The p21-activated Kinase 3 Implicated in Mental Retardation Regulates Spine Morphogenesis through a Cdc42-dependent Pathway*

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The p21-activated kinase 3 (PAK3) is one of the recently identified genes for which mutations lead to nonsyndromic mental retardation. PAK3 is implicated in dendritic spine morphogenesis and is a key regulator of synaptic functions. However, the underlying roles of PAK3 in these processes remain poorly understood. We report here that the three mutations R419X, A365E, and R67C, responsible for mental retardation have different effects on the biological functions of PAK3. The R419X and A365E mutations completely abrogate the kinase activity. The R67C mutation drastically decreases the binding of PAK3 to the small GTPase Cdc42 and impairs its subsequent activation by this GTPase. We also report that PAK3 binds significantly more Cdc42 than Rac1 and is selectively activated by endogenous Cdc42, suggesting that PAK3 is a specific effector of Cdc42. Interestingly, the expression of the three mutated proteins in hippocampal neurons affects spinogenesis differentially. Both kinase-dead mutants slightly decrease the number of spines but profoundly alter spine morphology, whereas expression of the R67C mutant drastically decreases spine density. These results demonstrate that the Cdc42/PAK3 is a key module in dendritic spine formation and synaptic plasticity.

The number, shape, and structure of dendritic spines changes in response to synaptic activity, and anomalies of spine morphogenesis are often associated with cognitive defects, such as mental retardation, Alzheimer disease, Down syndrome, autism, and schizophrenia (1, 2). Actin cytoskeleton, enriched in dendritic spines, plays a major role in spine dynamics and is under the control of Rho-GTPase signaling (3–5). Several genes that are implicated in nonsyndromic mental retardation are direct regulators or effectors of Rho GTPases (6).

The p21-activated kinase 3 (PAK3) is one of these recently identified genes implicated in X-linked nonsyndromic forms of mental retardation, and three mutations have been identified in independent families (7–9). PAK3 is a member of the PAKs group I, which are effectors of Rac1 and Cdc42 GTPases. Interestingly, inhibition of PAKs by expression of an inhibitory peptide in postnatal forebrains impaired synaptic plasticity (10). PAKs also play a fundamental role in cognitive deficits in Alzheimer disease (11, 12). Recent evidence supports a role for PAKs in spine morphogenesis: indeed inhibition of PAKs decreased the number of spines and altered their morphology (10). Concerning PAK3, reduction of its expression or expression of a PAK3 kinase-dead mutant induced abnormal elongated dendritic spines and decreased mature spine synapses (13, 14). PAK3 knockdown and knock-out experiments induce abnormal long term potentiation, indicating a major role for PAK3 in synaptic plasticity (13, 15).

How PAK3 regulates spinogenesis and synaptic plasticity is still unknown. To address this question, we introduced the three identified mental retardation mutations into the PAK3 gene and analyzed their effects on the functions of PAK3. Two mutations, R419X and A365E, are located in the kinase domain, whereas the third one, R67C, is present in the regulatory domain, at the N-terminal end of the p21-GTPase binding domain (PBD). We show that the two mutations R419X and A365E completely inactivate the catalytic function of the kinase. We also report that Cdc42 is the main activator of PAK3 and that the R67C mutation severely impairs PAK3 binding to Cdc42 and the subsequent activation of PAK3. Interestingly, whereas the mutated proteins are normally localized in dendritic spines, their expression in hippocampal neurons of organotypic slice cultures induces different phenotypes. Our data delineate a new pathway linking the Cdc42-GTPase to PAK3 in dendritic spine morphogenesis.

EXPERIMENTAL PROCEDURES

**Antibodies**—Antibodies to HA (12CA5) were purchased from Roche Diagnostics (Meylan, France). The anti-PAK3-N19

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The abbreviations used are: PAK, p21-activated kinase; PBD, p21-binding protein; GEF, guanine exchange factor; HA, hemagglutinin; CMV, cytomegalovirus; siRNA, small interference RNA.
antibody directed against the 19 N-terminal amino acids and the GFP antibody were purchased from Santa Cruz Biotechnology (Tebu, Le Perray en Yvelines, France); the monoclonal anti- 

**Plasmid Constructions**—All PAK3 constructs were derived from the mouse PAK3 gene, without alternatively spliced exon, herein named as PAK3 (16). All of the PAK3 mutants were generated from the pHA-PAK3a plasmid previously described (16) with Pfu polymerase (Promega, Charbonnieres, France), using procedures based upon the QuikChange protocol (Stratagene, Amsterdam, The Netherlands) and confirmed by sequencing. The mutations introduced into PAK3 include the three mutations responsible for mental retardation: R67C (oligonucleotide set: 5′-ccataagaagagagagagatgccccagagatcttcc-3′ and 5′-ggagagagagatcctgagctgcctctctctctctcttgag-3′) leading to the HA-PAK3a-R67C plasmid; A365E (oligonucleotide set: 5′-ggaagagagagatcctgagctgcctctctctctctctctgag-3′ and 5′-ggagagagagatcctgagctgcctctctctctctctctgag-3′) leading to the HA-PAK3a-A365E plasmid; R419X (oligonucleotide set: 5′-catctctgagaaagtaaatgaagcactatggtgggaac-3′ and 5′-gtcctccacctagctgcttactcttgcaggt-3′) leading to the HA-PAK3a-K297L plasmid. The PAK3 double mutant H78L-H81L was generated with the oligonucleotide set: 5′-ctctctctctctctctctctctctctctctctctctctctctctctctcagactttgag-3′ and 5′-gtgacgtcataaaacc-acaagatagtgcttaactgtggagaggag-3′), leading to the HA-PAK3-H78L-H81L plasmid. The HA-PAK3-K297L plasmid containing the mutation K297L leading to a kinase dead construct was previously described (16). The BamHI/XbaI fragments of the different HA-tagged PAK3 constructs described above were subcloned into the BamHI/XbaI-linearized pEGFP vector (Clontech, Ozyme, St. Quentin en Yvelines, France), to obtain GFP-tagged PAK3 cDNA expression plasmids were co-transfected at a ratio of 1:2. Imaging was carried out with a Leica (Mannheim, Germany) confocal microscope using the 488 nm band of an argon laser and the 561 nm band of a diode-pumped solid-state laser for excitation of GFP or RFP, respectively. Well differentiated and simultaneous transfected neurons were chosen, and images were acquired with a 63× objective (1.4 numerical aperture). Because dendritic spines often cross several z planes, we took z series stacks of 1–1.5 μm (typically 5–8 frames) from the bottom to the top of dendrites and used Image J software to generate image projections.

**Cell Culture, Transfection, and Confocal Imaging for COS7 Cells and Dissociated Hippocampal Neurons**—Culture and transfection of COS7 cells were done as previously described (16). Plasmid expression was limited to 30 h to avoid protein overexpression. Primary cultures of dissociated hippocampal neurons were prepared from embryonic day 18 (E18) rats as reported by Goslin and Banker (17). Cultures were transfected using Lipofectamine 2000 (Invitrogen) at 7 days in vitro (DIV) and fixed at DIV 21. Alternatively, transfection at DIV 18 gave rise to the same results. The neurons, cultured DIV 21, display spines that possess the characteristics of mature synapses. Indeed, in these cultures, we observed actin concentration in spines, PSD-95 aggregates in the spine head, and electrophysiological recordings show functional synaptic transmission (18). Simultaneous transfection of the RFP-actin plasmid was used to confirm the presence of spines in transfected neurons. RFP-actin and tagged GFP wild-type or mutant PAK3 cDNA expression plasmids were co-transfected at a ratio of 1:2. Imaging was carried out with a Leica (Mannheim, Germany) confocal microscope using the 488 nm band of an argon laser and the 561 nm band of a diode-pumped solid-state laser for excitation of GFP or RFP, respectively. Well differentiated and simultaneous transfected neurons were chosen, and images were acquired with a 63× objective (1.4 numerical aperture). Because dendritic spines often cross several z planes, we took z series stacks of 1–1.5 μm (typically 5–8 frames) from the bottom to the top of dendrites and used Image J software to generate image projections.

**Hippocampal Slice Cultures, Transfection, Image Acquisition, and Analysis**—Rat organotypic hippocampal slice cultures were prepared as described by Stoppini et al. (19). Cultures were maintained 11–12 days in vitro and transfected using a gene gun (Bio-Rad) technique according to the manufacturer’s instructions. Plasmid DNAs at a ratio of 20 μg of pCDNA3.1-EGFP to 40 μg of HA-PAK3 constructs were precipitated onto 10 mg of 1.6-μm gold microcarriers. Transfection of 10–30 cells per slice culture was reproducibly obtained, and expression was stable when 7–8 days. Control experiments showed that cells co-transfected with EGFP and DsRed constructs expressed green and red fluorescence in all cases (n = 7). Two days after transfection hippocampal organotypic cultures were submerged in a chamber perfused with an extracellular medium containing (in mM): 124 NaCl, 1.6 KCl, 2.5 CaCl₂, 1.5 MgCl₂, 24 NaHCO₃, 1.2 KH₂PO₄, 10 glucose, and 2 ascorbic acid, saturated with 95% O₂ and 5% CO₂, pH 7.4, temperature of 33 °C. Imaging of dendritic segments was performed through a 60× water immersion objective using a Visirot (Puchheim, Germany) spinning disk confocal system. Control experiments showed that repetitive imaging under those conditions did not affect the morphology or viability of transfected cells. Z-stacks of 50- to 200-μm-long dendritic segments were taken on secondary and tertiary dendrites of pyramidal neurons, and the characteristics of dendritic protrusions were measured using Metamorph software (Universal Imaging Corp., Downingtown, PA). The parameters analyzed were protrusion (spines and filopodia) density and length, measured from the limit of the dendrite to the tip of the protrusion.
Cell Lysis, Immunoprecipitation, Kinase Assay, Pulldown, and Immunoblotting Analysis—Transfected cells were lysed as previously described (16). In some experiments, to activate the kinase, cleared cell extracts of PAK3-transfected cells were incubated with increasing amounts of recombinant Cdc42-V12 in the presence of 25 μM ATP during 30 min at room temperature. Extracts were then immunoprecipitated by incubating them with 4 μl of 12CA5 anti-HA antibody and 50 μl of protein G-agarose (Sigma-Aldrich) overnight at 4 °C. After washing the immunocomplexes, aliquots were subjected to immunoblotting to ensure that PAK proteins were correctly expressed and immunoprecipitated. Protein samples were separated by 10% SDS-PAGE and transferred to a polyvinylidene difluoride membrane (Amersham Biosciences, GE Healthcare Europe, Orsay, France). Immunodetection was performed using the SuperSignal chemiluminescent reagent (Pierce, Perbio Science, Berrières, France). Quantification of chemiluminescence was performed after acquisition with a charge-coupled device camera GeneGnome (Syngene, Ozyme, Voisin le Bretonneux, France). using quantification software (GeneSnap and GeneTools, SynGene, Ozyme, Voisin le Bretonneux, France). Immunoblotting was performed as described (16).

For kinase reactions, immunoprecipitates were washed one 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 μM ATP and 5 μCi of [γ-32P]ATP (Amersham Biosciences, GE Healthcare Europe) for 20 min at 30 °C, in the absence or in the presence of 3 μg of H2B (Sigma-Aldrich) or 3 μg of myelin basic protein (MBP) (Sigma) 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 PhosphoImager (Amersham Biosciences).

Co-immunoprecipitation was performed as previously described (16). Briefly, COS7 cells were transfected with 5 μg of active GFP-tagged Rac1 or Cdc42 along with 5 μg of HA-tagged PAK3 constructs. Cell lysates in Robert’s lysis buffer were immunoprecipitated overnight with anti-HA-agarose conjugate. Immune complexes were separated by electrophoresis and transferred to polyvinylidene difluoride membranes. Western blotting analysis was performed with anti-PAK3 N19 antibodies to detect PAK3, anti-HA (12CA5) to identify HA-PAK3 and HA-ITSN proteins, anti-GFP antibodies to detect co-precipitated Cdc42 or Rac1 proteins, and anti-FLAG (F7425 from Sigma) to reveal FLAG-Tiam1. Pulldown assays to determine the active Cdc42 and Rac1 levels in cell lysates expressing inter- sectin or Tiam1 were performed using GST-PBD-PAK3 as described (16).

Two-hybrid Analysis—Transformants were grown on tryptophan/leucine drop-out agar plates (Clontech, San Francisco, CA) for 3 days at 30 °C. The interactions were assessed using liquid culture β-galactosidase assays with O-nitrophenyl β-D-galactopyranoside (Sigma-Aldrich) as the substrate, according to the Clontech protocol. β-Galactosidase activity was quantified by spectrophotometer readings and converted to β-galactosidase units by normalizing to the number of cells. The interaction of PAK3-wt with Cdc42-V12 was defined to be 100%. Experiments were repeated three times in triplicate.

RESULTS

Differential Effects of PAK3 Mental Retardation Mutations on Its Kinase Activity—The three mutations responsible for mental retardation are located in different functional regions of the PAK3 protein (Fig. 1A). The first mutation, located in the catalytic domain, generates a stop codon TGA at the position coding for arginine 419 (R419X), leading to a truncated protein, which is devoid of kinase activity (7). The second mutation, in the kinase domain, corresponds to a substitution of a conserved alanine residue to a glutamate (A365E) (9). The third mutation present at the extremity of the PBD domain mutates the arginine 67 to a cysteine residue (R67C) (8). The molecular defects associated with the A365E and R67C mutations have not yet been described. To assess the effects of the three mutations R419X, A365E, and R67C, on the biochemical properties of PAK3, we introduced them into the coding sequence of the PAK3 gene. We compared these new mutated forms to two well described PAK mutants: the kinase-dead mutant PAK3-K297L and a GTPase-deficient binding mutant PAK3-H78L-H81L (20, 21).

FIGURE 1. Mental retardation mutations of PAK3 differentially affect the kinase activity. A, PAK3 possesses the classic structure of the PAKs of the group I, i.e. a C-terminal kinase domain, a p21-binding domain (PBD) that interacts with Rho GTPases and, overlapping with this domain, an auto-inhibitory domain (AID) that inhibits the kinase activity in resting cells. The binding of the GTPases leads to the activation of the catalytic domain. Arrows indicate the localization of the three mutations R67C, A365E, and R419X, responsible for mental retardation, whereas arrowheads indicate mutations described in the literature: the H78L-H81L mutation located in the PBD disrupts the binding to GTPases, and the K297L mutation disorganizes the ATP binding pocket in the catalytic domain. Amino acid numbering is based on the mouse PAK3 sequence that we have previously cloned (accession number AJ496262). MRX30 and MRX47 correspond to the families identified by the X-Linked Mental Retardation Consortium. B, R419X and A365E mutations abolish kinase activity, whereas R67C mutation has no effect on the kinase activity. PAK3 constructs: wt, K297L kinase-dead mutant, and the three mental retardation mutants (R419X, A365E, and R67C) were co-transfected in COS7 cells with active V12 or inactive N17 mutants of GFP-Cdc42 constructs. PAK3 proteins were HA-immunoprecipitated 30 h later and tested in a kinase assay using MBP as a substrate (upper panel). Immunoprecipitated PAK3 proteins and expression of GTPases were controlled by immunoblotting using anti-PAK3 and GFP antibodies (middle and lower panels, respectively). Data shown are representative of three independent experiments. Note the electrophoretic shift (arrowhead) of the Cdc42-activated PAK3 wild-type and R67C proteins due to autophosphorylation (middle panel).
To analyze their respective kinase activity, the different PAK3 mutants were co-expressed in COS7 cells with constitutively active V12 or inactive N17 forms of Cdc42. Kinase assay were performed with HA-immunoprecipitated PAK3 proteins using the MBP as substrate (Fig. 1B). The wild-type PAK3 protein is strongly activated when co-expressed with Cdc42-V12, whereas no activity is detected when co-expressed with the inactive N17 form of the GTPase. As expected, the PAK3-K297L and the PAK3-R419X proteins had no detectable kinase activity. We also report here that the A365E mutation completely abolished the kinase activity of PAK3. Surprisingly, the PAK3-R67C protein showed the same kinase activity as the wild-type protein when it was co-expressed with Cdc42-V12 (R67C: 110 ± 12.4% compared with wild-type). Kinase assays measured by autophosphorylation confirmed these results (supplemental Fig. S1A). We observed similar activation of wild-type PAK3 and PAK3-R67C when they were co-expressed with Rac1-V12 (supplemental Fig. S1B). Thus, contrary to the R419X and A365E mutants, which are devoid of kinase activity, the kinase activity of the R67C mutant is similar to that of the wild-type PAK3 protein, in these experimental conditions.

PAK3 Binds More Efficiently Cdc42 than Rac1—Despite its full activation when Cdc42 was overexpressed, the location of the R67C substitution at the NH2-extracellularly of the PBD suggested that this mutation may impair the binding of PAK3 to the GTPases and its activation. We first quantitatively compared the interaction of constitutively active or inactive Rac1 and Cdc42 with the wild-type PAK3 protein using a liquid β-galactosidase two-hybrid assay (Fig. 2A). We verified that inactive forms N17 of Rac1 and Cdc42 did not bind PAK3 (Fig. 2A and supplemental Fig. S2). We also verified that the PAK3-K297L-H81L protein, impaired in GTPase binding, did not interact with Cdc42-V12 or Rac1-V12 (supplemental Fig. S2). As shown in Fig. 2A, the wild-type PAK3 protein interacts strongly with the active Cdc42 bait. In contrast, the interaction with the active form of Rac1 is very faint, ~2% of the interaction between PAK3 and Cdc42. Quantification of these different interactions are presented in supplementary data (supplemental Fig. S2C).

To confirm that the PAK3 protein binds more strongly Cdc42 than Rac1, HA-PAK3 co-expressed with active forms of GFP-Rac1 and GFP-Cdc42 were immunoprecipitated, and isolated complexes were analyzed for the presence of the associated GTPases (Fig. 2B). Because the interaction of PAK3 proteins with the GTPases could be regulated by autophosphorylation, we used kinase-dead mutants of PAK3 (K297L) (22). Quantification of co-immunoprecipitated GTPases showed that the amount of Cdc42 bound to PAK3 is 10-fold higher than Rac1 (the interaction with Rac1 reached 11 ± 0.58% of the normalized interaction with Cdc42) (Fig. 2C).

The R67C Mutation Decreases Cdc42 Binding to PAK3—We next analyzed the interaction of the PAK3-R67C protein with Rac1 and Cdc42 GTPases using the liquid β-galactosidase two-hybrid assay and co-immunoprecipitation. Interestingly, we show that the R67C mutation decreased the binding of PAK3 to Cdc42 (Fig. 3A). This interaction decreased to 70% compared with the interaction with wild-type PAK3. However, the binding of PAK3-R67C to Rac1 was not significantly different from PAK3-wt and remained faint.

To confirm this result, we examined GTPase co-immunoprecipitation with PAK3 proteins from co-transfected COS7 cells. We verified that Cdc42-V12 and Rac1-V12 did not bind the PAK3-H78L-H81L protein (Fig. 3, B and D). In agreement with the two-hybrid assays, we found a strong decrease of the amount of Cdc42 co-immunoprecipitated with PAK3-R67C (down to 40%) (Fig. 3C). However, in these conditions PAK3-R67C binds five times more Rac1 than the wild-type PAK3 protein does (Fig. 3, D and E). We also analyzed by co-immunoprecipitation the interaction of Cdc42 with the three kinase-dead mutants PAK3-K297L, PAK3-R419X, and PAK3-A365E. We found that these mutations did not impair the binding of PAK3 with this GTPase in agreement with the first characterization of the interaction of PAK3 with Cdc42 (supplemental Fig. S3) (7, 23). Altogether our results demonstrate that PAK3 binds Cdc42 more efficiently than Rac1 and that the R67C mutation significantly decreased the binding of PAK3 to Cdc42 and increased the binding to Rac1.

R67C Mutation Impairs Kinase Activation by Cdc42—Although the R67C protein is activated when Cdc42-V12 is co-expressed in COS7 cells (Fig. 1B), the interaction with this GTPase is weak (Fig. 3, A and B). This apparent discrepancy could be explained by the high expression level of Cdc42-V12,
which may bypass the loss of affinity of PAK3-R67C for Cdc42.

To more precisely control the amount of Cdc42 used to activate PAK3, we performed an in vitro kinase assay, by adding increasing amounts of GST-Cdc42-V12 to transfected cell lysates. Hence, lysates of COS7 cells expressing PAK3-wt or PAK3-R67C were incubated without Cdc42 or with a precise amount of GST-Cdc42-V12 protein before performing the kinase assay. As shown in Fig. 4A, PAK3-R67C is significantly less activated by Cdc42-V12 than the wild-type protein, under these conditions.

To analyze the in vivo activation of PAK3 by endogenous Cdc42 and Rac1, we took advantage of the ability of the guanine exchange factors (GEFs), intersectin and Tiam1, to promote specific activation of Cdc42 and Rac1, respectively (24, 25). We verified the specificity of the GEFs by monitoring the levels of active Cdc42 and Rac1 in a pulldown assay using GST-PBD-PAK3 to sequester active GTP-bound GTPases. The overexpression of Tiam1 caused an increase in endogenous levels of Rac1-GTP but not Cdc42-GTP, whereas the expression of intersectin stimulated the production of Cdc42-GTP and to a lesser extent Rac1-GTP (Fig. 4B). The activation of Rac1 by intersectin is due to an endogenous activation of Rac1 by Cdc42 (26). To analyze the activation of PAK3 by intersectin and Tiam1, we performed kinase assays with the standard substrate histone 2B. The same results were obtained with the substrate MBP (data not shown). We observed that the co-transfection of PAK3 wild type with the constitutively active form of intersectin induced an increase of its kinase activity. Interestingly, the R67C-mutated protein was not activated in these conditions (Fig. 4, C and D). However, the expression of the constitutively active form of Tiam1 did not activate either PAK3 wild-type or R67C mutant (Fig. 4, E and F). Taken together, our results prove that PAK3 is mainly activated by the intersectin-Cdc42 module and that the R67C mutation, which inhibits the binding of PAK3 to Cdc42, impairs PAK3 activation. Moreover, although the R67C mutation increases the binding of PAK3 to Rac1 (Fig. 3D), it doesn’t allow an activation of PAK3 by the Tiam1-Rac1 module. Thus, the main molecular defect of the R67C protein is its absence of activation by Cdc42 due to a decrease of its affinity to this GTPase.

PAK3 Mental Retardation Mutations Do Not Affect PAK3 Localization in Dendritic Spines—Because PAK3 is expressed in neurons (7, 13, 27) and several authors have reported its localization in dendritic spines (10, 28), we tested the hypothesis that PAK3 mutations may induce a mislocalization of the protein in dendritic spines of neurons. It has been reported that expression of a kinase-dead PAK3 protein in dissociated hippocampal neurons induces a dramatic impairment in dendritic spine formation (14). Indeed, we also observed a strong decrease in spine density in 21-day-old neurons expressing the kinase-dead PAK3-K297T mutant (Fig. 5A), and this impeded our study of spine localization of PAK3 mutants.

It has recently been shown that the overexpression of actin in hippocampal organotypic culture of neurons increases spine density (29). Therefore, we expressed actin to bypass the inhibition of spinogenesis induced by the expression of mutated PAK3 proteins and thereby we could determine the localization of PAK3 proteins in spines. GFP-tagged PAK3 wild-type or mutants were co-expressed in dissociated hippocampal neurons with the RFP-actin (Fig. 5B). The expression of RFP-actin also allows the visualization of the morphology of dendritic spines in

FIGURE 3. The R67C mutation decreased interactions of PAK3 with Cdc42 but increased interactions with Rac1. A, a hybrid interaction of Cdc42-V12 and Rac1-V12 with full-length wild-type PAK3 and R67C mutant. Interactions were measured and quantified as described in Fig. 2A. The figure shows the mean value of at least three independent colonies in triplicate. Comparison with Student’s t test: **, p < 0.01. B, in vivo interaction of active Cdc42 with PAK3 proteins. HA-tagged PAK3 proteins (wt, R67C, or H78L-H81L) were co-expressed in COS7 cells with active GFP-tagged Cdc42. Presence of the GFP-tagged Cdc42 protein in the immune HA-complexes was revealed by anti-GFP Western blotting (upper panel). PAK3 proteins were immunoprecipitated (IP) using anti-HA antibodies, and the amount of immunoprecipitated PAK3 proteins was controlled by using anti-PAK3 N19 immunoblotting (middle panel). The presence of GFP-GTPases in total cell lysates (TCL) was controlled by anti-GFP immunoblotting (lower panel). As noted in Fig. 1B, co-transfection of PAK3-wt and PAK3-R67C with Cdc42-V12 induces an electrophoretic shift (arrowhead). Data shown are representative of three independent experiments. C, quantifications of co-immunoprecipitation with Cdc42 from three independent experiments were standardized after acquisition of chemiluminescence with a charge-coupled device camera (histogram). Comparison with Student’s t test: *, p < 0.05. D, co-immunoprecipitation of active GFP-tagged Rac1 with HA-tagged PAK3 proteins was performed as described in Fig. 2B. Presence of GFP-tagged Rac1 protein in the immune complexes was revealed by anti-GFP Western blotting (upper panel). PAK3 proteins were immunoprecipitated (IP) using anti-HA antibodies and amount of immunoprecipitated PAK3 proteins was assessed by anti-PAK3 N19 immunoblotting (middle panel). Presence of GFP-GTPases in total cell lysates (TCL) was assessed by anti-GFP immunoblotting (lower panel). Note the electrophoretic shift when Rac1-V12 was overexpressed with PAK3-wt and PAK3-R67C (arrowhead). Data shown are representative of three independent experiments. E, quantifications of co-immunoprecipitation with Rac1 from three independent experiments were standardized after acquisition of chemiluminescence with a charge-coupled device camera a (histogram). Comparison with Student’s t test: **, p < 0.01.

Cdc42/PAK3 Module Regulates Spine Formation

Because PAK3 is expressed in
We observed a heterogeneous localization of GFP-PAK3-wt in the dendritic shaft and in the head of spines, where it partially co-localizes with RFP-actin (Fig. 5B). The three kinase-dead proteins K297L, R419X, and A365E and the two mutants affecting GTPase binding, H78L-H81L and R67C, displayed the same subcellular localization as the PAK3-wt protein. In these experimental conditions, we did not observe any defects or differences in the spine morphology and density. We conclude that the kinase activity and the binding to GTPases are not required for PAK3 spine localization and that the PAK3 mutations do not alter the targeting of the protein to spines.

Expression of Mutated PAK3 Proteins Differentially Affects Dendritic Spine Morphogenesis—We then analyzed the effects of expressing these different PAK3 mutants in pyramidal cells of hippocampal organotypic slice cultures, a model that is closer to the in situ situation for analysis of spine morphogenesis. For this, R419X, A365E, and R67C mutants were co-expressed with EGFP in pyramidal cells through biolistic transfection. Two parameters were analyzed using confocal microscopy; the density of dendritic protrusions (spine and filopodia) and protrusion length (measured from the limit of the dendrite to the tip of the protrusion). We verified the expression of HA-PAK3 proteins in GFP-positive neurons by HA immunofluorescence as previously described (data not shown; 13). As shown in Fig. 6B and consistent with previous results (13), expression of PAK3 carrying the R419X kinase-dead mutation resulted in a phenotype characterized by a loss of regular mushroom type spines and an increase in immature, elongated spines and filopodia-like protrusions (13). Overall, the protrusion density was only slightly, but not significantly reduced in these cells when compared with control, EGFP-transfected neurons (Fig. 6E; 0.81 ± 0.04 versus 0.90 ± 0.03; n = 7–32; p > 0.05) and the shift in protrusion length illustrated in Fig. 6F clearly indicated the

GFP-PAK3 expressing neurons. In these experimental conditions, the expression of the kinase-dead PAK3-K297L mutant does not inhibit dendritic spine formation (Fig. 5B, second line).
increased proportion of immature, elongated protrusions (mean length of protrusions: 1.52 ± 0.09 versus 1.14 ± 0.03 μm, n = 8–12; p < 0.01). Interestingly, expression of the A365E mutant (Fig. 6C) produced a phenotype comparable to the R419X mutant, although with a slightly different balance between the two types of alterations. As illustrated in Fig. 6E, the reduction in protrusion density was more marked in most A365E-transfected cells than had been observed with the R419X mutant (0.69 ± 0.07 protrusions/μm; n = 8; p < 0.05), whereas the increase in elongated immature protrusions was comparable, as indicated by the shift observed in the distribution of protrusion length (Fig. 6F; mean length: 1.50 ± 0.07 versus 1.14 ± 0.03 μm, n = 8–12; p < 0.05). Finally and most interestingly, expression of the R67C mutant, which affected the GTPase-binding region, resulted in a markedly reduced density in the spine density (Fig. 6D and E; 0.60 ± 0.05 protrusions/μm; n = 8; p < 0.01) without any changes in spine morphological characteristics and no shift in the distribution of spine length (Fig. 6F; mean length: 1.07 ± 0.03 μm). In these conditions, the presence of filopodia was very rare and nearly all protrusions were spines. Thus expression of the mental retardation PAK3 mutants differentially affected the mechanisms of spine morphogenesis.

**DISCUSSION**

**PAK3 Mutations Responsible for Mental Retardation Affect Different Properties of the PAK3 Protein**—One way to investigate how learning occurs and how memories are formed and sustained is through the identification of mutated genes associated with mental retardation (30). Defining the molecular and cellular consequences of mental retardation mutations is a step further toward understanding the mechanisms that are required for normal cognitive functions. We report here the characterization of three PAK3 proteins bearing mutations responsible for mental retardation, and we show that these mutations affect differently PAK3 functions.

The two mutations R419X and A365E, located in the catalytic domain, inactivate the kinase activity. This result was previously reported for the R419X-truncated protein, but was unpredicted for the A365E mutant (7, 9). This A365E mutation is located in a conserved subdomain named VIa inside the α helix E of the large lobe of the kinases (31). Several mutations associated with human pathologies were identified in this subdomain (see the KinMutBase web site) (32). We report here for the first time that a disease-associated mutation in this subdomain suppresses the kinase activity.

The Arg-67 residue is perfectly conserved in the PAK3 gene of vertebrates, i.e. in all mammalian, avian, amphibian, and fish genes analyzed. This arginine residue is also present in the two other PAKs of the group I (arginine 72 in PAK1 and 71 in PAK2), in PAK4 and -6, and corresponds to a conserved lysine in PAK5. It is located in the overlapping sequence of the polybasic region extending from Lys-58 to Arg-67 and the PBD domain covering residues from Lys-62 to Thr-108 (33). Several
mutations in the PBD domain abolish the binding to Rac1 and Cdc42 (34). Mutations in the polybasic region of the PAK1 protein have complex effects: neutral mutations of all three lysine residues Lys-66, Lys-67, and Lys-68 decrease the affinity toward Rac1 but not toward Cdc42, and reduce PAK1 activation by Cdc42 and Rac1 (35). In contrast, their mutation to basic residues has no effect. Importantly, it has been shown that the homologous residue of arginine Arg-67 in different GTPase effectors plays an important role in Cdc42 interaction (36). In this report, we show that the R67C mutation decreases the interaction with Cdc42 and, as a consequence, reduces the activation by Cdc42. We have observed that R67C mutation does not modify the two-hybrid binding to Rac1, whereas the same mutation increases the co-immunoprecipitation of PAK3 with this GTPase in COS7 cells. One explanation we propose is that the loss of Cdc42 binding to PAK3 could induce an increase of Rac1 interaction. Although this mutation increases the binding to Rac1, it does not induce the activation of PAK3 by a specific GEF for Rac1. This result is in agreement with published data showing that the binding of GTPases to PAK is not sufficient to trigger PAK activation (35).

Our results show that the C-terminal residue of the polybasic region governs PAK3 specificities toward GTPases, possibly by playing a role in the structural contacts between the PBD and the GTPases. Finally, the R67C mutation is responsible for a more severe phenotype than the two other mutations of PAK3 suggesting that the interaction of PAK3 with Cdc42 is crucial for synaptic plasticity. Indeed, PAK3 could have kinase-independent functions, such as playing a scaffolding role (37), and kinase-dependent functions, both depending on Cdc42 binding. 

**Cdc42 Is the Main Activator of PAK3**—PAK3 was believed to be activated by both Cdc42 and Rac1 GTPases (23, 38). In agreement with this, we found that co-transfection of PAK3 with either Cdc42-V12 or Rac1-V12 induced a strong activation of the kinase. However, when we activated PAK3 by endogenous GTPases, we found that only Cdc42 was able to activate PAK3. Indeed intersectin, a specific GEF for Cdc42, activated PAK3 via the endogenous Cdc42 GTPase, whereas Tiam1, a specific GEF for Rac1, did not. The discrepancy observed between endogenous activation versus co-transfection, may be caused by the exogenous GTPase expression in the latter condition, which overrides the weak affinity of PAK3 for Rac1. We report here that the wild-type PAK3 protein binds efficiently Cdc42 and faintly Rac1, which is in agreement with the notion that GTPase interaction is necessary for PAK activation (39). Excitingly, we found that the mental retardation R67C mutation inhibits its activation by the intersectin/Cdc42 pathway confirming that the Cdc42/PAK3 module is the one implicated in cognitive functions.

**PAK3 Mental Retardation Mutations Do Not Alter Spine Localization of the PAK3 Protein**—Because PAK3 mutations may induce a mislocalization of PAK3 responsible for synaptic defects, we analyzed the localization of the mental retardation PAK3 mutants in mature differentiated neurons. Interestingly, we demonstrate here that none of the kinase-dead PAK3 mutants presented defects in spine and dendritic localization, suggesting that spine localization is independent of kinase activity. However, the presence of phosphorylated, *i.e.* active PAK3, in dendritic spines was demonstrated both by biochemical methods and after isolation of post-synaptic densities (10, 14). Thus, we propose that PAK3 phosphorylation is not required for proper targeting. We also show that interaction with GTPases is not necessary for spine localization, because the R67C mutant and the H78L-H81L proteins display the same localization as the wild-type protein. PAK3 may be recruited to spines through interaction with other partners, such as the PIX-GIT complex (14). To conclude, we demonstrate for the first time that mental retardation PAK3 mutations do not induce protein mislocalization in spines, suggesting that cognitive defects in mental retardation patients, associated with PAK3 mutations, are mainly due to a functional defect of PAK3.

**Role of a Cdc42/PAK3 Pathway in Spine Formation**—It was previously shown that the kinase activity of PAK3 is required for the formation of mature dendritic spines (13, 14). Expression of PAK3-R419X and PAK3-siRNA in rat hippocampal organotypic slice culture resulted in the formation of morphologically abnormal spines, with a decrease of mature dendritic spines and an increase in elongated, immature protrusions (13). We report here that expression of the kinase-dead PAK3-A365E mutant induced a similar phenotype, whereas expression of PAK3-R67C, defective in Cdc42 binding and in activation by Cdc42, led to a pronounced decrease of spine density. Altogether these results show that both inactivation of the kinase activity (R419X and A365E) and defect of binding and activation by Cdc42 (R67C) led to spine anomalies, suggesting that PAK3 binding to Cdc42 and PAK3 kinase activity are both necessary for spinogenesis. The R67C mutation only affects spine density suggesting that this mutation could alter different mechanisms in spine number regulation, *i.e.* spine initiation or stabilization of newly formed spines. Concerning the second hypothesis, the protrusions would present morphological anomalies or appear as remnant structures, because the spine maintenance is perturbed. However, we did not observe such anomalies, and thus we favor the hypothesis that the R67C mutation impairs spine initiation.

We propose a model in which PAK3 may act at two different steps during spine formation, at the initiation of spines and at spine maturation (Fig. 7). The binding of Cdc42 to PAK3 could contribute to the formation of a signaling complex involved in the initiation of the growth of new protrusions. It is well known that Cdc42 governs filopodia formation in several cell types, and because PAK3 is implicated in spinogenesis, we could hypothesize that Cdc42 initiates filopodia formation via the activation of PAK3 (40). On the other hand, the kinase activity might be necessary for the stabilization and maturation of newly formed protrusions, as also suggested by the small size of the post-synaptic densities found on immature spines (13). The elongated filopodia observed in neurons expressing kinase-dead mutants may correspond to precursors of spines blocked in spine maturation and could represent a transitory step in a dynamic process (41). The role of Cdc42 in spine formation is poorly understood (4, 5). However, in addition to PAK3, Cdc42 has several effectors implicated in spine morphogenesis such as IRSp53 or N-WASP (42, 43). The study of Cdc42 in the PAK3
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signaling pathway involved in spine formation, using pharmacological approaches or active and inactive Cdc42 mutants, has not been possible, because other effectors than PAK3 would also be affected. However, a PAK3 mutant such as the R67C mutant we described here, selectively defective in Cdc42 binding, constitutes a powerful tool to study the role of Cdc42/PAK3 module in spinogenesis. The identification of upstream regulators and downstream targets of the Cdc42/PAK3 module will be of major interest to understand how this pathway regulates synaptic plasticity and how it is impaired in mental retardation associated disease. Finally, in patients with mental retardation, the loss of PAK3 function is not compensated by the other PAK proteins, confirming the idea of a specific role of PAK3 in synaptic plasticity. The identification of these PAK3-specific functions constitutes a challenge to understand the signaling pathways of neuronal plasticity.

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