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Missense Mutation in PAK3, R67C, Causes X-Linked Nonspecific Mental Retardation

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X-linked mental retardation is a very common condition that affects approximately 1 in 600 males. Despite recent progress, in most cases the molecular defects underlying this disorder remain unknown. Recently, a study using the candidate gene approach demonstrated the presence of mutations in *PAK3* (p21-activating kinase) associated with nonspecific mental retardation. *PAK3* is a member of the larger family of *PAK* genes. PAK proteins 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 screened 12 MRX pedigrees that map to a large region overlying Xq21-q24. Mutation screening of the whole coding region of the *PAK3* gene was performed by using a combination of denaturing gradient gel electrophoresis and direct sequencing. We have identified a novel missense mutation in exon 2 of *PAK3* gene (R67C) in MRX47. This confirms the involvement of *PAK3* in MRX following the report of a nonsense mutation recently reported in MRX30. In the MRX47 family, all affected males show moderate to severe mental retardation. No seizures, statural growth deficiency, or minor facial or other abnormal physical features were observed. This mutation R67C is located in a conserved polybasic domain (AA 66–68) of the protein that is predicted to play a major role in the GTPases binding and stimulation of Pak activity. Am. J. Med. Genet. 93:294–298, 2000. © 2000 Wiley-Liss, Inc.

KEY WORDS: X-linked mental retardation; PAK3; Xq23

INTRODUCTION

X-linked mental retardation (XLMR) is a very common condition that affects about 1 in 600 males. It can be syndromal or nonspecific (MRX). Until recently, *FMR2* was the only gene known to be involved in MRX [Gecz et al., 1996; Gu et al., 1996]. Over the past 2 years, despite the complexity of the extensive genetic heterogeneity, positional cloning efforts based on either the investigation of balanced X;autosome translocations, deletion mapping, or candidate gene strategy allowed to identify, so far, four different genes involved in MRX [Chelly, 1999]. The first one, called *oligophrenin 1*, encodes a rhoGTPase-activating protein [Billuart et al., 1998]. The second gene is *GDI1*, a rab-GDP-dissociation inhibitor, implicated in synaptic vesicle cycling and neurotransmitters release [D'Adamo et al., 1998]. The third gene, *PAK3*, is a member of the larger family of p21-activating kinase (PAK) [Allen et al., 1998], and the fourth gene, *IL1RAPL* (interleukin-1 (IL-1) receptor accessory protein like) encodes a 696-amino acids protein that has significant homology to IL-1 receptor accessory proteins [Carrié et al., 1999].

PAK3, implicated in family MRX30 [Allen et al., 1998], is a member of the family of PAK genes that also includes human *PAK1, PAK2,* and *PAK4* [Sells and Chernof, 1997; Abo et al., 1998]. PAK proteins are

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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. In the MRX30, the mutation R419X produces premature termination, leading most likely to a loss of function of PAK3.

In order to evaluate the frequency of PAK3 mutations among mapped familial cases of MRX, we screened for point mutations mentally retarded individuals from 12 different MRX pedigrees, including a large French family (MRX47), which are linked to the Xq21-q24 region [des Portes et al., 1997]. Screening of the whole coding region of the *PAK3* gene was carried out by using a combination of denaturing gradient gel electrophoresis (DGGE) and direct sequencing analyses. We identified a novel missense mutation R67C in the propositus of the MRX47 family.

MATERIALS AND METHODS

Patients

We analysed 12 extended families with idiopathic mental retardation (10 nonspecific and two syndromal). Linkage analyses had previously shown the most likely location of the disease in Xq21-q24 region in each family either by exclusion mapping (lod score <2) or by linkage analysis (lod score >2) (Table I). Fragile X syndrome was excluded by Southern-blot analysis using DNA digested with *Eco*RI/*Eag*I endonucleases and StB12-3 probe corresponding to the FRAXA locus [Rousseau et al., 1991]. Ethics committee approval and patient or family consent was obtained.

Mutation Analysis

DGGE. DNA was extracted from peripheral-blood leukocytes or lymphoblastoid cells, and the 14 exons and the flanking intronic sequences of the *PAK3* gene were separately polymerase chain reaction (PCR)-amplified from genomic DNA using primers, listed in Table II, with psoralen clamps. DGGE conditions were chosen according to the Meltmap program, kindly provided by L. Lerman and colleagues. The denaturants were 7 M and 40% formamide, and gels run at 60°C [Myers et al., 1987; Bienvenu et al., 1995]. PCR products were subjected to electrophoresis as described in Table III.

Mutation identification. PCR products showing an abnormal migration pattern on DGGE analysis were directly sequenced on an automated sequencer (ABI 377, Perkin-Elmer Applied Biosystems, Foster City, CA) using the dye-terminator method. Usually sequence variations are checked by restriction analysis of genomic DNA. Exon 4 of the PAK3 was analysed by direct sequencing of PCR products (4F 5′ gaatgaaacattctttatga 3′ and 4R 5′ aacagttttcaatttttcac 3′).

RESULTS

Summary of genetic data in the 12 families with mental retardation mapped to the Xq21-q24 region are shown in Table I [The European XLMR Consortium, 1999]. Coding exons of the *PAK3* gene were screened for mutations by DGGE in 12 affected males. No abnormalities were detected in all exons except exon 2. The screening showed the presence in one patient with MRX of an abnormal migration pattern of exon 2. The sequence of the PCR product of exon 2 showed a C to T substitution at cDNA position 199 (Fig. 1). This change causes arginine (CGC) to cysteine (TGC) substitution at amino acid 67 of the PAK3 protein. As the missense mutation destroys a unique restriction *Hha*I site, this event was used to study the segregation of this base substitution in the entire family of this patient. Figure 2 shows co-segregation between the mutation and the mental retardation phenotype. This base substitution was absent in 110 normal controls.

This missense mutation is located in a polybasic region upstream of the Cdc42/Rac interactive binding (CRIB) domain. It has been shown that mutations of the basic residues to neutral residues reduce Rho-GTPases binding to Pak and reduce or abolish Pak activation by Rho-GTPases [Knaus et al., 1998].

In this MRX47 family, all affected males (III-5, III-8, III-12, IV-2, IV-3, IV-7) show moderate to severe mental retardation. Clinical and neuropsychological data of the affected males were previously reported [des Portes et al., 1997]. No seizures, statural growth deficiency, neurological impairment, or minor facial or other physical anomalies were observed.

DISCUSSION

In order to evaluate the frequency of *PAK3* mutations among mapped familial cases of MRX, we have carried out a systematic analysis of the *PAK3* gene in

TABLE I. Summary of Genetic Data in Mentally Retarded Families Mapped to Xq23 and Screened for Mutation in Xq23 [Moraine et al., 1994; The European XLMR Consortium, 1999]

Locus	Family number	Z_{max}	Flanking markers
Xq22.1-26.1	MRX35	2.41	DXS178-HPRT
Xq22.3-q25	MRX47	3.96	DXS1105-DXS1059
Xq12-q25	MRX61	3.51	DXS135-DXS737
Xq21.3-q23	MRX67	2.36	DXS458-DXS424
Xq22.1-q27.2	$T19_{(+GH\ deficiency)}$	2.96	DXS178-DXS292
Xq21.33-q25	MRX62	2.23	DXS458-DXS737
Xp11.4-Xq23	T40	1.51	DXS556-DXS1001
Xq22.1-q26	$L17_{(+spastic\ paraplegia)}$	1.5	DXS1214-DXS990
Xq23-qter	L25	1.5	DXS424-Xqter
Xq21.3-q26.2	F6	0.7	DXS1217-DXS1062
Xq24-q25	F15	1.5	DXS1220-DXS1047
Xp22.3-q26.1	N9	1.41	DXS989-HPRT

TABLE II. Parameters for Amplification of the *PAK3* Gene Fragments

Fragment	Sequences of primers[a]	Length (bp)	Annealing temperature (°C)
Exon 1	1F:5′Pso-gacctcttctccctcaactc3′ 1R:5′ttttcaatgcccggccactg3′	288	45
Exon 2	2F:5′Pso-cccaaataaatacatgttaac3′ 2R:5′ttacaaaacaaaacaaggagc3′	170	50
Exon 3	3F:5′cattctttccctttggttgt3′ 3R:5′Pso-caccttgataccacagact3′	266	50
Exon 5	5F:5′Pso-acctgatctttaaactttgt3′ 5R:5′tttggaaatagtcctttgtg	190	50
Exon 6	6F:5′Pso-tgttttaattgcagagcttt3′ 6R:5′gaaagtaatcatggcaagaa3′	250	50
Exon 7	7F:5′ttcttctctccccacccatc3′ 7R:5′Pso-catctttttatatttcaaat3′	134	50
Exon 8	8F:5′Pso-cttgattgcttattcttttg3′ 8R:5′ataaaaatcaggattgaacg3′	144	50
Exon 9	9F:5′ttcttgtattttaattgcca3′ 9R:5′Pso-aagtacataataagacaaaa3′	188	45
Exon 10	10F:5′Pso-agcgtcataaggcaaagtct3′ 10F:5′cggtggtcttctcagctaga3′	194	49
Exon 11	11F:5′ttgtgatataattaaaactt3′ 11R:5′Pso-tctaataccacctgaaccaa3′	180	45
Exon 12	12F:5′gggcttattttaactggctt3′ 12R:5′Pso-acctgacttctcttctgact3′	261	50
Exon 13	13F:5′atgtatgtgctgaatggatt3′ 13R:5′Pso-gcctctctttgcaattgtcc3′	225	50
Exon 14	14F:5′Pso-ctctttttccttcctttttgc3′ 14F:5′gaggtgtaaggcttgcagtc3′	140	49

[a]Pso, Psoralen-TA.

12 patients with MRX, and we have screened, using DGGE, the whole coding region of this gene. Upon analysis of exons 1 through 14, we have identified a novel missense mutation that predicts a change of an arginine to cysteine (R67C) that represents a nonconservative amino acid substitution. This novel missense mutation is likely to be pathogenic because: 1) it cosegregates with the disease trait in the large family MRX47 previously localized in Xq23.3-q24 with a 2-point lod score >3 [des Portes et al., 1997]; 2) it changes a conserved residue during evolution (Fig. 3); and 3) it was not found in 110 control chromosomes.

The p21-activated kinases (PAKs) are evolutionarily conserved regulators of the actin cytoskeleton organization [Sells and Chernof, 1997]. The yeast Pak Ste20 regulates polarized cell growth in response to mating pheromone, and mammalian Paks reorganize the actin cytoskeleton when over-expressed in tissue-culture cells. Pak is a protein that regulates its proper activity through interaction between the N-terminal regulatory region and the C-terminal kinase domain. The regulatory region contains binding sites for at least three types of signaling proteins. An N-terminal proline-rich sequence (PXXP) binds to Nck; a CRIB motif binds to GTP-bound forms of Cdc42 and Rac; and a proline-rich

TABLE III. Parameters for DGGE Conditions

Fragment	Gradient (%)	Running time (h) at 160 V
Exon 1	30–80	10
Exon 2	30–80	6.8
Exon 3	20–70	8.1
Exon 5	30–80	6.6
Exon 6	30–80	8.75
Exon 7	20–70	5.6
Exon 8	20–70	6.3
Exon 9	20–70	7.5
Exon 10	30–80	5.6
Exon 11	20–70	5
Exon 12	30–80	8.1
Exon 13	30–80	9
Exon 14	30–80	5.6

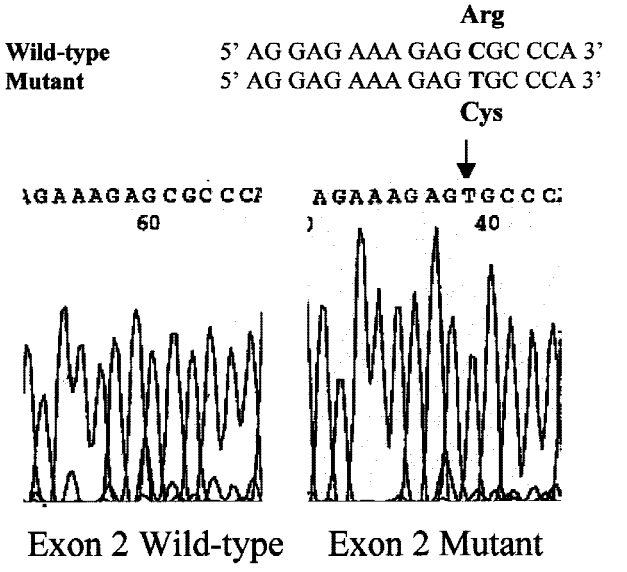

Fig. 1. Fluorescence sequence analysis of exon 2 of the *PAK3* gene. The arrow indicates the position of the mutation R67C (C to T at position 199). The number, below the sequence, corresponds to nucleotide positions along the electrophoregram of the sequenced fragment and not to the position of the region along the gene.

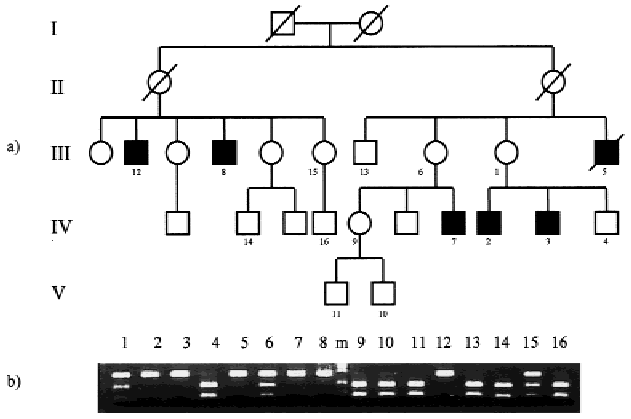

Fig. 2. **a:** Pedigree of family MRX47 and results of *Hha*I restriction enzyme analysis of exon 2 amplified samples. **b:** Digestion by *Hha*I yields one fragment (164 bp) when the mutation R67C is present (III-3, III-7, IV-7 affected males). Restriction enzyme analysis confirms the sequence interpretation and indicates co-segregation of the mutation with the phenotype in family MRX47. m, 100-bp DNA ladder (Promega, Madison, WI).

motif is constitutively bound to Pix, a guanine nucleotide exchange factor specific to Rac and Cdc42 [Obermeier et al., 1998]. In the inactive state, an autoinhibitory sequence adjacent to the CRIB motif inhibits Pak kinase activity.

The novel missense mutation is located in a polybasic region upstream of the CRIB domain. PAK1, 2, and 3 have 100% sequence identity in both the CRIB domain and the polybasic domain (AA 66–68 in human PAK1). It has been shown that mutation of the basic Lys residues of Pak1 to neutral Gln residues (K66–68Q and K66–68L) reduces Rac1 and Rac2 binding to Pak1 and reduces or completely abolished Pak1 activation by Rac1, Rac2, or Cdc42. These results suggest that the polybasic region (66–68) plays an important role in Pak activation by Rac1, Rac2, and Cdc42 proteins [Knaus et al., 1998]. Our novel missense mutation R67C alters the basis charge of the region and should alter the GTPase binding and stimulation of Pak activity. This result confirms that the positively charged domain is likely to be important for the biological activity of the PAK isoforms.

This finding, together with previous reports [Billuart et al., 1998; D'Adamo et al., 1998; Chelly, 1999] raises the issue of the cellular bases underlying mental retardation resulting from dysfunction of pathways regulated by Rho-GTPases proteins. The Rho-GTPases, in particular RhoA, Rac1, and Cdc42 act as molecular switches, which integrate various intracellular signaling pathways to mediate a coordinated behaviour of the actin cytoskeleton. The cellular functions of the Rho proteins have mainly been studied in fibroblasts, where RhoA directs the formation of stress fibers, Rac1 is involved in lamellipodia formation, and Cdc42 stimulates formation of filopodia. For example, Rho is an inhibitor of neurite outgrowth, whereas Cdc42 acts upstream of Rac1 and promotes neurite outgrowth by regulating the formation of growth cone filopodia and lamellae [Kozma et al., 1997]. Thus, neurite outgrowth appear to be guided through the combined operation of different guidance mechanisms (short- and long-range attraction, and short- and long-range repulsion), and the outcome of any particular guidance decision appears to reflect the balance of attraction and repulsion operating at the decision point. Abnormalities in the coordinated effects of these Rho proteins on the organization of the actin cytoskeleton could result in alteration of growth cone morphology and network formation of neurons. Thus, mutations in a gene like *PAK3* could modify binding of activated Cdc42/rac to Pak, change the dynamics of the actin cytoskeleton, and/or

Gene	Sequence
	# #
PAK1 HUMAN	ILPG--DKTNKKKEKE**R**PEISLPSDFEHTIHVG
PAK 2 HUMAN	IFSG-TEKGSKKKEKE**R**PEISPPSDFEHTIHVG
PAK3 HUMAN	IFPGGGDKTNKKKEKE**R**PEISLPSDFEHTIHVG
PAK65 HUMAN	IFSG-TEKGSKKKEKE**R**PEISPPSDFEHTIHVG
RATBETA-PAK	IFPGGGDKTNKKKEKE**R**PEISLPSDFEHTIHVG
RATALPHA-PAK	ILAG--DKTNKKKEKE**R**PEISLPSDFEHTIHVG
RATMUK2	ILAG--DKTNKKKEKE**R**PEISLPSDFEHTIHVG
MUSMUSC-PAK-B	IFPGGGDKTNKKKEKE**R**PEISLPSDFEHTIHVG
MUSMUSC-PAK3	IFPGGGDKTNKKKEKE**R**PEISLPSDFEHTIHVG
ORYCTOCUN-PAK1	IFSG-TEKGSKKKEKE**R**PEISPPSDFEHTIHVG
XENOPUS-PAK1	KKKG--QKNNKKDKKD**R**LEISSPFGYEHIYHVG
	* * ** * * *** * ** ***

Fig. 3. Multiple alignment of a part of the N-terminal domain of Pak proteins. The site of mutation is shown in bold; the CRIB motif is underlined; # corresponds to the binding-inactivating mutations K66-68L, K66-68R, and K66-68Q of PAK-1 [Knaus et al., 1998]; * corresponds to residues conserved in all proteins.

affect neurite outgrowth, neuronal morphogenesis, or establishment of neuronal connections. Disruption of these processes could be a cellular basis for mental retardation. In summary, our data further confirm that mutations in the *PAK3* gene are associated with MRX. Other proteins involved in this pathway (i.e., Nck (an SH3/SH2 adaptor protein) and Pix) are probably potential candidate genes for mental retardation.

However, as for the previously described genes, such as *OPHN1*, *GDI1*, *IL1RAPL* [Billuart et al., 1998; D'Adamo et al., 1998; Allen et al,. 1998; Carrié et al., 1999], mutations in *PAK3* seem to be very rare, and the absence of mutations in most families mapped in genetic intervals that encompass the position of each gene is most surprising. These data suggest the presence of at least one additional gene in each genetic interval, making the number of genes involved in mental retardation at least double that previously estimated number.

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