ARL4, an ARF-like Protein That Is Developmentally Regulated and Localized to Nuclei and Nucleoli*

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ADP-ribosylation factors (ARFs) are highly conserved ~20-kDa guanine nucleotide-binding proteins that participate in both exocytic and endocytic vesicular transport pathways via mechanisms that are only partially understood. Although several ARF-like proteins (ARLs) are known, their biological functions remain unclear. To characterize its molecular properties, we cloned mouse and human ARL4 (mARL4 and hARL4) cDNA. The appearance of mouse ARL4 mRNA during embryonic development coincided temporally with the sequential formation of somites and the establishment of brain compartmentation. Using ARL4-specific antibody for immunofluorescence microscopy, we observed that endogenous mARL4 in cultured Sertoli and neuroblastoma cells was mainly concentrated in nuclei. When expressed in COS7 cells, ARL4-T34N mutant, predicted to exist with GDP bound, was concentrated in nucleoli. Yeast two-hybrid screening and in vitro protein-interaction assays showed that hARL4 interacted with importin-α through its C-terminal NLS region and that the interaction was not nucleotide-dependent. Like ARL2 and -3, recombinant hARL4 did not enhance cholera toxin-catalyzed auto-ADP-ribosylation. Its binding of guanosine 5′-O-(thiotriphosphate) was modified by phospholipid and detergent, and the N terminus of hARL4, like that of ARF, was myristoylated. Our findings suggest that ARL4, with its distinctive nuclear/nucleolar localization and pattern of developmental expression, may play a unique role(s) in neurogenesis and somitogenesis during embryonic development and in the early stages of spermatogenesis in adults.

The Ras superfamily of ~20-kDa guanine nucleotide-binding proteins (or GTPases) contains more than 100 gene products that have been grouped into five subfamilies, i.e. the Ras, Rab, Rho, Ran, and ARF families. The ARF1 (ADP-ribosylation factor) family, which is most different from the other groups, comprises at least six ARFs and six ARF-like (ARL) proteins (reviewed in Refs. 1 and 2). Although ARFs and ARLs are very similar in amino acid sequences, most ARLs, but not ARF1, apparently do not activate the cholera toxin ADP-ribosyltransferase. They differ also from ARFs in having demonstrable GTPase activity and different conditions that favor guanine nucleotide binding. Both ARFs and ARLs are widely distributed in eukaryotic organisms from yeast to human, consistent with evolutionary conservation of their biological functions.

ARFs play an important role in intracellular membrane trafficking, although there remains much to be learned. The biological functions of ARLs are still unclear, although some are expressed in a tissue- and/or differentiation-specific pattern (3–7). ARL1 was localized in the Golgi complex of normal rat kidney cells (8) and Saccharomyces cerevisiae (9), consistent with a function in vesicular trafficking. Unlike the lethal phenotype of double null alleles of arf1 and arf2, however, knock-out of the yeast ARL1 gene was not lethal (9). Expression of rat ARL4 was reported to be cell differentiation-dependent (4). Its role in adipocyte metabolism and sperm production was suggested (10) and nuclear localization of transiently expressed protein was demonstrated (11).

To obtain additional clues to its physiological role(s), we investigated the expression, subcellular localization, and biochemical properties of ARL4. As reported here, mouse ARL4 (mARL4), which is abundant in testis, is developmentally regulated during mouse embryogenesis. The mARL4 mRNA appears transiently, progressing in a rostro-caudal direction in day 8.5 to day 10.5 embryos, which coincides temporally with the appearance of somitomeres in the same locations. Endogenous mARL4 in Sertoli (TM4) and neuroblastoma (Neuro 2A) cells was mainly localized in nuclei. When expressed in COS7 cells, ARL4-T34N, a mutant predicted to be GDP-bound, was localized to nucleoli. This may be the first report of a small GTPase localized in nuclei in a nucleotide-dependent manner. Data from yeast two-hybrid and in vitro protein interaction analyses revealed that hARL4 interacted with the NLS-receptor, importin-α, through a bipartite nuclear localization signal (NLS) in its C-terminal region and that this interaction was not nucleotide-dependent. To our knowledge, no other small GTPase has been implicated in the regulation of somite...
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**Table I**

| Name   | Sequence *(5' → 3')* |
|--------|----------------------|
| ARL-R1 | (T/D)CT/TG/AC/GT/CCAGTCGAC/AC/GT/AC/GT/AC/GT/TCCA |
| ARL-R2 | (T/C)TCA/A/AC/GT/AA/GA/AC/GT/GG/AC/GT/AC/GT/TA/AC/GT/TT |
| ARLaA | GAATTCGAGAAATGGGCTGAGCAAG |
| ARL4B | TCTAGATTTCTTTTTCTTTGTCAGCCCA |
| ARL4C | TCGUGGTAGTGGTGTGGAGAAATATTGGACCT |
| ARL4D | AGTGGCCTTAATTTCTCCAGACCACCTACATCCCAGA |
| ARL4E | TCTGGGATGTGAGGTGGTCTGGAGAAATTAAGGCCACT |
| ARL4F | AGGGTATAAACAGGATCCTTCTTTTCAGCACAGTCCA |
| ARL4G | TCTAGATTTCTTTTTCTTTGTCAGCCCA |

* Underlines bases introduce EcoRI site.
* Underlined bases introduce XbaI site.
* Underlined base introduces Q79L point mutation.

For the preparation of the His-tagged fusion protein, the hARL4 PCR product was cloned into the expression vector pET15b (Novagen), yielding pET15b-His-hARL4. For the nonfusion protein, the hARL4 PCR product was digested with NdeI and BamHI, purified, and annealed to expression vector pET7/Nde (18), yielding pET-ARL4, which was used to transfect BL21(DE3) (9). Cell pellets were harvested and His-tagged fusion protein was isolated on Ni2+-NTA resin (Qiagen, Chatsworth, CA) by standard methods. The purity of the His-tagged hARL4 was assessed by SDS-PAGE.

Fractionation by Differential Centrifugation—Nuclear (N), crude cytosol (C), and membrane (M) fractions were prepared as described previously (20, 21). Briefly, confluent TM4 cells were scraped and homogenized in HES buffer (20 mM HEPES, pH 7.4, 1 mM EDTA, 250 mM sucrose) plus 1 mM phenylmethylsulfonyl fluoride and a mixture of protease inhibitors (leupeptin, aprotinin, chymostatin, antipain, and pepstatin, each 1 μg/ml) at 4 °C by 10 strokes in a ball-bearing homogenizer. The cell lysate was centrifuged at 400 × g for 10 min to eliminate unbroken cells, nuclei, and cell debris. The supernatant was centrifuged (150,000 × g, 1 h) at 4 °C to generate cytosolic (C) and membrane (M) fractions. To obtain the nuclear fraction, cell pellet containing unbroken cells, nuclei, and cell debris was dispersed in 1 ml of Tris-buffered saline, transferred to a microcentrifuge tube, and centrifuged for 15 s in a Microfuge. Tris-buffered saline was removed and the pellet was suspended in 400 μl of cold buffer A (10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride) by gentle pipetting in a yellow tip. The cells were allowed to swell on ice for 15 min, after which 25 μl of a solution containing 10% Nonidet P-40 were added and the tube was vigorously vortexed for 10 s. The homogenate was centrifuged for 30 s in a Microcentrifuge, and the nuclear pellet (N) was collected (21).

Cell Culture and Transient Transfection—Sertoli TM4 cells were grown at 37 °C on glass coverslips (18-mm diameter) in 12-well dishes for 16 h before processing at room temperature. Mouse neuroblastoma cells, Neuro 2a (ATCC: CCL-131), were grown in Dulbecco’s modified Eagle’s medium (Life Technologies, Inc.) supplemented with 10% fetal bovine serum, 2 mM glutamine, and 100 units/ml each of penicillin and streptomycin. The cells were subcultured by trypsinization (0.05%(w/v) trypsin, with 1% EDTA), and plated in growth medium in a humidified 5% CO2 incubator at 37 °C every 2 to 3 days. The medium was then replaced with fresh growth medium the day after transfection, and cells were harvested 30 to 36 h later for analysis.

Indirect Immunofluorescence Staining and Immunohistochemistry—

Experimental Procedures—Isolation of Mouse and Human ARL4 cDNA—Mouse ARL4 cDNA was synthesized by polymerase chain reaction (PCR) from a mouse ARL4 cDNA library. PCR-based cloning methods were used to obtain cDNA segments, from which a composite sequence of the full-length coding region was assembled (12). A probe composed of degenerate oligonucleotides (ARL-R1 and ARL-R2) (Table I) corresponding to part of the consensus sequences WDVGGQE and KLRPLWK in human and mouse ARL4 cDNA as described previously (9). All PCR products were purified, subcloned, and sequenced by the dideoxy chain termination method (13). The nucleotide sequence of mouse ARL4 has GenBank accession number U76546. Human ARL4 cDNA was isolated by the same procedures. The identical nucleotide sequence was deposited under GenBank accession number U73906.

Northern Analyses—Blots with RNAs from adult mice and mouse embryos at several stages of development (CLONTECH) were hybridized with mARL4-specific probes as described previously (12). A blot with samples of poly(A)+ RNA (2 μg) from testes of 5-, 10-, 15-, 20-, 25-, 30-, and 60-day-old rats was kindly provided by Dr. Ian Okazaki (National Institutes of Health). TM4 (Sertoli) and MA10 (Leydig) cells were grown and harvested as described (14, 15). Total RNA was extracted and reverse-transcribed using a reverse transcriptase kit (Life Technologies, Inc.). The identity of the RT-PCR product was confirmed by Southern blotting with an mARL4 cDNA probe.

In *Situ* RNA Hybridization—mARL4 RNA probes were prepared from pCRII-mARL4 constructed by PCR amplification of the adult probe. In situ hybridization using digoxigenin-labeled RNA probe was performed as described by Cheng et al. (16). Briefly, 14-μm cryosections of whole-mounted embryos were fixed with 4% paraformaldehyde for 1 h, followed by overnight hybridization at 70 °C with the digoxigenin-labeled probe (1 μg/ml) in hybridization buffer (50% formamide, 5 mM sodium citrate, and 1% SDS containing yeast RNA (50 μg/ml) and herring sperm (50 μg/ml)). After three stringent post-hybridization washes, the hybridized sections were incubated for 10 min to eliminate unbroken cells, nuclei, and cell debris. The supernatant was centrifuged, and the nuclear pellet (N) was collected (21).

Expression and Purification of Recombinant Proteins—The entire open reading frame of human ARL4 was obtained by PCR, using primers that incorporated unique Ndel and BamHI sites, respectively, at the initiating methionine and 6 base pairs downstream from the stop codon.

Development. The specific spatial and temporal expression of mouse ARL4 mRNA in the central nervous system during later embryonic stages suggests that ARL4 might also be involved in neurogenesis or cortical histogenesis. Thus, ARL4 may have a physiological role(s) in vertebrate somite formation, and central nervous system differentiation, as well as in the early events of gametogenesis.

**EXPERIMENTAL PROCEDURES**

**Isolation of Mouse and Human ARL4 cDNA**—Mouse ARL4 cDNA was synthesized by polymerase chain reaction (PCR) from a mouse ARL4 cDNA library. PCR-based cloning methods were used to obtain cDNA segments, from which a composite sequence of the full-length coding region was assembled (12). A probe composed of degenerate oligonucleotides (ARL-R1 and ARL-R2) (Table I) corresponding to part of the consensus sequences WDVGGQE and KLRPLWK in human and mouse ARL4 cDNA as described previously (9). All PCR products were purified, subcloned, and sequenced by the dideoxy chain termination method (13). The nucleotide sequence of mouse ARL4 has GenBank accession number U76546. Human ARL4 cDNA was isolated by the same procedures. The identical nucleotide sequence was deposited under GenBank accession number U73906.

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**In *Situ* RNA Hybridization—**mARL4 RNA probes were prepared from pCRII-mARL4 constructed by PCR amplification of the adult mouse ARL4 cDNA and cloning of the product into the pCRII vector. In situ hybridization using digoxigenin-labeled RNA probe was performed as described by Cheng et al. (16). Briefly, 14-μm cryosections of day 12.5, 14.5, 17.5, or 19.5 mouse embryos were incubated serially with 6% H2O2, 1 × PBS (PBS with 0.1% Tween 20), protease K, and 4% paraformaldehyde for fixation. Prehybridization was performed at 70 °C for 2 h, followed by overnight hybridization at 70 °C with the digoxigenin-labeled probe (1 μg/ml) in hybridization buffer (50% formamide, 5 mM sodium citrate, and 1% SDS containing yeast RNA (50 μg/ml) and herring sperm (50 μg/ml)). After three stringent post-hybridization washes with 50% formamide, 2 × SSC, slides were incubated (2 h, room temperature) with anti-digoxigenin antibody conjugated with alkaline phosphatase (Roche Molecular Biochemicals) diluted 1:2000, and colored by incubation with substrate nitro blue tetrazolium chloride/5-bromo-4-chloro-3-indolyl phosphate. Inspection and photography were performed with an Olympus microscope. In situ RNA hybridization with whole mounted embryos was performed as described by Cheng and Flagman (17).

Expression and Purification of Recombinant Proteins—The entire open reading frame of human ARL4 was obtained by PCR, using primers that incorporated unique NdeI and BamHI sites, respectively, at the initiating methionine and 6 base pairs downstream from the stop codon.
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Cells were fixed with 4% paraformaldehyde in PBS-Ca ++ -Mg ++ (0.6 mM CaCl₂ and 0.5 mM MgCl₂ in 1 × PBS) for 15 min, incubated with 0.1% Triton X-100 and 0.05% SDS in PBS-Ca ++ -Mg ++ for 4 min, and in the same buffer containing 0.2% bovine serum albumin for an additional 15 min, followed by incubation with primary antibodies; i.e. affinity purified anti-ARL4 peptide, mouse anti-p58 (Sigma), mouse anti-β-COP (Sigma), or mouse anti-C23 (nucleolin, Santa Cruz) in the same blocking solution for 40 min. After three washes with PBS-Ca ++ -Mg ++ , cells were incubated with secondary antibody, Alexa 594-conjugated anti-rabbit IgG antibody, or Alexa 488-conjugated anti-mouse IgG antibody (Molecular Probes), washed three times with PBS-Ca ++ -Mg ++ , mounted on Mowiol (supplemented with Hoechst 33258), and examined with a Zeiss Axioshot equipped for epifluorescence according to standard procedures (22). Primary antibodies previously depleted of anti-ARL4 activity by incubation with purified recombinant hARL4 were used as control. Immunohistochemistry was performed using an avidin-biotin system, the ABC Vectastain Elite kit (Vector Laboratories, Burlingame, CA). Representative, mouse embryos were fixed in 4% paraformaldehyde and processed for OCT block preparation. 5-mm cryostat sections of coronally positioned 14-dpc embryos were collected. After several rinses with phosphate-buffered saline, pH 7.4 (PBS), endogenous peroxidases were quenched by adding 6% hydrogen peroxide in PBS for 15 min at room temperature. After washing with PBS, sections were treated with 20% normal goat serum for 30 min, washed with PBS, incubated with the anti-ARL4 antibody for 1 h at room temperature, and then washed with PBS. Biotinylated goat anti-rabbit antibodies conjugated to Cy-3 were used as a secondary antibody. After washing with PBS and addition of 0.05% 3,3′-diaminobenzidine tetrahydrochloride. Color development was monitored and stopped by dilution with water. Sections were dried, mounted, and inspected by light microscopy (Olympus Co.).

Yeast Two-hybrid Screen and Assay—Yeast strains (L40), plasmids (pBTM116 and pVP16), and library for the yeast two-hybrid screen were obtained from Dr. H. Shih. The genotype of the S. cerevisiae reporter strain L40 is MATa trp1 leu2 his3 lys2::lexA-HIS3 URA3::lexA-lacZ (23). Yeast strains were grown at 30 °C in rich media, followed by washing with PBS and addition of 0.05% 3,3′-diaminobenzidine tetrahydrochloride. Color development was monitored and stopped by dilution with water. Sections were dried, mounted, and inspected by light microscopy (Olympus Co.).

ARL4 mRNA in mouse tissue and embryos and rat testis. Blots containing poly(A) + RNA from adult mouse tissues (A) or whole embryos at different developmental stages (B) were hybridized with a random-primed, 32P-labeled mARL4 cDNA probe. Hybridization with a β-actin probe was a control for sample loading. C, a blot containing poly(A) + RNA from rat testes (15 days), midpubertal (20 and 25 days), late pubertal (30 days), and adult (60 days) rat testis was hybridized with a random-primed, 32P-labeled rat ARL4 cDNA probe and a GAPDH cDNA as control.

Primer ARL4G. Plasmids pLexA-ARL1, pLexA-ARL3, pLexA-ARL4, pLexA-ARL4-Q79L, pLexA-ARL4-T34N, and pLexA-ARL4-dC, constructed, respectively, by inserting a PCR-generated fragment of the ARL1, ARL3, ARL4, ARL4-Q79L, ARL4-T34N, or ARL4-dC cDNA into the EcoRI site of the pBTM116 plasmid, were used to express the ARL4 as a fusion protein with the DNA-binding domain of LexA. For two-hybrid screening, the yeast reporter strain L40, which contains the reporter genes lacZ and HIS3 downstream of the binding sequences for LexA, was transformed with pLexA-ARL4-Q79L and a human testis pACT2 cDNA library (CLONTECH) by the lithium acetate method (24), and subsequently treated as described (23). Double transformants were plated with synthetic medium lacking histidine, leucine, tryptophan, uracil, and lysine. Plates were incubated at 30 °C for 3 days. His+ colonies were patched on selective plates and assayed for β-galactosidase activity by a filter assay (23). Plasmid DNA was prepared from colonies displaying a HIS + /lacZ + phenotype by electrotransformation of HB101 cells and used to re-transform the L40 strain containing the appropriate pLexA-ARL1, pLexA-ARL3, pLexA-ARL4, pLexA-ARL4-Q79L, pLexA-ARL4-T34N, pLexA-ARL4-dC, and pLexA-lamin, to test for specificity. For assay of β-galactosidase activity, transformants were grown in histidine-containing medium, lysed, and assayed as described (23).

In Vitro Interaction of ARL4 and Importin-α—Recombinant human ARL4 mutants (ARL4-Q79L, ARL4-T34N, and ARL4-dC) were PCR amplified from the NdeI site upstream of the initiator methionine codon. PCR fragments were ligated into the pT7Blue Blunt vector (Novagen). The pT7Blue (ARL4) plasmids were digested with NdeI and BamHI and the ARL4 fragments were ligated in-frame to the PET-15b expression vector (Novagen). To synthesize importin-α as an N-terminal GST fusion protein, importin-α cDNA was amplified by PCR using primers containing NotI sites at both 5′- and 3′-ends. After ligation into pT7Blue Blunt vector, the NotI sample of each fragment was subcloned into the same sites of GST fusion vector pGEX-4T1 (Amersham Pharmacia Biotech) to generate pGEX-importin-α. Transformed Escherichia coli strain BL21 were grown and recombinant protein was prepared as described previously (9). The soluble fraction of E. coli expressing GST or GST-importin-α was incubated with glutathione-Sepharose 4B beads (Amersham Pharmacia biotech) for 30 min at room temperature. The beads were then washed four times with PBS, and finally suspended in PBS. GST and GST-importin-α immobilized on glutathione-Sepharose beads were quantified by SDS-PAGE with Coomassie Blue staining.

Each hARL4 construct was expressed in E. coli, and 750 μl of each soluble fraction were incubated at 4 °C for 1 h with 10 μg of GST or GST-importin-α immobilized on glutathione-Sepharose beads. After washing five times with ice-cold washing buffer (25 mM Tris-HCl, pH 8.0, 100 mM NaCl, 10 mM MgCl₂, 1 mM dithiothreitol), beads plus 25 μl of SDS sample buffer were boiled for 10 min and a 15-μl sample of each supernatant was subjected to SDS-polyacrylamide gel electrophoresis.

FIG. 1. ARL4 mRNA in mouse tissue and embryos and rat testis. Blots containing poly(A) + RNA from adult mouse tissues (A) or whole embryos at different developmental stages (B) were hybridized with a random-primed, 32P-labeled mARL4 cDNA probe. Hybridization with a β-actin probe was a control for sample loading. C, a blot containing poly(A) + RNA from rat testes (15 days), midpubertal (20 and 25 days), late pubertal (30 days), and adult (60 days) rat testis was hybridized with a random-primed, 32P-labeled rat ARL4 cDNA probe and a GAPDH cDNA as control.
FIG. 3. In situ hybridization of ARL4 mRNA in mouse embryos at day 14.5–17.5 pc. Pairs of sections of each embryo were reacted with the mARL4 antisense probe (A–C) or stained with hematoxylin-eosin (D–F). Day 14.5 embryo (A and D) is a transverse section near the level of the nasopharynx. Structures that appear to show specific reactivity include neuronal stoma around the central canal of the spinal cord (arrows), the dorsal horn (arrowheads), the ventral horn (asterisks), and the root ganglion (red arrowhead). Abbreviations are: c.p., cartilage primordium; c.a., common carotid artery; e, esophagus; f, larynx. Day 16.5 embryo (B and E) is a sagittal section. The root ganglion (red arrowhead) and spinal cord (arrows) show mARL4 reactivity. Abbreviations are: Int, intestine; Liv, liver; Kid, kidney. Day 17.5 embryo (C and F) is a coronal section through the head. Generalized mARL4 reactivity in the brain parenchyma is detected, especially in the neopallial cortex (arrows).

RESULTS

Developmentally Regulated Expression of mARL4—mARL4 mRNA was much more abundant in testis than in other adult mouse tissues (Fig. 1A). A prominent ~1.4-kilobase transcript along with a lesser one of ~4.0-kilobase was observed in mouse tissues. On Northern blot analysis of mouse embryos at different stages of development, the level of mARL4 mRNA was highest on embryonic day 7 and was 90% lower by day 11 (Fig. 1B). Northern blot analysis of whole testis preparations collected from prepubertal (5 and 10 days), early pubertal (15 days), mid-pubertal (20 and 25 days), late pubertal (30 days), and adult (60 days) rats revealed a major rat ARL4 mRNA of 1.4 kilobases that was first detectable in mid-pubertal testis and was more abundant in the mature 30- and 60-day-old testis possessing fertilizing activity (Fig. 1C). In situ hybridization with frozen sections of testis and epididymis from 3- and 6-month-old mice identified mARL4 RNA mainly localized in the basal region of seminiferous tubules (i.e. spermatogonia/spermatocytes and Sertoli cells); it was not detected in the androgen-producing Leydig cells (data not shown), as reported by Jacobs et al. (10). Similarly, in cultured cell lines, RT-PCR showed that Sertoli cells (TM4), but not Leydig cells (MA10), had mARL4 transcripts, consistent with the results of in situ hybridization, although combined RT-PCR and Southern analysis did reveal small amounts of mARL4 mRNA in the Leydig cell line (data not shown).

In Situ Hybridization Analysis of ARL4 RNA in Mouse Embryos—Abundant mARL4 mRNA was detected in 7-dpc (days post-coitus) mouse embryos (Fig. 1B). In situ hybridization of mouse embryos at 8–10 dpc and tissue sections of embryos at later stages was performed to determine whether mARL4 is expressed in a tissue-specific manner during embryonic development. mARL4 mRNA was prominent at the earliest time point analyzed, day 8.5 at the 10–12 somite stage (Fig. 2A), localized specifically in pairs of somites and at the junction of forebrain and midbrain (Fig. 2A, red and black arrows, respectively). In 9.0-dpc embryos, mARL4 mRNA was present in more caudal somites, coinciding with the sequential formation of new somites (Fig. 2B, red arrow). At the 25–29-somite stage (day 9.5 embryo), mARL4 mRNA was concentrated at the junction of midbrain and hindbrain (Fig. 2C, arrowhead) and in the caudal somites (Fig. 2C, red arrow); it had disappeared from the first 10–12 pairs of somites and the forebrain-midbrain junction.

Later in embryonic development (Fig. 3, A and D), mARL4 mRNA was detected in the neuronal stoma around the central canal, the dorsal and ventral horns of the spinal cord, and the root ganglia (day 14.5 embryo). It was still present in the spinal cord and root ganglia of day 16.5 embryos (Fig. 3, B and E). In day 14.5 to 17.5 embryos, the brain parenchyma contained less mARL4 mRNA than did the spinal cord. A prominent zone of mARL4 mRNA was detected, however, in the neopallial cortex of the day 17.5 embryo (Fig. 3C). mARL4 mRNA decreased with maturation and was undetectable in the spinal cord of the neonatal mouse, although trace amounts were still discernible in the brain parenchyma (data not shown).

Subcellular Localization of ARL4—To identify the intracellular location of the ARL4 protein, we generated ARL4-specific antibodies against a unique peptide sequence (residues 138–154) of human ARL4. Predicted amino acid sequences of human and mouse ARL4 differ only at position 14 (data not shown). The affinity-purified antibodies proved to be sensitive and specific for detection of both human and mouse ARL4 proteins. Immunoblotting with this antiserum detected ARL4 in low nanogram amounts, while no reaction was detected with 100 ng of other recombinant ARLs (Fig. 4A).
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To assess the subcellular distribution of ARL4 in mouse Sertoli TM4 cells, homogenates were fractionated by differential centrifugation. Nuclear (N), membrane (M), and cytosol (C) fractions were separated and ARL4, proliferating cell nuclear antigen, and tubulin (cytoplasmic marker) in subcellular fractions were identified by Western blot analysis (Fig. 4B). Using affinity-purified anti-ARL4 antibodies, endogenous ARL4 was detected in the nuclear fraction, whereas immunoprecipitated ARL1 was in the cytoplasmic fraction (Fig. 4B). ARL3 was not detected in the TM4 cells. The specific ARL1, ARL3, and ARL4 antibodies used did not cross-react with other ARls. Immunodetection of ARL4 was abolished by prior incubation of the antisera with recombinant ARL4 (data not shown). By immunofluorescence microscopy, endogenous mARL4 of Sertoli TM4 and neuroblastoma (Neuro 2a) cells were distributed mainly over the nucleus in a fine punctate pattern (Fig. 4C). No immunoreactivity was detected after incubation of antibody ARL4-B mainly with purified recombinant ARL4 (Fig. 4C, e) or with preimmune serum (not shown). Nuclei were stained with the DNA-binding dye H33258 (Fig. 4C, f, j, and k) and Golgi with anti-β-COP antibodies (Fig. 4C, c, g, and k).

We have tried, thus far without success, to demonstrate nuclear localization of ARL4 in mouse fetal tissues. In the day 14 embryo, only weak staining was observed in the spinal cord and root trunks by immunohistochemistry, and none in the adjacent tissues derived from myotome and sclerotome (Fig. 4D). This is consistent with the low level of ARL4 mRNA in the day 11 mouse embryo (Fig. 1B), and makes it difficult to establish mARL4 localization in nuclei.

Two-hybrid Interaction of Importin-α with ARL4 C-terminal Nuclear Localization Signal NLS—To identify molecules that might act as downstream effectors of ARL4, we used plasmid pLexA-ARL4-Q79L to express the putatively constitutively active mutant of ARL4 (ARL4-Q79L) as bait in a yeast two-hybrid screen of human testis cDNA library (23). Plasmids that were associated with β-galactosidase production were identified from a screen of approximately 4 × 10^6 colonies. The DNA sequence of each library insert was determined and eight distinct genes were chosen for further analysis. One of these, importin-α (karyopherin α2; accession number NM_002266) (28, 29), was further characterized to support the observations on ARL4 localization. Five different fragments (residues 1–530, 135–530, 152–350, 233–530, and 235–530) of importin-α were fused to LexA-ARL4 to test whether interaction of hARL4 and importin-α is dependent on this sequence. We also constructed wild type ARL4 and ARL4-T34N (predicted to be GDP-bound mutant) to test whether interaction of hARL4 and importin-α is nucleotide-dependent. All of the LexA fusion ARL proteins were expressed in yeast and detected by antibodies against LexA or against specific ARls (Fig. 5B). In the yeast two-hybrid assay, transformants containing interacting proteins that transactivate two reporter genes, HIS3 and LacZ, exhibit β-galactosidase activity and can grow on minimal medium lacking histidine. As illustrated in Fig. 5C, LexA-ARL4, LexA-ARL4-Q79L, and LexA-ARL4-T34N, but not LexA-ARL4-dC, LexA-ARL1, or LexA-ARL3, interacted with the Gal4AD-importin-α fusion protein and activated the reporter genes.

To confirm that the interactions between ARL4 and importin-α are direct, an in vitro GST pull-down assay was used. Recombinant His-tagged hARL4 and its mutants were produced in E. coli, and the soluble bacterial proteins were incubated with immobilized GST-importin-α in vitro. As shown in
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Fig. 5. Interaction of hARL4 with importin-α in the two-hybrid system. A, diagrammatic comparison of wild type (WT) hARL1, hARL3, and hARL4 and three hARL4 mutants with the total number of amino acids on the right. Positions 188 to 200 contain the bipartite NLS of ARL4. ARL-dC lacks 11 of those amino acids (190 to 200) at the C terminus. Positions of the mutations Q79L and T34N are also indicated. B, expression of LexA-ARL fusion proteins. Yeast reporter strain L40 was transformed with the indicated LexA construct, pBTM116 (LexA only), or pLexA-lamin. Samples (20 μg) of cell lysates were subjected to SDS-PAGE in a 10% gel. Proteins were transferred to nitrocellulose and reacted with anti-LexA antibodies (upper panel), or specific anti-ARL1 (16), anti-ARL3 (see Footnote 2), or anti-ARL4 antibodies as indicated (lower panel), followed by detection using the ECL system. Positions of protein standards (kDa) are on the left.

C, interaction of hARL4 and mutants with importin-α in the two-hybrid system. The yeast reporter strain L40 was co-transformed with pACT2-importin-α and the indicated pLexA-ARL construct or pLexA-lamin. Co-transformants were plated on synthetic histidine-containing medium lacking leucine, tryptophan, uracil, and lysine (His- plate, middle panel). Colonies from His+ plates were assayed for ß-galactosidase activity by a filter assay to test for specificity (lower panel). Colonies from His+ plates were also patched on His- selective plates lacking histidine, leucine, tryptophan, uracil, and lysine (His- plate, middle panel). D, in vitro interaction of GST-importin-α with various hARL4 constructs. Recombinant His-tagged ARL4, ARL4-Q79L, ARL4-T34N, ARL4-dC, and ARL1 were synthesized in E. coli, and 750 μl of soluble fraction from each batch of cells, as indicated, were incubated with 10 μg GST or GST-importin-α immobilized on glutathione-Sepharose beads at 4°C for 1 h. After washing five times with ice-cold washing buffer, 25 μl of SDS sample buffer was added to each batch of beads, followed by boiling for 10 min. From each preparation, a sample (15 μl) was subjected to SDS-polyacrylamide electrophoresis in 12% gel. A sample (7.5 μl) of the indicated E. coli soluble fraction, in the first lane, is followed by GST-bound and GST-importin-α-bound samples in each group of three lanes.
function (11), we wanted to examine further whether the subcellular localization of ARL4 was dependent on GTP or GDP binding. COS7 cells transiently expressing GFP-ARL4, -ARL4-Q79L, -ARL4-T34N, and -ARL4-dC were inspected by fluorescence microscopy (Fig. 6A). ARL4 appeared to be located in nuclei and partially in nucleoli (Fig. 6A, a-c, arrows). ARL4-T34N, but not ARL4-Q79L, appeared to be more concentrated in nuclei (Fig. 6A, i-k, arrows). The nucleolar localization of ARL4-T34N was confirmed by coinmunostaining with nucleolin, a marker for nucleoli. ARL4-T34N was colocalized with nucleolin, confirming its nucleolar localization (Fig. 6A, q-t). ARL4-dC, not seen in nuclei, was in part co-localized with the Golgi marker p58 (Fig. 6A, o and p). Subcellular distribution of transiently expressed hARL4 and its mutants in homogenates of COS7 cells was also examined. ARL4, ARL4-Q79L, and ARL4-T34N were detected mainly in the nuclear fraction with very little in the membrane fraction (Fig. 6B). ARL4-dC, however, appeared mainly in the membrane fraction, confirming the results of fluorescence microscopy.

**Functional Properties of ARL4 Protein**—The hARL4 fusion protein, like ARL2 and ARL3, failed to stimulate auto-ADP-ribosylation of the cholera toxin A1 protein (data not shown). It did bind GTPγS in a concentration-dependent manner that reached a steady state within 60 min at 30 °C. Phospholipids that increased GTPγS binding by hARF1, however, markedly decreased binding by hARL4 (Fig. 7). To determine whether hARL4 could be myristoylated, hARL4, hARL1, and yARL3 were co-expressed in E. coli with yeast N-myristoyltransferase. As shown in Fig. 8, hARL4 was myristoylated as was hARL1, which has been reported (9); yARL3, previously shown not to be myristoylated (26), served as a negative control. Thus, the biological function of ARL4, like those of ARFs and other proteins, may be influenced by myristoylation.

**DISCUSSION**

We have characterized the expression, subcellular localization, and biochemical properties of a highly conserved small GTPase, ARL4. RNA blot hybridization revealed more than one species of ARL4 mRNA, which might serve different biological functions in different cells. The existence of multiple mRNAs has been reported also for human ARL1 (7), ARL3 (5), and ARFs (reviewed in Refs. 1 and 2). Our data clearly indicate that the expression of mARL4 mRNA is developmentally regulated and are consistent with involvement of the protein in early events of spermatogenesis, somitogenesis, and the embryogenesis of the murine central nervous system. By indirect immunofluorescence and biochemical techniques, we show that localization of ARL4 in nuclei is influenced by nucleotide binding.

ARL4 mRNA, which was detected in numerous adult organs, was most abundant in the spermatogonia and/or Sertoli cells of adult testis. No reactivity was detected in the epididymis by in situ hybridization, suggesting that ARL4 is not involved in the activation of motility or the maturation of spermatids induced by the ciliated epithelium of epididymis. The mRNA for rat ARL4, which differs in only one amino acid from mouse ARL4, was reported to be abundant in testis and 3T3-L1 adipocytes (10).

In mouse embryo, the pattern of appearance of ARL4 mRNA proceeding in a rostro-caudal direction, coincided temporally with the appearance of somites during embryonic days 8.5–10.5. Somites form in a pairwise fashion within the presomitic mesoderm following gastrulation. In the mouse embryo, somite pairs are laid down in a rostro-caudal progression with a total of 65 somite pairs formed during embryogenesis. Somitogenesis is the basis of the segmented body plan and precursor to the axial skeleton, the dermis of the back, and all striated muscles of the adult body. Proteins proposed to participate in somitogenesis include c-hairy-1, notch/delta, and the eph family (30–33). The function of c-hairy-1 is suggested to be that of a molecular clock determining vertebrate segmentation as a result of transient waves of expression in chicken presomitic mesoderm that move rostrally with a periodicity corresponding to the time required to form one somite (90 min). A caudal-rostral wave of c-hairy-1 expression is repeated during the formation of each somite.

The notch/delta family was initially identified as a group of genes encoding cell-surface proteins that define neuronal cell fate and subsequently demonstrated to participate in prefiguring somite units. Notch/delta is expressed in a metaxic pattern in the presomitic mesoderm, thereby establishing boundaries of each somite segment during somitogenesis (39–42). Unlike c-hairy-1 or notch/delta mRNAs, mARL4 mRNA was evenly distributed in each somite pair. Its appearance moves caudally as the embryo develops, as does the pattern of eph family signaling in somitogenesis (34). No other GTPase has apparently been implicated in somitogenesis. It will be important to
investigate a possible link between the eph receptor tyrosine kinase family and the ARL small GTPase family in signal transduction pathways that determine cell fate during embryogenesis.

mARL4 mRNA was detected in the embryonic central nervous system at the earliest time examined (8.5-dpc mouse embryo), around the junction between the forebrain and midbrain. In 9.5-dpc mouse embryos, that earlier concentration of mARL4 had disappeared, and mARL4 was, instead, localized at the midbrain-hindbrain junction (Fig. 2). We interpret this change to mean that mARL4 may play a role in the establishment of central nervous system compartmentalization analogous to its function in the segmentation of somitogenesis. Indeed, Ephrin-A1, Ephrin-B2, and the receptor EphA4 are expressed in an iterative manner in the developing somites and in a gradient along the anterior-posterior axis of the developing midbrain (16, 34).

At later stages of embryonic development, mARL4 was found in specific central nervous system structures, which included the neopallial cortex (future neocortex), ventricular zone of the cerebrum, and the spinal cord. The expression zones exactly matched those of neuronal cell bodies (perikaryon), which contain abundant rough endoplasmic reticulum, ribosomes, vesicles, and inclusions that are thought of as regions of mRNA concentration. A more comprehensive study will be required to define precisely the subcellular localization of mARF4 in individual cells.

Compared with ARL1 and ARL3, ARL4 contains an additional C-terminal putative bipartite NLS, i.e. K\textsuperscript{148}RRKVMLQQKQKKQ\textsuperscript{200} (Fig. 5A). Importin-\(\alpha\) (karyopherin-\(\alpha\)) is a protein of 530 amino acids that interacts directly with NLS. The C-terminal region of importin-\(\alpha\) recognizes the NLS-containing protein (35). Interaction of hARL4 with importin-\(\alpha\) apparently depends on its C-terminal NLS, since ARL4-dC lacking 11 amino acids at the C terminus failed to induce \(\beta\)-galactosidase in the yeast two-hybrid assay. hARL4 interacted with five different recombinant importin-\(\alpha\) constructs (residues 1–530, 135–530, 152–530, 233–530, and 235–530), each of which contains the NLS-binding site in the C-terminal region. The \textit{in vitro} interaction experiments using GTPase-defective (ARL4-Q79L) and GTP-binding defective (ARL4-T34N) mutants of ARL4, as well as ARL4-dC, confirmed that interaction of hARL4 with importin-\(\alpha\) is not GTP-dependent and does require its C-terminal NLS.

Immunofluorescence microscopy and subcellular fractionation analyses revealed that endogenous mARL4 of Sertoli TM4 and neuroblastoma Neuro-2a cells was localized mainly in nuclei, in a punctate pattern (Fig. 4). Although, transiently expressed GFP-ARL4 fusion protein was reported to be localized in nuclei (11), we found that some of the transiently expressed GFP-ARL4 was localized to nucleoli. Moreover, nucleolar concentration of ARL4-T34N appeared to depend on its GDP-bound conformation, since ARL4-Q79L was not similarly concentrated. The function of the nucleolus as a factory for assembling ribosomal subunits is well established, but one of the more surprising findings of the past decade is the discovery of a variety of macromolecules in the nucleolus with no apparent ribosomal function (reviewed in Refs. 36 and 37). The nucleolus also seems to play a role in nuclear export, sequestration of the p53 inhibitor Mdm2, and controlling aging. In these novel events, the nucleolus serves as a privileged site for both recruitment and exclusion of regulatory complexes. The nucleolus may serve as a “sequestration center” for proteins that are to be kept inactive. This notion raises the possibility that some of the many proteins that have been localized to the nucleolus may be stored there in anticipation of eventual release. Interestingly, a p34\textsuperscript{cdc2} homolog was localized to the nucleoli of neurons and glia in the mitotically quiescent murine central and peripheral nervous systems (38). Recently, three cell-cycle regulators, Cdc 14, Mdm2, and Pch2, have been identified whose activity is regulated by sequestration in nucleoli (reviewed in Ref. 39). Furthermore, nucleolar localization of the tumor suppressor protein p19ARF, with concomitant sequestration of the p53 inhibitor Mdm2, is disrupted by tumor-associated mutations and may be key for p53 activation (reviewed in Ref. 36). To our knowledge, ARL4 may be the first small GTPase reported to be localized in nucleoli. Although, the physiological significance of the presence of ARL4 in nucleoli is not understood, it will be important to determine whether ARL4 can participate in the regulation of nucleolar sequestration of proteins.

Of eight proteins that interacted with hARL4 in the yeast two-hybrid screening, one that interacted with ARL4-Q79L, but not ARL4WT or ARL4-T34N, in the two-hybrid system is the Sec7-domain of a known guanine nucleotide-exchange protein. It will be interesting to learn whether this guanine nucleotide-exchange protein, although believed to have an extra-nuclear distribution, can translocate into nuclei to activate ARL4. Six of the proteins that interacted with ARL4 have a nucleolar localization and one of these is involved in the dynamics of the inner nuclear membrane and lamina. The small GTPase Ran, which plays a key role in nuclear transport, was recently reported to function also in mitosis by regulating microtubule nucleation and/or growth (40). The nuclear envelope of higher eukaryotes is a dynamic structure that breaks down during mitotic prometaphase and reforms during anaphase and telophase (41). During nuclear envelope breakdown, the nuclear lamina and pore complexes disassemble, and the nuclear membranes vesiculate. During reassembly, nuclear membranes are targeted to the daughter chromosomes where they fuse to enclose the chromatins. The nucleus then grows by protein import through newly assembled pore complexes and the fusion of additional vesicles. Our studies demonstrated that ARL4, like ARF’s, can be amino terminally myristoylated, consistent with a function dependent upon its reversible association with specific intracellular membranes that is influenced by myristoylation as well as guanine nucleotide binding. It has been suggested that a non-ARF GTPase is required for nuclear fusion and mitotic membrane disassembly (42), and we also speculate that ARL4 may have such a role in novel nuclear membrane trafficking and/or signaling cascades during embryonic development.

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