponds to synaptosomes with mitochondria, synaptic vesicles and plasma membrane; the synaptosomal (Syn) fraction was obtained after purification from a 7.5% and 14% Ficoll gradient and the postsynaptic density (PSD) fraction was obtained after detergent extraction of the synaptosomes.
Supplemental Fig. S4: Two-hybrid analysis of the PAK3 splice variant interactions. N-terminal portion of the different splice variants of PAK3 fused to GAD bait were co-expressed in yeast with the C-terminal moieties of PAK1 and PAK3 fused to Lex. Growth on histidine-medium indicates an interaction which was measured by the β-galactosidase assay. Quantifications were done relatively to the PAK3a-Nter/PAK3-Cter interaction. The presence of the alternatively spliced exons c and b induces a strong decrease of the interactions between the C-terminal moieties of PAK1 and PAK3, suggesting that the inserts modify structures involved in dimer formation. However this decrease is less pronounced toward PAK1 than PAK3, reinforcing the idea that the splice variants heterodimerize with PAK1 in vivo. Yeast growth on histidine minus media (Histidine test) indicates a protein-protein interaction (+). β-galactosidase activity was expressed relative to the PAK3a-Nter/PAK3a-Cter interaction. Comparison with Student t test: *, p<0.05, ***, p<0.001, n=3.
The p21-activated Kinase PAK3 Forms Heterodimers with PAK1 in Brain Implementing Trans-regulation of PAK3 Activity
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