PAK3 mutation in nonsyndromic X-linked mental retardation

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Nonsyndromic X-linked mental retardation (MRX) syndromes are clinically homogeneous but genetically heterogeneous disorders, whose genetic bases are largely unknown. Affected individuals in a multiplex pedigree with MRX (MRX30), previously mapped to Xq22, show a point mutation in the *PAK3* (p21-activated kinase) gene, which encodes a serine-threonine kinase. PAK proteins are crucial effectors linking Rho GTPases to cytoskeletal reorganization and to nuclear signalling. The mutation produces premature termination, disrupting kinase function. MRI analysis showed no gross defects in brain development. Immunofluorescence analysis showed that PAK3 protein is highly expressed in postmitotic neurons of the developing and postnatal cerebral cortex and hippocampus. Signal transduction through Rho GTPases and PAK3 may be critical for human cognitive function.

Introduction

Human mental retardation reflects a diversity of potential causes, but a substantial proportion is due to mutations in specific genes. Many genetic causes of mental retardation, such as storage or mitochondrial disorders, reflect metabolic defects in the neuron that presumably cause relatively nonspecific defects in neuronal function; these disorders provide little direct insight into the biology of human learning and memory, or into potential therapeutics. Other genetic causes of mental retardation include grossly definable disorders of brain development[1], which result in a brain that lacks the normal stereotyped patterns of organization and connectivity required for normal brain function. In contrast, mental retardation that is accompanied by grossly normal brain development, and few or no other signs or symptoms, is referred to as nonsyndromic mental retardation. These disorders promise to identify a number of genes that are required for normal cognitive function.

Several X-linked forms of non-syndromic mental retardation (MRX) have been mapped due to the ease of gene mapping on the X chromosome. MRX syndromes are clinically homogeneous but genetically diverse. The heterogeneity of MRX complicates positional cloning efforts. The lack of distinctive clinical features for MRX syndrome causes each syndrome to be represented by essentially a single pedigree. At present more than 60 such MRX pedigrees have been mapped to a variety of loci on the X chromosome. It has been estimated that these 60 pedigrees define at least 8–10 genetic loci[2,3], although the actual number is unknown and is probably higher[4].

The first gene for MRX, *FMR2*, was identified in 1996 (ref. 5), and two additional genetic forms of MRX have been found. Whereas *FMR2* encodes a nuclear protein that may regulate transcription, the other two MRX genes may function as critical regulators of pre- or postsynaptic function. For example, *GDI1*, which encodes a GDP-dissociation inhibitor for Rab3a, is mutated in two MRX pedigrees that map to Xq28 (ref. 4). Rab3a is a small G protein that regulates vesicular transport through the secretory pathway; thus *GDI1* mutations may alter the exocytic events associated with synaptic transmission.

Another gene found to be mutated in MRX pedigrees mapping to Xq12 encodes oligophrenin, a protein that includes a GTPase activation domain (GAP) for Rho GTPases. GAP proteins stimulate the intrinsic GTPase activity of small G proteins, so that inactivation of GAP proteins potentially causes constitutive activity of the corresponding G protein. Oligophrenin shows GAP activity for Rho, Rac and Cdc42, which are G proteins implicated in the control of actin cytoskeletal organization and cell shape and motility[6]. The Rho GTPases have been implicated directly in the control of axon outgrowth in a variety of organisms[7–10], as well as in the control of the shape and size of dendrites[11] and dendritic spines, which are a frequent site of synapses in vertebrate neurons[12]. Therefore, the implication of a RhoGAP in human mental retardation suggests that the Rho GTPases may be directly involved in the synaptic and dendritic mechanisms of neuronal plasticity.

Four MRX pedigrees (MRX23, MRX30, MRX35, MRX47) and two other more distinctive neurological syndromes (Table 1) map to a large genetic region overlying Xq21–q24. Recent efforts at identification of genes from this region that are expressed in brain[1,13] suggested *PAK3* as a positional candidate gene for these six disease loci. PAK3 was originally cloned as a protein that binds Rac and Cdc42 (refs 14,15). *PAK3* is a member of the larger family of p21-activating kinase (PAK) genes that also includes human *PAK1*, *PAK2* and *PAK65*, and yeast Ste20. PAK proteins are highly conserved in their amino acid sequence, and have been implicated as critical downstream effectors that link Rho GTPases to the actin cytoskeleton and to MAP kinase cascades, including the c-Jun amino-terminal kinase (JNK) and p38. We show here that *PAK3* is specifically mutated in affected males with MRX30.

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Results

Cloning and sequencing of *PAK3*

A fragment of *PAK3* was originally identified as the end of a cosmid (H1, Fig. 1*a*,*b*) derived from YAC 148D1 from the ICRF YAC library, during the construction of a physical map of Xq21–q23 (ref. 13). To allow screening of *PAK3* as a candidate gene for neurological disorders, we obtained the full-length cDNA by a combination of RT-PCR, 3′-RACE and sequencing of ESTs obtained by BLAST searching (Fig. 1*c*). This approach allowed the construction of sequence corresponding to the entire 1,635-bp open reading frame (ORF) of *PAK3* (Fig. 1*c*). Cosmid sequencing, either directly or using vectorette PCR (ref. 16), allowed the design of PCR primers to amplify each coding exon of *PAK3* from genomic DNA.

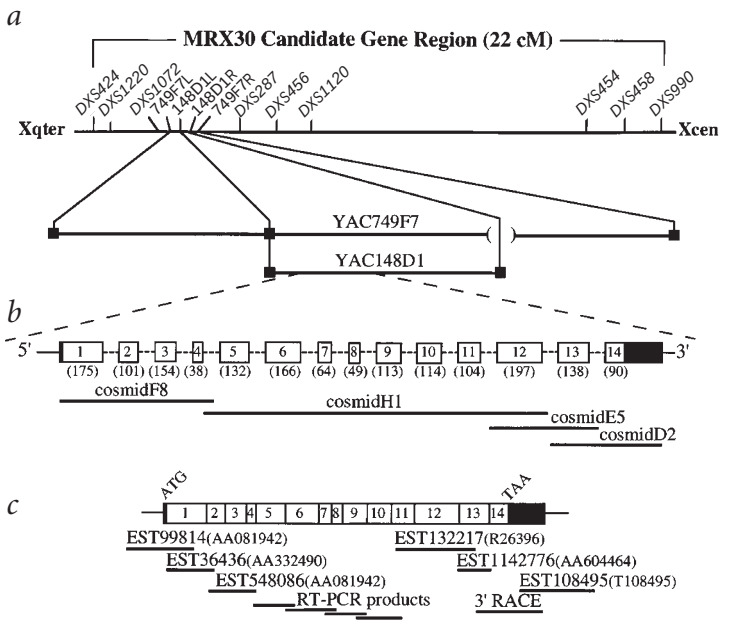

Fig. 1 Genomic organization of human *PAK3* and cDNA contig. ***a***, A physical map of Xq21–q23 (ref. 13), representing the candidate region for MRX30 (ref. 17). Represented above the chromosome are the published markers used for linkage analysis[17], and YAC-end STS used to develop the YAC contig around *PAK3* (ref. 13). Boxes indicate the presence of the YAC-end STS in the YAC, whereas parentheses indicate the absence of that particular marker. ***b***, Cosmids generated from YAC 148D1 and a cosmid contig of *PAK3*. The numbered boxes indicate the exons and the filled boxes indicate untranslated regions that were part of exons. The size of the exons is indicated in parentheses, but the exact size of *PAK3* introns was not determined. ***c***, Human *PAK3* cDNA contig with exons indicated. The sequence matches multiple anonymous EST's (GDB accession numbers indicated in parentheses), and shows regions of *PAK3* identified by RT-PCR or 3′-RACE.

Mutational analysis of *PAK3*

We analysed five neurological disease loci for mutations in *PAK3* by PCR amplification of the coding exons and subsequent single stranded conformational polymorphism (SSCP) analysis and/or sequencing of the PCR products, and identified a deleterious mutation in one of these disorders, MRX30 (ref. 17). The mutation was present in all affected males of the pedigree, whereas all unaffected carrier females were heterozygous for the mutation (Fig. 2*a*,*b*). None of the normal females or males from this pedigree, nor any of more than 45 normals studied, showed the mutation. The mutation represents a C-to-T transition, changing an arginine codon (CGA) at aa 419 to a stop codon (TGA). Human *PAK3* cDNA is extremely homologous to mouse[14] and rat (originally called *βPak*; ref. 15) *Pak3*, showing greater than 92% identity at the nucleotide level, and more than 98% identity at the amino acid level to either rodent protein. All PAK proteins show very high amino acid conservation (>95%) in the 11 domains required for normal kinase activity (I–XI) and in the Cdc42/Rac interactive binding (CRIB) motif. In addition, there are several potential SH3-binding domains, the first of which is required for binding of PAK1 to the adapter protein Nck (ref. 18). The premature stop codon seen in the MRX30 pedigree occurs in segment VII of the kinase domain, in a region that is more than 95% identical and essentially 100% conserved at the amino acid level, not only between rodent and human PAK3, but between all known PAK family members. The mutation truncates the predicted protein in a region essential for normal kinase function[19], and removes a threonine (T421) that corresponds to a site of autophosphorylation (T423) during PAK1 activation by Rac (ref. 20).

No *PAK3* mutations were found in affected individuals with MRX23 (ref. 21), MRX35 (ref. 22), X-linked epilepsy and mental retardation limited to females[23], or X-linked nonprogressive congenital cerebellar hypoplasia[24] (Table 1). These disorders are clinically heterogeneous and several are, in fact, clinically distinct from MRX30. Hence, the disorders are probably genetically heterogeneous, as is the case for other MRX syndromes studied[4,25]. Each syndrome is mapped to a very broad region of the X chromosome (10–30 cM) overlapping the location of *PAK3*. We cannot rule out that *PAK3* mutations outside the coding region may account for some of the other disorders. As MRX pedigrees are only defined by their map position, and as MRX pedigrees that map to Xq22–q24 are rare, the frequency with which *PAK3* mutations cause MRX could not be determined.

Analysis of the MRX30 mutation

When the MRX30 mutation is introduced into *Pak3* cDNA and transfected into COS cells, the cDNA produces a stable, albeit truncated, protein (Fig. 3*a*,*b*). When assayed for PAK kinase activity, either as PAK autophosphorylation or as phosphorylation of myelin basic protein (MBP), the MRX30 mutation abol-

Table 1 • Disorders mapped to Xq23

Disorders	Location	*PAK3* mutation	Reference
MRX23	Xq23–q24q	—	Gregg *et al.* 1996
MRX30	Xq21.3–q24	yes	Donnelly *et al.* 1996
MRX35	Xq22–q26	—	Gu *et al.* 1995
MRX47	Xq22.3–q24	NT	des Portes *et al.* 1997
X-linked epilepsy and mental retardation limited to females (EFMR)	Xq21–q23	—	Ryan *et al.* 1997
X-linked nonprogressive congenital cerebellar hypoplasia	Xq21–q23	—	Illarioshkin *et al.* 1996

Disorders including Xq22–23 in the candidate interval that were analysed for *PAK3* mutations. The disorders are listed and the approximate map location determined by linkage mapping is indicated. This table represents a complete list, to our knowledge, of all neurological disorders mapping to this region. NT, not tested. —, no mutation found.

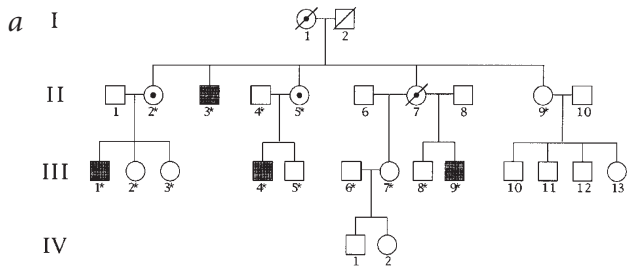

Fig. 2 Pedigree of the MRX30 family and mutation analysis of *PAK3* in MRX30. ***a***, Adapted pedigree of MRX30 (ref. 17) showing unaffected females as white circles, and phenotypically unaffected, obligate carrier females as a circle with a dot. Unaffected males are indicated by open squares and affected males are shaded. Asterisks indicate all individuals on which linkage data and DNA sequence analysis of *PAK3* were performed. Individuals III2, III3 and III7 do not carry the mutation. ***b***, Mutation analysis of *PAK3* in MRX30. PCR products from exon 12 were sequenced to identify the presence or absence of a C→T point mutation at nt 1255. Normal males or females have a C, affected males have a T and obligate heterozygous carrier females have both the T and C.

ished measurable kinase activity (Fig. 3*a*). In contrast, the MRX30 mutation leaves the CRIB domain intact, and mutant PAK3 protein still binds activated Cdc42 (Fig. 3*c*) and activated Rac (data not shown).

To determine whether the mental retardation in males that carry the *PAK3* mutation represents a gross structural disorder of brain development, we used MRI to analyse the most severely affected male with MRX30 (Fig. 2*a*, II-3). This individual was also microcephalic, although several other affected males in the MRX30 pedigree were not[17]. MRI analysis showed that, as expected, the brain was small, but it was generally well-formed with normal gross architecture of the cortex and hippocampus (data not shown). In addition, the white matter and major fiber tracts, such as the internal capsule, anterior commissure and corpus callosum, showed no detectable abnormalities. Although more subtle defects in neuronal development cannot be ruled out, MRI analysis suggests *PAK3* is not absolutely required for neuronal proliferation, migration, or cortical gyration. Therefore, the observation that the *PAK3* mutation produces mental retardation may reflect a later requirement for *PAK3* in axon outgrowth or function of the adult cortex.

Expression of *PAK3* in brain

PAK3 mRNA is highly expressed in the fetal human brain (Fig. 4*a*), but is not expressed at detectable levels in other fetal organs[14]. *PAK3* is encoded on a large, approximately 10-kb transcript, with a second, less abundant 8-kb transcript seen with some probes. The rat orthologue of *PAK3* (*βPAK*; ref. 15) is encoded on a similarly large transcript, despite the relatively small ORF. The significance of the evolutionarily conserved large size of the *PAK3* transcript is unknown. A PAK3-specific antiserum detects only a single, approximately 60–65-kD band that is expressed in cerebral cortical brain homogenates across a wide range of developmental stages, as well as showing persistent expression in adult cortex (Fig. 4*b*).

In stained sections of neonatal mouse brain, Pak3 immunoreactivity is detected in neuronal dendrites and axons of postmitotic neurons of the cerebral neocortex and hippocampus, but immunoreactivity is absent from the dividing cells of the ventricular zone (VZ) and subventricular zone (SVZ; Fig. 5*a*,*b*). Pak3 immunoreactivity in the adult rat brain (Fig. 5*e*,*f*) is ubiquitous in neurons of the cerebral cortex, hippocampus and most other parts of the brain, but is absent in myelinated white matter tracts.

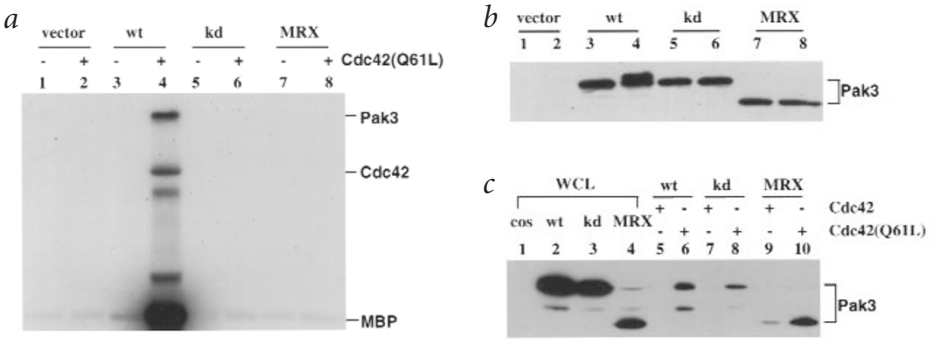

Fig. 3 The MRX30 mutation encodes a stable, truncated protein without kinase activity but with preserved Cdc42 binding. ***a***, The MRX30 mutation abolishes PAK kinase activity. COS cells were transfected with empty vector (vector), wild-type Pak3 (wt), kinase dead Pak3 due to a K297R point mutation (kd), or the MRX30 truncation created by site-directed mutagenesis (MRX). An *in vitro* kinase assay[14] was used to measure autophosphorylation of Pak3 (Pak3), or phosphorylation of myelin basic protein (MBP) in the presence or absence of recombinant Cdc42 containing an activating point mutation, Cdc42(Q61L). Wild-type Pak3 shows kinase activity, whereas the kd and MRX30 allele show no detectable autophosphorylation or MBP phosphorylation. ***b***, The MRX30 mutation creates a truncated protein. A western blot (probed with anti-Pak3 antiserum) of the cell lysates used in (*a*) shows that wt Pak3 protein runs as a doublet due to phosphorylation (lane 4), whereas the kd allele runs as a singlet. The MRX30 allele runs as a single band that is smaller in size, as expected, but apparently stable. ***c***, Preserved binding of Cdc42 to wt Pak3, kd Pak3 and Pak3 carrying the MRX30 mutation. Binding assay was performed as described[14] and again shows expression of a truncated PAK3 protein in whole COS cell lysates (WCL) transfected with mPak3 carrying the MRX30 mutation (lane 4), but preserved binding of constitutively active Cdc42 to wt Pak3, kd Pak3 and the MRX30 allele. Similar results were obtained with binding of activated Rac (data not shown).

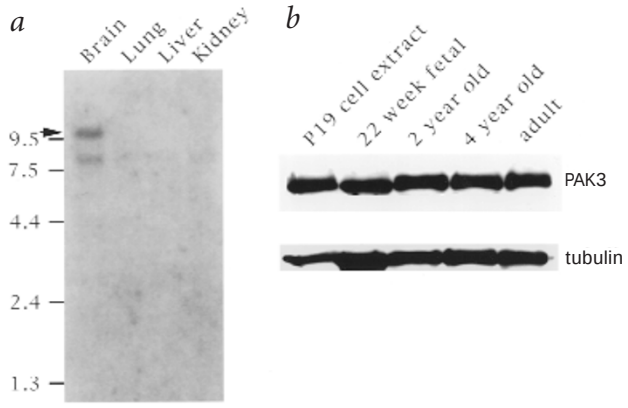

Fig. 4 Expression analysis of *PAK3* mRNA and protein. ***a***, A commercial (Clontech) multiple-tissue northern blot prepared from polyA-selected RNA from 20-week-old human fetus and hybridized with EST99814 (Fig. 1), which contains part of the first coding exon and part of the 5' UTR of *PAK3*. There is hybridization to a predominant 10-kb brain-specific transcript, as well as to a less abundant 8-kb transcript. Hybridization of this same blot with cDNA probes from the *PAK3* coding region shows a single hybridizing band at 10 kb (data not shown). ***b***, Western-blot analysis of PAK3 in equivalent amounts of total protein extracts prepared from cultured P19 cells, and from human cortex at several developmental time points as indicated. PAK3 is expressed throughout the period from 22 weeks gestation until adulthood at comparable levels. The lower image shows western-blot analysis of the same filter used in (*b*), using anti-tubulin antibody to control for protein loading.

The immunohistochemical distribution of Pak3 closely matches previously published rat *in situ* hybridization data, suggesting that *PAK3* mRNA is expressed in developing and adult hippocampus and cortex, especially in postmitotic neurons in the adult dentate gyrus and cortical layers II/III and V (ref. 15). Staining in hippocampal and cortical neurons (Fig. 5*e*,*f*) and in cerebellar Purkinje cells (data not shown) appears prominently in the cell soma and proximal dendrites, but there is little or no staining of synaptic terminals or dendritic spines. Although staining at synaptic terminals cannot be ruled out, PAK3 protein appears to be predominantly localized to the postsynaptic neuron. In cultured neuronal cells, PAK3 immunoreactivity is distributed in a diffuse pattern in the cell soma and neuritic processes (Fig. 5*d*). These data suggest that PAK3 is localized in axons, somata and dendrites of developing neurons, and in soma and dendrites of adult neurons as well.

Discussion

We report that a point mutation in *PAK3* is present in affected members of a multiplex pedigree with MRX30. Together with evidence implicating signalling through Rho GTPases and PAK in the control of neuronal shape and growth, these data suggest that Rho GTPase and PAK signalling may be critical for neuronal connections that underlie human cognitive function.

What are the neurobiological roles of PAK3? PAK proteins appear to be major effector proteins that link Rac and Cdc42 to the actin cytoskeleton[20] as well as to transcriptional activation through kinase pathways acting through p38 and JNK (ref. 26). Perhaps, as Rac activation is critical for axon initiation[8], axon elongation[8,12] and axon targeting[7,10], PAK3 may be necessary for the normal development of axonal connections, leading to aberrant or absent axonal connections in MRX30 patients. Alternatively, as Rac signalling also appears to regulate the size and number of dendritic spines[12] and the remodelling of dendrites[11], PAK3 may be necessary for dendritic development or for the rapid cytoskeletal reorganizations in dendritic spines associated with synaptic plasticity[27]. Given the predominant localization of PAK3 immunoreactivity in the adult in postsynaptic profiles, it seems more likely that the locus of PAK3 effects is in the postsynaptic cell; however, this bears further study.

As several of the actions of PAK proteins depend on their kinase activity, the MRX30 mutation may cause a simple loss of biochemical function or may even represent a dominant interfering construct. PAK activation has been shown to produce activation of the JNK1 and p38 MAP kinase pathways[26,28], probably *via* a MEKK, and this MAP kinase activation appears to depend upon the kinase activity of PAK (refs 26,29). MAP kinase activation leading to alterations in nuclear transcription has also been directly implicated in synaptic plasticity[30]. Thus, the role of PAK3 could be primarily *via* modulating transcription in the postsynaptic neuron. An engineered mutation in p65PAK (called PAK/RD), which lacks the kinase domain but contains the CRIB region, resembles the MRX30 allele[31]. Co-expression of PAK/RD suppressed the activation of the TPA response element and cell transformation caused by constitutively activated alleles of Ras and Rac (ref. 31). By analogy, the MRX30 allele of PAK3 could inhibit multiple signalling pathways through Ras and Rac. The MRX30 mutant PAK3 could even interfere with pathways that use distinct PAK proteins, by titrating out activated Rac and thereby inhibiting MAP kinase activation.

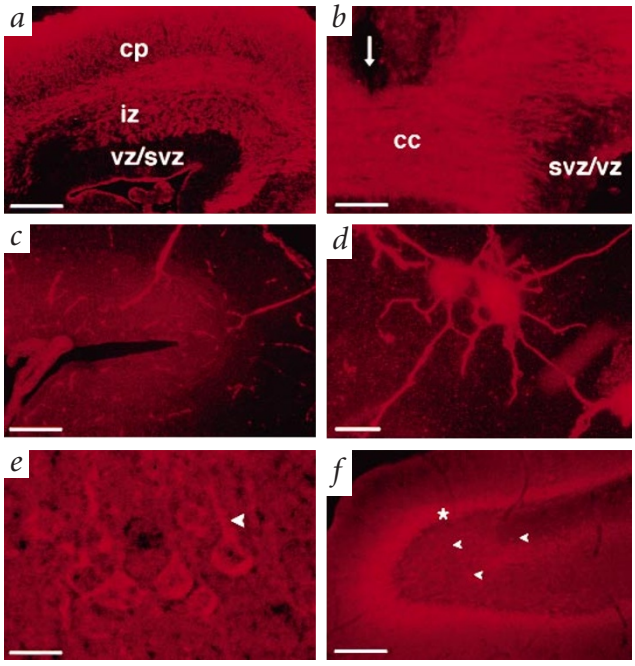

Fig. 5 Immunohistochemical analysis of Pak3 protein expression in developing mouse and adult rat cortex. ***a***, Immunofluorescence of a coronal section of P0 mouse cerebral cortex stained with antiserum specific for Pak3. Pak3 immunoreactivity is seen in neurons of the cortical plate (cp), but there is no staining of the dividing cells of the VZ and SVZ. Immunoreactivity is intense in cortical axons in the subcortical white matter (iz). ***b***, Higher magnification of immunofluorescence for Pak3 in a coronal section of P0 mouse cerebral cortex showing intense staining in the axons of the corpus callosum (cc), with staining extending into the growth cones of the axons. The midline is indicated by the arrow; the SVZ/VZ shows no staining. Scale bar, 500µm in (*a*) and 100 µm in (*b*). ***c***, Negative control for immunofluorescence by omission of primary antiserum, coronal section. Scale bar, 500 µm. ***d***, Immunofluorescence of cultured E17 mouse cortical neurons showing the subcellular localization of Pak3 immunoreactivity. Immunofluorescent staining shows a diffuse distribution throughout the soma and neurites. Scale bar, 20 µm. ***e***, Immunofluorescence of cerebral cortex showing the subcellular localization of Pak3 immunoreactivity in the adult. Pak3 immunoreactivity is strong in primary dendrites of pyramidal neurons (arrowhead), and shows little evidence for staining of synaptic terminals. Scale bar, 40 µm. ***f***, Immunofluorescence of adult rat dentate gyrus showing preferential staining of cell somata of granule cells (asterisk), as well as neurons scattered in the hilus (arrowheads). Scale bar, 1 mm.

However, many of the downstream effects of PAK proteins on the actin cytoskeleton do not appear to depend upon the intrinsic kinase activity of PAK. For example, kinase-deficient PAK1 proteins are still capable of inducing neurite outgrowth in PC12 cells[32], or inducing widespread cytoskeletal reorganization[20]. PAK1 appears to bind Nck and perhaps other adapter proteins through the SH3 binding domains at their N termini[18,33,34]. Nck binding is facilitated by Rac binding to the CRIB domain of PAK; Rac activates PAK1 by inducing autophosphorylation as well as by facilitating protein-protein interactions through the N terminus of PAK, as well as by altering the subcellular localization of PAK1 (refs 18,20,33–35). Kinase-independent actions of PAK3 may not be blocked by truncation of the kinase domain. In fact, the MRX30 truncation may create a PAK3 that is constitutively active for kinase-independent functions. Thus, further study of the MRX30 mutation may provide important insights into the biology and neurobiology of PAK gene function.

Methods

Patient samples. Clinical data and blood samples were obtained after receiving informed consent, in accordance with protocols for human studies approved by the Beth Israel Deaconess Medical Center Institutional Review Board. Clinical data and diagnostic categorization of the patients used in this study are summarized (Table 1). MRI analysis was performed using standard sequences. DNA from peripheral blood lymphocytes was prepared using Qiagen columns.

Physical map of the *PAK3* locus. The region surrounding the *PAK3* locus was mapped as described [1,13]. Cosmids H1, F8, E5 and D2 containing *PAK3* were obtained by subcloning YAC 148D1 from the ICRF library (HGMP), using the SuperCos 1 vector (Stratagene) as described[1].

Identification of the *PAK3* ORF. A fragment of *PAK3* sequence was originally obtained from end-sequencing of cosmid H1 (ref. 1). Additional *PAK3* sequence was obtained by sequencing cDNA deposited as ESTs that matched the DNA sequence of *PAK3* (Fig. 1*c*). Additional cDNA sequence was also obtained from human fetal brain cDNA (Clontech) by 3′-RACE and by RT-PCR using *PAK3*-specific primers, or by using one *PAK3*-specific primer and one degenerate primer designed to rodent *Pak3* sequences, and later re-confirming the human *PAK3* sequence by re-amplification and re-sequencing.

Ligation-mediated PCR. Intronic sequences of *PAK3* were determined by direct sequencing of cosmids F8, H1, E5 and D2 using exon-specific primers or by the bubble vectorette technique[16]. The captured intronic sequences were used to design primers to amplify the entire exon using the Primer program v. 3.0 available from the Whitehead Institute/MIT Center for Genome Research (http://www-genome.wi.mit.edu).

Mutation detection. To examine the 14 exons of *PAK3*, patient DNA was amplified using the following primers: exon 1, forward, 5′–GACCTCTTCTCCCTCAACTCCTT–3′, reverse, 5′–GCCCGGCCACTGATACTTTACT–3′ (268 bp); exon 2, forward, 5′–GTGGATGCCAAAACTATACCCC–3′, reverse, 5′–CATGGCAATTCTTGCTTGAAAA–3′ (234 bp); exon 3, forward, 5′–TAGCATGGGCTTCTTGGTGA–3′, reverse, 5′–CGATTTCAAATGTCCCACAGAC–3′ (394 bp); exon 4, forward, 5′–CTGGTGTGGTCTCTGAATGAAA–3′, reverse, 5′–TGGAAACACCAATCATGTGAAA–3′ (149 bp); exon 5, forward, 5′–TTCCTCCTATCCCTACATGCTT–3′, reverse, 5′–TGACTATTCCAAAGGGGATCTT–3′ (295 bp); exon 6, forward, 5′–TTGACTCCACACTCTTTCCTTG–3′, reverse, 5′–TGAGAAACACTCCCGTGTAGAA–3′ (274 bp); exon 7, forward, 5′–CTCTTCCACCTCCTCCTCTTTT–3′, reverse, 5′–GAAAGGCCAATACTGAGAATGC–3′ (258 bp); exon 8, forward, 5′–GTTTGCTAATTTTCCTCCCCTC–3′, reverse, 5′–CACCTATGTCAACCCAGTGCTA–3′ (270 bp); exon 9, forward, 5′–TGTTAAATGAACGAATCCCAAA–3′, reverse, 5′–TTTGGGGACATAGAGTGAAAC–3′ (290 bp); exon 10, forward, 5′–CTGAGGCCTGGGAATATCAG–3′, reverse, 5′–AAGAACTGCCAATTTCGGTG–3′ (245 bp); exon 11, forward, 5′–TCCCTGCCCAAAATTTATCT–3′, reverse, 5′–TGGTCACAATATTCAGGAACACA–3′ (352 bp); exon 12, forward, 5′–TTTGGGCTTATTTTAACTGGC–3′, reverse, 5′–GCCTTTCTTCTTTTCCTGGG–3′ (284 bp); exon 13, forward, 5′–TGTATGTGCTGAAATGGATTTTTC–3′, reverse, 5′–GCCTCTCTTTGCAATTGTCCT–3′ (210 bp); exon 14, forward, 5′–TCAAAAGGGCTGCTTGAAAA–3′, reverse, 5′–TCATGAGGGAGATGGTGAGG–3′ (211 bp). PCR was performed using standard protocols. A 'hot start' was followed by 35 cycles of 92 °C for 30 s, 60 °C for 1 min and 72 °C for 2 min. For SSCP analysis, PCR products were denatured and electrophoresed on 6% polyacrylamide gels run at 30 W for 2–4 h at 4 °C, and developed using the Silver staining system (Promega). PCR products from affected males in each pedigree (except EFMR) were also directly sequenced and analysed on an ABI377 automated DNA sequencer.

Plasmids. The plasmids J3HmPak3, J3HmPak3K297R (kinase dead), GST-Cdc42 and GST-Cdc42Q61L have been described[14,20,26]. A plasmid containing the MRX30 mutation (J3HmPak3R419ter) was generated by site-directed mutagenesis using the Quick Change kit (Stratagene), using the oligonucleotides 5′–CTCCTGAGCAAAGTAAATGAAGCACTATGGTGGG–3′ and 5′–CCCACCATAGTGCTTCATTTACTTTGCTCAGGAG–3′. These oligonucleotides incorporate the C→T transition that converts aa 419 from Arg to a termination codon.

Protein and kinase analysis. Cell culture and transfections were performed as described[14], and mPak3 kinase activity and binding to Cdc42 and Rac was determined as described[14,26], using the constitutively active mutant, GST-Cdc42(Q61L).

Northern and western analysis. A commercial fetal tissue northern blot (Clontech) was probed with EST99814 (Fig. 1*c*) labelled with [α–32P] CTP using random priming as described[1]. PAK3 western-blot analysis of total protein (100 μg) from fresh frozen human brain homogenates was performed as described[14].

Immunofluorescence analysis of PAK3. Neonatal (P0) mouse brains or adult rat brains were fixed by perfusion with 4% paraformaldehyde in PIPES buffer (pH 7.0), followed by immersion in phosphate-buffered saline that contained 30% sucrose until the brains had sunk. Brains were then frozen in OCT compound and sectioned on a freezing microtome (30 μm). Sections were incubated overnight with a primary rabbit anti-mouse antiserum (diluted 1:50–100) raised to the N-terminal 13 aa from mouse Pak3 (ref. 14; Upstate Biotechnology). The initial 13 aa of Pak3 show at least 3 of 13 divergent amino acids *versus* other PAK family members. The anti-PAK3 antiserum recognizes a single band, and detects no other PAK proteins in COS cells (Fig. 3). Sections were then rinsed with PBS and incubated with Cy3-labelled anti-rabbit secondary antibody (diluted 1:200), rinsed several times in PBS, mounted in Aquamount and examined under an Olympus fluorescent microscope. Negative controls were prepared by omission of the primary antiserum.

Neuronal cell culture. Cultured neurons for immunofluorescence were prepared as described[36].

GenBank accession numbers. Human *PAK3*, AFO68864; mouse *Pak3*, V39738 and rat *Pak3* (*βPak*), U33314. Other human PAK family members include: *PAK1*, U24152; *PAK2*, U24153; and *PAK65*, U25975.

Acknowledgements

We are grateful to the patients who participated in this study. We thank the following individuals for providing DNA samples from pedigrees that ultimately did not show PAK3 mutations: S. Illarioshkin, S. Claes, S. Fritz, J.-J. Cassiman, S. Ryan and R. Gregg. We thank J. Gonzalez, E. Olsen and S. de la Calle for help with immunohistochemistry, Y. Feng and F. Watson for help with COS cell transfection and western blots, J. Fox for help with SSCP, T. Young-Poussaint for reviewing the MRI and S. Hong for technical assistance. K.M.A. was supported by an NRSA from the NIMH (MH10691) and by the Goldenson/Berenberg fellowship. J.G.G. was supported by an NSADA from the NINDS (5K12NS01701) and by an educational grant from Sigma Tau Pharmaceuticals. C.A.W. was supported by the Human Frontier Science Program and NIH RO1 NS 35129 from the NINDS. C.A.W. is a Scholar of the Rita Allen Foundation.

Received 13 July; accepted 10 August, 1998.

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