ARTICLE

Functional analysis of ARHGAP6, a novel GTPase-activating protein for RhoA

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Microphthalmia with linear skin defects (MLS) is an X-linked dominant, male-lethal syndrome characterized by microphthalmia, aplastic skin and agenesis of the corpus callosum, and is caused by the deletion of a 500 kb critical region in Xp22.3. Our laboratory isolated a novel rho GTPase-activating protein (rhoGAP) gene named ARHGAP6 from the MLS region. ARHGAP6 contains 14 exons encoding a 974 amino acid protein with three putative SH3-binding domains. Because exons 2–14 are deleted in all MLS patients, we hypothesized that ARHGAP6 may be responsible for some of the phenotypic features of MLS. We pursued two approaches to study the function of ARHGAP6 and its role in the pathogenesis of MLS: gene targeting of the rhoGAP domain in mouse embryonic stem cells and in vitro expression studies. Surprisingly, loss of the rhoGAP function of Arhgap6 does not cause any detectable phenotypic or behavioral abnormalities in the mutant mice. Transfected mammalian cells expressing ARHGAP6 lose their actin stress fibers, retract from the growth surface and extend thin, branching processes resembling filopodia. The ARHGAP6 protein co-localizes with actin filaments through an N-terminal domain and recruits F-actin into the growing processes. Mutation of a conserved arginine residue in the rhoGAP domain prevents the loss of stress fibers but has little effect on process outgrowth. These results suggest that ARHGAP6 has two independent functions: one as a GAP with specificity for RhoA and the other as a cytoskeletal protein that promotes actin remodeling.

INTRODUCTION

The rho family of small GTP-binding proteins provides a critical link between membrane receptors, the cytoskeleton and the nucleus. Overexpression of rho proteins in cultured cells creates characteristic morphological alterations, including the extension of filopodia, formation of stress fibers or the induction of membrane ruffles (1). Conversely, rho inactivation by dominant-negative mutations or enzymatic inhibitors rapidly causes microfilamentous structures to collapse (2). Strict spatial and temporal regulation of rho GTPases is critical for developmental or physiological processes that require cytoskeletal plasticity.

GTPase-activating proteins (GAPs) are crucial components of the regulatory machinery for rho GTPases. Rho-specific GAPs (rhoGAPs) bind directly to activated GTPases, hydrolyze GTP to GDP and switch off signal transduction. However, rhoGAPs are not only inhibitory, but can also transduce separate signals or function as accessory effectors of rho GTPases. The 140 amino acid GAP domain is often only one segment of a large and multifunctional rhoGAP (3). In mammals, the possible combination of at least 14 rho GTPases and 12 rhoGAPs lends the system exquisite diversity and specificity, because a single GAP can interact differentially with several rhoGAPs (4).

RhoGAP domains isolated from N-chimerin, Graf and p190 elicit cytoskeletal changes in cultured cells, and the functions of the full-length proteins apparently are eliminated by mutations that abolish GAP activity (5–7). These observations suggest that the primary cellular functions of many rhoGAPs are accomplished through the inactivation of rho GTPases and that defects caused by loss-of-function mutations in rhoGAPs and constitutive activation of rho GTPases may be similar (8).

Other observations suggest that GAP proteins may have additional signal transduction functions that are required for normal development. Point mutations or small deletions in the GAP domain of the NF1 gene cause neurofibromatosis. However, patients with larger deletions that remove the entire gene display learning impairments even in the absence of neuronal tumors (9). Analysis of NF1-deficient mice revealed a previously unknown role for NF1 in the morphogenesis of neural crest-derived tissues (10). In vitro studies have implicated the N-terminus of NF1 in signaling functions that do not require GAP activity (11,12).
date, no other GAP gene has been inactivated in mice, and the biological roles of most rhoGAP family members are not known. 

ARHGAP6, which encodes a novel 974 amino acid rhoGAP, is deleted in patients with microphthalmia with linear skin defects (MLS) syndrome, a dominant, male-lethal disorder characterized by eye, skin and central nervous system (CNS) malformations (13). Direct mutational analysis of MLS patients is not possible because ARHGAP6 is always contained within the Xp22 terminal deletions that cause MLS syndrome. As an alternative approach to determine whether loss of ARHGAP6 function contributes to MLS, we generated mice with a targeted deletion of the rhoGAP domain. Based on neurobehavioral and neuropathological analysis, Arhgap6 mutant mice do not differ significantly from their wild-type littermates and do not show features of MLS syndrome. These results suggest that loss of the rhoGAP function of ARHGAP6 does not contribute to the MLS phenotype. Transfection studies show that ARHGAP6 inactivates rhoA signaling and produces additional effects on the actin cytoskeleton that are independent of its GAP activity.

RESULTS

Isolation of the ARHGAP6-coding sequence and identification of novel splice variants

The published sequence of ARHGAP6 was 2.0 kb shorter than the principal mRNA species (4.2 kb) and lacked a suitable Kozak initiation site (14). To isolate additional coding sequence, a 275 bp RT–PCR product containing exons 1–3 was hybridized to a human fetal kidney cDNA library. Five positive clones (HFK-1 to -5) were isolated, sequenced and assembled into a 5156 bp cDNA contig. The complete ARHGAP6 human fetal kidney cDNA sequence contains an 875 bp 5′-untranslated region (5′-UTR), a 2921 bp open reading frame (ORF) and a 1360 bp 3′-UTR.

ARHGAP6 is expressed ubiquitously as a 4.2 kb transcript with additional 2.6, 5.0 and 6.0 kb isoforms in skeletal muscle and kidney, as previously described (14). Sequence analysis of fetal kidney, cerebellar and lung cDNAs suggests that the coding region of ARHGAP6 undergoes extensive alternative splicing. The HFK contig contains a 1462 bp 5′ sequence (exon 1a) including a novel 800 bp ORF in-frame with exon 2. This sequence is not present in HFK-2 (exon 1b) or in a fetal brain cDNA (exon 1c), which contain unique, untranslated 5′ exons.

RT–PCR amplification of total RNA from lymphoblasts as well as five adult human tissues shows that at least three alternative 5′ ends of ARHGAP6 are spliced to the invariant central nine exons of the gene. The longest isoform is most abundant in all tissues examined.

Alternative splicing at a cryptic splice donor site within exon 12 bypasses a stop codon and extends the ORF by 248 novel amino acids. dbEST searches identified five expressed sequence tags (ESTs) from fetal heart (accesion no. 111376), pregnant uterus (768489) and fetal liver/spleen libraries (997642, 292405 and 292407), which are identical to the 5′-UTR of the HFK sequence. Expression of the alternative 3′ ends was compared by RT–PCR using specific primers for each isoform. The short isoform with the stop codon in exon 12 is widely expressed, but the longer isoform containing exon 14 is not detectable in fetal brain (data not shown). These results suggest that alternative splicing of the 3′ coding sequence is developmentally regulated and may play a role in the function of the gene in the CNS.

The complete coding sequence of mouse Arhgap6 revealed that, similarly to the human gene, exon 11 is alternatively spliced out (14). The mouse Arhgap6 gene is also expressed as a prominent 4.2 kb transcript with fainter 2.4 and 6.0 kb isoforms at all embryonic stages (data not shown). The 4.2 kb transcript appears to be the solitary isoform in embryonic stem (ES) cells as well as in adult brain, heart and lung, and a faint 6.0 kb transcript is visible in adult kidney. The results suggest that the alternative splicing and expression pattern of Arhgap6 are conserved in the mouse.

The longest isoform of ARHGAP6 encodes a 974 amino acid protein with a 150 amino acid rhoGAP domain and three proline-rich motifs with putative SH3-binding sites (15) (Fig. 1A). If exon 1a is alternatively spliced out, a 771 amino acid protein could be translated from an initiation site in exon 2 (Fig. 1B). Alternative splicing of exons 11 and 12 truncates the C-terminus of the protein by 338 or 249 amino acids, respectively (Fig. 1B). Without the stop codon-containing exons, the predicted protein sequence of the mouse Arhgap6 gene is 84% identical and 88% similar to the human sequence. The C-terminal coding sequence is not highly conserved, with the exception of a polyproline motif and a region with homology to the WASP protein family (amino acids 878–974, in bold in Fig. 1A) that share >90% identity.

Characterization of the genomic structure

The exon–intron boundaries were sequenced using cosmid DNA as genomic template or determined by computer alignment of the cDNA contig with genomic sequence of three bacterial artificial chromosomes (BAC) clones, 602M16 (132 kb), 512P14 (125 kb) and 590F6 (110 kb), which contain the 3′ and 5′ ends of the ARHGAP6 gene, respectively. ARHGAP6 spans >500 kb of genomic DNA and contains 14 exons (Fig. 1C). The intron–exon boundaries and the sizes of the exons and introns in the newly identified coding sequence were determined by sequence alignment and deposited in GenBank (14). Exon 1a is entirely co-linear with a 1.4 kb genomic sequence in BAC 590F6. A potential promoter with a TATA box 860 bp from the 5′ end of the transcript and a 423 bp CpG island in the intervening sequence, marking the probable transcription initiation site, was identified by sequence analysis. Exons 12 and 14 contain multiple stop codons and polyadenylation signals. Each splice junction conforms to the vertebrate consensus.

Exons 1a and 2 of ARHGAP6 are separated by two contiguous BAC clones (27C22 and 285115) and one gap of undeetermined size in the physical map (50–100 kb) totaling >280 kb of genomic sequence (Fig. 1C). By Southern analysis, the ARHGAP6 gene spans the proximal boundary of the MLS critical region, with patient breakpoint BA325 located between exons 1a and 2. The 10 kb amelogenin gene (AMELX) is contained within the first intron of ARHGAP6 –40 kb centromeric of exon 2 and is transcribed in the opposite orientation (16). Exon 1b is separated from exon 2 by a 9 kb intron with consensus splice sites, and exon 1c is separated from the AMELX promoter by only 2930 bp of genomic sequence.

Generation of Arhgap6 null mice

To evaluate the in vivo function of Arhgap6 and its role in the MLS phenotype, a portion of the Arhgap6 coding region was deleted using homologous recombination in ES cells (Fig. 2A). The targeting vector was designed to delete a 5.5 kb genomic fragment containing exons 6–8, which encode the N-terminal 118 amino acids of the 140 amino acid rhoGAP domain (Fig. 2B).
High-percentage male chimeras (>60% agouti coat color) were obtained for three ES clones, which were transmitted onto a 129 Sv/Ev background. All genotypes were observed in the expected Mendelian ratios (Fig. 2C).

To confirm that the homologous recombination event disrupts Arhgap6 expression, northern analysis of total RNA from the brain, heart, lungs and kidneys of wild-type male mice and putative mutant male mice was performed (Fig. 3A). A 4.2 kb...
transcript was detected in each tissue of wild-type mice, but not in Arhgap6 mutant mice. Mice with the targeted allele express a 3.8 kb transcript whose intensity is ~50% of wild-type levels. This may reflect mRNA instability or reduced transcription caused by the targeting event. The 400 bp reduction is consistent with the deletion of exons 6–8 in the rhoGAP domain. Hybridization of the same blot with a probe from within the rhoGAP domain detects splicing around the inserted cassette is expected to create a frameshift between the remaining exons 5 and 9 (indicated by *). Correct targeting was diagnosed by Southern analysis of BamHI- or ApeI-digested genomic DNA with the appropriate diagnostic probes (bars). (C) Southern analysis of targeted clones and germline transmission of the mutation. Targeted XY ES cells (–) show a single 12 kb BamHI fragment (left) or a 10.5 kb ApeI fragment (right). The wild-type (+) sizes are 14 and 22 kb, respectively. (D) Outcome of matings between chimeric males and wild-type females to produce heterozygous female offspring (+/−) and wild-type male offspring (+) in the F1 generation. These siblings were mated to produce the F2 generation (right) including mutant male mice (−).

Arhgap6 mutant mice do not display histological or behavioral abnormalities

Arhgap6 mutant male and female mice were indistinguishable from their wild-type littermates at birth and thereafter. There were no detectable differences between the external appearance, cage behavior and body weight of mutant (n = 15) and wild-type (n = 13) animals. Two 3-month-old mutant males and two wild-type littersmates were evaluated histopathologically. Analysis of brain, eyes, heart, lungs, liver, kidney, testes, intestine, skeletal muscle and skin revealed no obvious abnormalities. No abnormalities of ossification or skeletal morphogenesis were seen in mutant E17 embryos (n = 4) compared with wild-type littersmates (n = 4) (data not shown).

Because the corpus callosum and septum pellucidum are abnormal in several MLS patients, we evaluated hematoxylin and eosin-stained serial coronal sections at 4–5 levels through the corpus callosum, hippocampal commissure and dorsal fornix of adult wild-type (n = 16) and mutant (n = 16) animals. Two animals of each genotype were stained using the Sevier–Munger method (17). No significant differences in the thickness or contralateral projection of the fibers were observed. Normal development of the cerebellum, cortex and hippocampus was revealed by staining with cresyl violet and immunohistochemistry using antibodies against glial fibrillary acidic protein, calbindin and the GABA receptor β2 subunit in at least four animals of each genotype. No lesions or reactive gliosis were detected in the mutant animals in comparison with equal numbers of wild-type controls. These results suggest that the architecture of the CNS is grossly intact in Arhgap6 mutant animals. We also evaluated renal function in view of the abundant expression of Arhgap6 in the kidney. Routine chemistry and urinalysis were normal and there was no evidence of proteinuria.

Thirteen mutant and seven wild-type animals were evaluated using a behavioral test battery to screen for defects in several domains of CNS function including basic sensory/motor abilities, learning and memory (18). To test further the role of Arhgap6 in cognition, a separate set of male mutant (n = 9) and wild-type
(n = 9) mice were evaluated in a conditional alternation task (19), which can be used to assess conditional working memory processes. Basic sensory/motor responses, locomotor activity, anxiety-related responses, sensorimotor gating and analgesia-related responses of the mutant animals were not significantly different from those of their wild-type littermates (P > 0.5).

Arhgap6 mutant mice did not display any defects in motor coordination or skill learning deficits on the rotarod test (Fig. 4A). In addition, the performance of Arhgap6 mutant mice in the conditioned fear test, Morris water task (Fig. 4B) and conditional alternation task was similar to that of the wild-type controls, indicating that the processes mediating several forms of learning and memory performance are not disrupted by the Arhgap6 mutation.

ARHGAP6 is a GAP for RhoA but not for Rac1 or Cdc42

To assess the GAP activity of ARHGAP6, a fragment of the mouse Arhgap6 cDNA encoding the SH3-binding and rhoGAP domains (residues 279–660 of the full-length protein) was expressed as a glutathione (GST)–fusion protein in Escherichia coli and purified through a GST–Sepharose column. The recombinant protein was incubated with [γ-32P]GTP-loaded RhoA, Rac1 and Cdc42 and assayed for its ability to stimulate the hydrolysis of GTP. As shown in Figure 5A, ARHGAP6 activates the intrinsic hydrolytic activity of RhoA but not of Rac1 or Cdc42. In a parallel analysis, the rhoGAP domain of p50rhoGAP displayed catalytic activity toward all three GTP-binding proteins, as was demonstrated previously (20). The results suggest that RhoA is the preferred in vitro substrate for ARHGAP6.

To determine whether ARHGAP6 shows in vivo specificity for any member of the rho family, the full-length cDNA was transfected as an N-terminal Xpress-tagged or C-terminal green fluorescent protein (GFP)–fusion protein into COS-7 and HeLa cells. Cells were scored for green fluorescence and double-labeled with anti-actin antibodies (red) to visualize the cytoskeleton. Agonists for RhoA [sphingosine-1-phosphate (SPP)] or Cdc42 (bradykinin) were added to the culture media and assayed for their effects on the morphology of transfected and untransfected cells. Serum-starved cells contain fewer actin stress fibers, correlating with the reduced expression of RhoA. Exposure to SPP, which rapidly restores the stress fibers, is a useful index for RhoA activation. Whereas 44/51 untransfected cells formed intense stress fibers after a 30 min exposure to SPP, all 50 ARHGAP6-expressing cells that were examined did not form stress fibers under the same conditions \( \chi^2 = 61.3, P < 0.01 \) (Fig. 5D and E). Prevention of stress fiber formation after SPP treatment by overexpression of ARHGAP6 demonstrates that the protein hydrolyzes and inactivates RhoA. In a parallel experiment, ARHGAP6 did not inhibit filopodial extension by bradykinin (data not shown), supporting its specificity for RhoA in vivo and confirming the results of the GAP assay.

Nine amino acids within the rhoGAP domain are conserved in all rhoGAP family members, including an invariant arginine that participates directly in the hydrolysis reaction and stabilizes the GTP/GDP transition state. Mutations that replace the arginine with another amino acid caused a 230- to 540-fold reduction in the in vitro rate of GTP hydrolysis by p50rhoGAP and abolished the morphological effects of two other RhoA-specific GAPs, Graf and p190, in cultured cells (5,6,21). The conserved arginine (R433) in ARHGAP6 was mutated to a glycine with a single base change (CCG → CGG) in exon 6. To determine whether the mutation affects rhoGAP activity in vivo, the mutant protein was assayed for its ability to antagonize SPP-mediated stress fiber

\( \chi^2 = 61.3, P < 0.01 \) (Fig. 5D and E).
ARHGAP6 regulates the actin cytoskeleton

Microinjection of rho GTPases into mammalian cells remodels the actin cytoskeleton to create stress fibers (RhoA), membrane ruffles (Rac1) or filopodia (Cdc42). When grown in serum-containing medium, HeLa or COS-7 cells expressing ARHGAP6 undergo similar morphological changes characterized by cell retraction, loss of adhesion to the coverslip and the extension of branched and beaded cytoplasmic processes. The protein appears to be concentrated in bead-like protruberances near the tips of the processes (arrows), and the extent of the cell body retraction is revealed by confocal microscopy (Fig. 6A). Double-labeling experiments with anti-actin antibodies indicate that actin filaments form the core of the processes induced by ARHGAP6 (Fig. 6B). These results suggested that disruption of the actin cytoskeleton by ARHGAP6 may perturb cellular morphology. Actin stress fibers are linked to the extracellular matrix through focal adhesions, which anchor cells to the growth surface. The loss of adhesion seen in transfected cells suggested that focal adhesions may also be disrupted by overexpression of ARHGAP6. Focal adhesions were visualized in transfected HeLa cells by co-staining with a monoclonal anti-focal adhesion kinase (FAK) antibody. The punctate staining pattern at the periphery of untransfected cells disappears in cells expressing ARHGAP6 (Fig. 6C). Overexpression of ARHGAP6 produced similar morphological effects in 90% of HeLa or COS-7 cells (n = 50) examined, although the length of the processes varied slightly between cell types.

Function of N- and C-terminal domains of ARHGAP6

To determine whether rhoGAP activity is sufficient to create the morphological effects, several deletion mutants were expressed in HeLa and COS-7 cells. ARHGAP6 contains a central putative SH3-binding motif and rhoGAP domain flanked by extensive proline-rich N- and C-terminal domains of unknown function. Transfection of the isolated SH3-binding and rhoGAP domains results in a uniform cytoplasmic distribution of the protein without any effect on the actin cytoskeleton (Fig. 6D). An N-terminal 454 amino acid peptide truncated between the second SH3-binding domain and the rhoGAP domain co-localizes with cortical actin fibers but does not affect cellular morphology (Fig. 6E). A C-terminal deletion mutant, which is truncated immediately after the rhoGAP domain, behaves similarly to the full-length protein (Fig. 6F). These results suggest that rhoGAP activity is necessary but not sufficient for the function of ARHGAP6 and implicate a region near the N-terminus in the association of the protein with the cytoskeleton.

To determine whether rhoGAP activity is essential for process formation by ARHGAP6, the catalytically inactive rhoGAP mutant was expressed in HeLa cells that were grown in serum-containing medium. The mutant protein produced distinctive morphological effects. Whereas stress fibers completely disappear from cells transfected with the wild-type protein and the cells retract from the coverslip (Fig. 6G), transfection of the R433G construct produces beaded processes (arrows) but does not cause depolymerization of stress fibers or retraction of the cells (Fig. 6H). Eighty-six percent of HeLa cells (n = 48) transfected with the wild-type construct exhibited the retracted phenotype compared with only 18% (n = 50) of HeLa cells transfected with the R433G mutant (χ² = 54.06, P < 0.05). These results suggest that ARHGAP6 has two independent functions as a GAP with specificity for RhoA and as a cytoskeletal protein that promotes actin remodeling. The point mutation selectively abolishes the ability of ARHGAP6 to promote retraction of transfected cells without affecting process outgrowth.

ARHGAP6 induces the active extension of cytoplasmic processes

The cytoplasmic processes in ARHGAP6-transfected cells may be created by retraction of actin stress fibers from extended positions, leaving threads of cytoplasm attached to the cell body, or active growth driven by polymerization of actin filaments. To
distinguish between these possibilities, HeLa cells were
monitored by video microscopy in a thermoregulated flow
chamber between 6 and 9 h after transfection with the wild-type or
R433G ARHGAP6 cDNA. The video data indicate that the
extensions are created by the continuous protrusion of filopodial-
and lamellipodial-like structures (Fig. 7A). The ARHGAP6–GFP
fusion protein is concentrated at sites of dynamic actin
polymerization near the tips of the outgrowing processes.
Extrusion of processes was accompanied by refractive waves that
propagated across the length of the cells and by retraction of the
opposite surface of the cells. Cells expressing the GAP-deficient
mutant protein did not retract from the coverslip and exhibited a
distinctive morphology characterized by peripheral microspikes
and increased numbers of processes with greater tortuosity
(Fig. 7B). The rate of cell movement was similar in both cases.
Cells transfected with an empty GFP vector maintained a cuboidal
morphology and did not extend processes (data not shown). These
observations confirm that ARHGAP6 affects cell morphology
and motility by promoting the continuous elongation of processes
and simultaneous retraction of the cell body.

DISCUSSION

MLS syndrome is a dominantly inherited, male-lethal disorder
characterized by developmental defects of the brain, eyes and skin
(13). Most MLS patients are heterozygous for terminal deletions
of Xp22.3 that include ARHGAP6, a novel rhoGAP gene (22).

The longest isoform of ARHGAP6 encodes a 974 amino acid
protein with three potential SH3-binding motifs, the 140 amino
acid rhoGAP domain and a 371 amino acid C-terminus of
unknown function. ARHGAP6 is a good candidate to cause MLS
because it occupies one-third of the critical region, and mutations
in another rhoGAP gene (oligophrenin) were discovered recently
in patients with non-syndromic mental retardation (MRX) (22).
To characterize ARHGAP6 as a candidate gene for MLS syndrome, two approaches were taken: generation of mice with a targeted mutation of Arhgap6 and functional analysis of the recombinant protein in cultured cells. Our results suggest that the rhoGAP activity of Arhgap6 is not involved in the pathogenesis of MLS syndrome, but provide evidence that the N-terminus of the protein plays a novel role in its regulation of cell morphology and actin polymerization.

The coding sequence of ARHGAP6 is interrupted by a large intron that spans at least 280 kb of genomic DNA between exons 1a and 2 and contains the amelogenin gene (AMELX). Other vertebrate genes with variations of this unusual structure have been described, including the NF1 gene and the human factor VIII gene (23,24). Intriguingly, both of these genes are subject to extensive alternative splicing, harbor multiple transcripts within a single large intron and are targets of frequent chromosomal rearrangements in the human disorders type 1 neurofibromatosis and hemophilia. Several hypotheses for the significance of the arrangement have been proposed, including antisense regulation of the larger transcript and co-expression of genes with similar functions (25).

With the apparent absence of abnormalities in the mutant animals, Arhgap6 may be added to a growing list of putative neurodevelopmental genes that unexpectedly failed to create detectable defects when mutated in mice, including Hprt and Ziprol, which encodes a zinc finger transcription factor involved in cerebellar granule cell proliferation (26,27). Several loss-of-function mutations in Drosophila, Caenorhabditis elegans and yeast create no apparent defects; this is even more likely in Caenorhabditis elegans and yeast create no apparent defects; this is even more likely in vertebrates given the abundance of homologous gene families or potential compensatory pathways (28). The diversity of vertebrate rhoGAPs suggests that functional redundancy may also modify the phenotype of Arhgap6 mutants.

The targeted Arhgap6 allele putatively encodes a truncated protein of 470 amino acids that lacks the rhoGAP domain and the alternatively spliced C-terminus. Exons 1–5, which remain intact in the mutant animals, encode two potential SH3-binding domains. N-terminal regions of other GAP proteins such as p120rasGAP, p190 and α-chimerin have been implicated in membrane interactions and signaling functions that do not depend on intact GAP activity (7,29,30). The N-terminus of p190 undergoes tyrosine phosphorylation and interacts with p120rasGAP to regulate ras signal transduction and cell growth (5). ARHGAP6 also contains a tyrosine phosphorylation site in addition to several protein interaction motifs outside the rhoGAP domain. These observations suggest that targeted deletion of the rhoGAP domain may not cause a complete loss of Arhgap6 function.

Mutations in the rho pathway involving a RacGAP (oligophrenin) and an effector for Rac (PAK3) recently were implicated in human MRX (22,31). ARHGAP6 is expressed in fetal brain, and at least one MRX locus that overlaps the genomic position of ARHGAP6 was identified recently in Xp22.31 (32). These observations suggest that mutation of ARHGAP6 may cause MRX, and its deletion as part of a contiguous gene defect could contribute to mental retardation in MLS syndrome. However, a thorough neurobehavioral study of Arhgap6 mutant mice failed to reveal any abnormalities. The results of other studies, including behavioral analysis of mice deficient for the fragile X syndrome protein Fmr1p, suggest that mutations causing mild or moderate mental retardation in humans may be difficult to detect in mouse models (33,34). Without more sensitive assays or evidence for the in vivo function of ARHGAP6, our results do not exclude ARHGAP6 as a candidate gene to cause MRX or some CNS manifestations of MLS syndrome.

Functional analysis of ARHGAP6 in cultured cells identified several unique characteristics of this novel rhoGAP. Transfected cells lose actin stress fibers, retract from the coverslip and extend branching, beaded cytoplasmic processes. In contrast to other rhoGAPs such as p190 and Graf, inactivation of the rhoGAP domain does not abolish the morphological effects of ARHGAP6 outgrowth (5,6). The mutation specifically disrupts the ability of ARHGAP6 to antagonize rhoA-mediated stress fiber formation but does not significantly affect process outgrowth. These results suggest that ARHGAP6 contains at least two actin-organizing functions, which may be partially or completely independent of its rhoGAP activity. Similar morphological effects were observed in cells expressing N(α-1)-chimerin, a 38 kDa brain protein with in vitro racGAP activity which, paradoxically, stimulates rac and Cdc42 in vivo (7,35). GTPase stimulation or inhibition by
chimerins depends on lipid binding by an N-terminal domain, which interacts with the racGAP domain (35). The N-terminus of ARHGAP6 appears to target the rhoGAP domain to cytoskeletal compartments near activated rho GTPases and is critical for ARHGAP6 function. The rhoGAP domain of ARHGAP6 is highly homologous to the chimerin family, and one could envisage a similar dual role for ARHGAP6 in GTPase signaling.

The function of ARHGAP6 as a regulator of cell morphology and the cytoskeleton is consistent with a role for this novel rhoGAP in the pathogenesis of several neurodevelopmental disorders that are linked to Xp22.3. The results presented here show that the rhoGAP activity of ARHGAP6 does not contribute to the pathogenesis of MLS syndrome. Generation of mutant mice that lack the transcription initiation site or exon 1a, or deletion of the entire coding region may be the only approach to resolve the role of this gene in the MLS phenotype. Further mutation analysis of ARHGAP6 and experiments to clarify its in vivo functions are in progress.

MATERIALS AND METHODS

Southern analysis and library screening

Probes were radiolabeled using random oligohexamer priming with [α-32P]dCTP (36) and purified through a Sephadex G-50 column. The mouse 129 Sv/Ev genomic library (Allan Bradley, Baylor College of Medicine, Houston, TX) and the human fetal kidney cDNA library (Clontech, Palo Alto, CA) screens were hybridized to a mouse developmental northern blot (Clontech) as well as northern blots containing RNA prepared from an Arhgap6 mutant animal and a wild-type littermate as previously described (14). Commercial northern blots containing mRNA from E7–E17 stage mouse embryos (#7762-2; Clontech) and from adult mouse tissues (#7762-1; Clontech) were hybridized under the same conditions. For RT–PCR, 1 μg of total RNA was reverse transcribed using the Superscript II first-strand cDNA synthesis kit (Life Technologies, Gaithersburg, MD) with random hexamer primers (Amersham Pharmacia Biotech, Uppsala, Sweden). PCRs were performed in an MJ DNA engine tetrad (MJ Research, Watertown, MA) with an initial denaturation step of 94°C for 3 min, 33 cycles of 94°C for 1 min, 60°C for 1 min, 72°C for 1 min, and a final 5 min extension step at 72°C. The alternative 5′ ends were amplified using forward primers in exon 1a, 5′-GAGCGGGGCGGGAGG-GGCGTGTG-3′; exon 1b, 5′-AAGATTCTAGTGCGGAGGTC-3′; or exon 1c, 5′-CGGATACACATGTAACCTT-GAGGTC-3′. A reverse primer in exon 2, 5′-GAGACTCTTGATGGGAGGACTGACC-3′. The alternative 3′ ends were amplified using a forward primer in exon 12, 5′-AGGA-AGCCCTGACATGCTG-3′, or with reverse primers in exon 12, 5′-CCAGGCTCCAGTTACCCCTCC-3′ or exon 14, 5′-TGGACATTGCCATCTGGTGG-3′. The reactions contained 10 mM Tris–HCl pH 8.3, 50 mM KCl, 1.5 mM MgCl₂, 250 μM dNTPs, 0.625 U of Taq polymerase and 1 μM each primer.

Generation of mice with a targeted mutation in Arhgap6

With a 1158 bp probe from adult human retina cDNA, two overlapping positive clones were isolated from a mouse 129 Sv/Ev genomic library. A restriction map of the region indicated that the clones contain 30 kb of the mouse Arhgap6 locus including exons 5–9. The Arhgap6 targeting vector was constructed by inserting 6.0 kb EcoRI–SalI and 5.5 kb BamHI–SalI fragments of cloned strain 129 Sv/Ev genomic DNA into the PL13 vector (a gift from Allan Bradley). A homologous recombination event inserts exon 1 and 2 of a hypoxanthine–guanine phosphoribosyltransferase (hprt) minigene, a loxp site and a neomycin-resistance cassette while deleting 5.5 kb of the Arhgap6 locus including three exons encoding the rhoGAP domain (codons 417–555 of the murine Arhgap6 cDNA sequence). The targeting vector was linearized by digestion with KpnI (25 μg) and electroporated into HPRT-deficient AB2.1 129 Sv/Ev ES cells using a GenePulser (Bio-Rad, Hercules, CA) set at 300 V and 500 μF. Cells were plated onto a G418-resistant embryonic fibroblast feeder layer. After 24 h, selection was initiated for 10 days using 175 μg/ml G418 and 0.2 μM FIAU. A total of 288 surviving colonies were transferred in triplicate into 96-well plates. Cells from one plate of each clone were trypsinized and stored in cryo-preservation medium at −80°C. DNA from surviving ES clones was prepared and analyzed according to our published protocols (40). Three ES cell clones carrying the mutant allele were injected into the inner cell mass of C57Bl/6j blastocysts. Chimeric mice with a high contribution to coat color from 129 Sv/Ev were bred for germline transmission. For genotyping, genomic DNA isolated from ES cells or mouse tails was digested with BamHI for hybridization with the 5′ diagnostic probe or with Apal for the 3′ diagnostic probe.

Histological analysis

Tissues were fixed in 4% formaldehyde, dehydrated and embedded in paraffin. Sections were cut at 10 μm thickness, mounted, stained with hematoxylin and eosin or with silver using the Sevier–Munger method (17), and examined at 20–63× magnification using an Axioskope microscope (Carl Zeiss,
Thornwood, NY) Skeletons were prepared from E17.5 embryos and stained with alizarin red and alcin blue as described (41).

Immunohistochemical staining was performed on mouse brain sections according to our published protocol (42).

**Neurobehavioral tests**

Animals from a C57Bl/6J–129 Sv/Ev hybrid genetic background were analyzed at 3–4 months of age. Mice were evaluated using a battery of behavioral tests in the order presented: neurological screen for gross sensory/motor deficits, 30 min open-field test, 10 min light–dark box test, four trials of rotarod testing using an accelerating rotarod, acoustic startle response (120 dB) and prepulse inhibition of the startle response, habituation of the acoustic startle response, contextual and auditory-cued conditioned fear, hidden platform version of the Morris water task, and hot plate analgesia test. Tests were separated by 1 week intervals, except for the conditioned fear and Morris tests which were separated by 2–4 weeks. Details of the testing equipment and procedures were described previously (18). The conditional alternation test was performed in a water-filled T maze using the methods described by Paylor et al. (19). One- and two-way analyses of variance tests were used to analyze most of the behavioral results. Newman–Keuls and simple effects tests were used for follow-up comparisons. The data from the neurological screen were analyzed using Fisher’s exact probability tests.

cDNA expression constructs

A human cDNA containing the complete coding sequence of ARHGAP6 was constructed by ligation of two overlapping fetal kidney clones (HKF-3 and -4). The N-terminal coding sequence was amplified from this template using primers 7258 (5′-AGGGATCCAGAGGCTGCTCCACAGCGTC-3′) and 7260 (5′-AGAGCTCGAGGCAAGGAAATTGATGAAAGC-3′) and subcloned as a BamHI–XhoI fragment into pcDNA3.1 (Invitrogen, Carlsbad, CA) for expression as an Xpress-tagged fusion protein (pcDNA-Nterm). To express the full-length protein (pcDNA-FL), two restriction fragments derived from the C-terminal coding sequence of HKF-3 were ligated sequentially into pcDNA-Nterm. A 1.2 kb EcoRI–XhoI fragment from pcDNA-FL containing exons 13 and 14 was subcloned into a separate pcDNA3.1 vector to create pcDNA-Cterm. A 1.1 kb EcoRI restriction fragment from a mouse fetal brain cDNA clone containing exons 4–10, including the SH3 and rhoGAP domains, was also subcloned into pcDNA3.1 to create pcDNA-GAP. For production of a bacterial GST–Arhgap6 fusion protein, the same mouse cDNA was subcloned into the pGEX-5X-3 vector (Amersham Pharmacia Biotech). The GFP–Arhgap6 fusion construct was created by subcloning the N-terminal Xpress tag and coding sequence from pcDNA-FL into the pEGF-N1 expression vector (Clontech) in three overlapping restriction fragments. This construct expresses 963 amino acids of the ARHGAP6 coding sequence fused to GFP at a Sall site 11 amino acids from the C-terminus. The integrity of all constructs was verified by DNA sequencing. Western analysis of protein extracts from COS-7 and HeLa cells was performed using our previously published protocols (42) and antisera to either the Xpress, FLAG or GFP epitopes.

**GTPase activation assay**

Rac1, Cdc42 (G25K isotype) or RhoA were expressed as GST–fusion proteins in *E.coli* and purified from glutathione–Sepharose beads by thrombin cleavage as described previously (43). The purified proteins were dialyzed against 15 mM Tris pH 7.5, 150 mM NaCl, 5 mM MgCl2, 0.1 mM diithothreitol and concentrated by centrifugation with a Centricon-10 spin column (Amicon, Beverly, MA). GST–p50rhoGAP (amino acids 198–439) and GST–ARHGAP6 (amino acids 279–660) were eluted from the beads with 5 mM reduced glutathione and concentrated with a Centricon-10 column. The assay was performed using 5 µg of purified Cdc42, Rac1 or RhoA, 0.2 µM of purified GST–Arhgap6 (amino acids 279–660) and GST–p50rhoGAP fusion proteins (amino acids 198–439) and 10 µCi of [γ-32P]GTP (6000 Ci/mmol; NEN Life Sciences, Boston, MA) as previously described (20). Concentrations of GST–fusion proteins were determined by spectrophotometry and SDS–PAGE. Similar data were obtained in three independent experiments.

**Cell culture and transfection**

HeLa and COS-7 cells (American Tissue Culture Collection, Manassas, VA) were maintained in OptiMem (Life Technologies, Gaithersburg, MD) containing 5% fetal bovine serum in an atmosphere of 5% CO2 at 37°C. For transfections, 105 cells were plated onto glass coverslips in 6-well plates, transfected with Lipofectamine (Life Technologies) using 1 µg of plasmid DNA per well and incubated with the transfection mixture for 4 h without serum or antibiotics. Cells were rinsed and incubated in serum-containing medium for an additional 24–48 h before fixation and staining. For growth factor treatment, cells were plated in serum for 4–7 days and then serum starved for 16 h. Cells were trypsinized, resuspended in serum-free medium containing 0.5 mg/ml soybean trypsin inhibitor (Sigma, St Louis, MO), rinsed once and replated onto fibronectin-coated glass coverslips (Becton Dickinson, Bedford, MA) for transfection as above. At 8–12 h post-transfection, cells were stimulated with 100 ng/ml bradykinin (Sigma) for 10 min at 37°C or with 5 µM SPP (Sigma) for 30 min at 37°C. Immediately after the incubation period, the cells were fixed and processed for immunofluorescent staining. Transfected and untransfected cells were scored for stress fibers or filopodia by double-labeling with anti-actin antibodies.

**Immunofluorescence**

Cells were rinsed twice in calcium- and magnesium-free phosphate-buffered saline (PBS) and fixed using 4% formaldehyde in PEM buffer (50 mM K-PIPES, 5 mM EGTA, 2 mM MgCl2, pH 6.8) with 0.2% Triton X-100 for 10 min at room temperature. After post-fixation in ice-cold methanol, the cells were rinsed three times in PBS and allowed to rehydrate in PBS at room temperature for at least 30 min. Cells were incubated with the primary antibodies anti-FAK at 1:2000 dilution (Sigma), monoclonal anti-actin JLA20 at 1:200 (Sigma) or anti-epitope antibodies [anti-Xpress 1:500 (Invitrogen)] or anti-FLAG 1:500 (Eastman Kodak, Rochester, NY) in PBS for 1 h at room temperature or overnight at 4°C. Following three washes in PBS, the secondary antibodies [anti-rabbit or anti-mouse IgG conjugated with either fluorescein isothiocyanate (FITC), Texas Red or biotin (1:500; Vector Laboratories, Burlingame, CA)]
were applied to the cells for 1 h at room temperature. Biotin-conjugated antibodies were detected by a third incubation with Cy3-conjugated Extravidin (1:300; Sigma). Nuclei were counterstained with 4,6-diamidino-2-phenylindole (DAPI, 1 µg/ml). The coverslips were inverted onto slides and mounted with Vector Shield medium (Vector Laboratories) for visualization.

Live video microscopy

Live microscopy was performed on cells transfected with a wild-type ARHGAP6–GFP fusion construct, the mutated (R435G) ARHGAP6–GFP construct or an empty GFP vector as the control. Cells were grown on 40 mm coverslips in 60 mm plates and transfected with 2 µg of each test plasmid using Lipofectamine as above. Following a 4 h incubation with the transfection mixture, cells were allowed to recover for 6–9 h and were transferred to a live cell chamber (Bioptechs, Butler, PA). Cells were maintained at 37°C in Dulbecco’s modified Eagle’s medium with 5% stripped fetal bovine serum, which was recirculated using a peristaltic pump. In order to minimize photo damage, cells were imaged at suboptimal exposure using neutral density filters, to allow only 32% of the total light, and 1 s exposure times. Optical sections were limited to ~20 per cell and images were taken at 3 min intervals for 1–2 h or overnight at 10 min intervals.

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