Identification, Characterization and Cloning of myr 1, a Mammalian Myosin-I

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Abstract. We have identified, characterized and cloned a novel mammalian myosin-I motor-molecule, called myr 1 (myosin-I from rat). Myr 1 exists in three alternative splice forms: myr 1a, myr 1b, and myr 1c. These splice forms differ in their numbers of putative calmodulin/light chain binding sites. Myr 1a–c were selectively released by ATP, bound in a nucleotide-dependent manner to F-actin and exhibited amino acid sequences characteristic of myosin-I motor domains. In addition to the motor domain, they contained a regulatory domain with up to six putative calmodulin/light chain binding sites and a tail domain. The tail domain exhibited 47% amino acid sequence identity to the brush border myosin-I tail domain, demonstrating that myr 1 is related to the only other mammalian myosin-I motor molecule that has been characterized so far. In contrast to brush border myosin-I which is expressed in mature enterocytes, myr 1 splice forms were differentially expressed in all tested tissues. Therefore, myr 1 is the first mammalian myosin-I motor molecule with a widespread tissue distribution in neonatal and adult tissues.

The myr 1a splice form was preferentially expressed in neuronal tissues. Its expression was developmentally regulated during rat forebrain ontogeny and subcellular fractionation revealed an enrichment in purified growth cone particles, data consistent with a role for myr 1a in neuronal development.

MYOSIN-Is differ from conventional muscle and non muscle myosins (myosin-IIs) by having only one heavy chain and a single motor domain (for review see 7, 24, 54). The myosin-I motor domain is homologous to the motor domain of conventional myosin. This domain exhibits actin-activated Mg^{2+}-ATPase activity and produces force along actin filaments (2, 25, 34, 64). However, the heavy chain of myosin-I molecules lacks the extended α-helical tail domain of the conventional myosin heavy chain and is not capable of self-assembling into dimers and bipolar filaments. Instead, the tail domains of myosin-Is are thought to contain targeting signal(s) for membrane subdomains and to determine what kind of cargo is being moved. Several myosin-Is have been localized to specific membranous compartments within cells (3, 18, 43, 66). Therefore, myosin-I motor molecules were proposed to move membranes along actin filaments. The movements of membranes powered by myosin-Is may include organelle movements (1, 36) and cell shape changes (17).

Myosin-Is have been first discovered in the soil amoeba *Acanthamoeba castellanii* (29, 31, 39, 55), and more recently several myosin-Is have been cloned from the slime mold *Dictyostelium discoideum* (30, 62). The only well characterized vertebrate myosin-I is brush border myosin-I, originally known as 110-kD calmodulin complex (10, 47). Associated with its heavy chain are 3-4 calmodulin molecules as light chains. Brush border myosin-I forms lateral links between the plasma membrane and the actin bundle within the microvilli of intestinal brush border cells (12, 43). The expression of brush border myosin-I is virtually restricted to intestinal brush border cells (19, 28). Very recently, the purification of a myosin-I from bovine adrenal medulla and brain was reported (4).

We hypothesized that myosin-I molecules play important roles in neuronal cells because actin-based cell motility is a fundamental prerequisite for neuronal development (46, 60). Functional connections between nerve cells develop through extensive neuronal shape changes. Furthermore, the development of neuritic processes involves intense membrane trafficking between neuronal cell bodies and elongating axons and dendrites. Shape changes and membrane trafficking are functions in which protozoan myosin-Is have been implicated (1, 17). Therefore, we probed the developing nervous system for the presence of myosin-I(s).

In this communication, we report on the identification of a novel mammalian myosin-I protein. To identify mammalian myosin-Is expressed in the developing nervous tissue, an antibody was generated against a synthetic peptide encompassing a conserved sequence of the (invertebrate) myosin-I motor domain which is sufficiently different from conventional myosin. Based on this myosin-I consensus antibody, we iden-

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tified, characterized and cloned myr 1a–c (first myosin I from rat), a novel myosin-I from rat tissue. We provide evidence that myr 1a–c are related to brush border myosin-I, but serve functions not restricted to microvilli. Alternatively spliced 29 residue repeats in the regulatory domain may endow myr 1a–c with different regulatory properties. A partial and preliminary account of this work has been published in abstract form (Bähler, M., 1990. *J. Cell Biol.* 111:167*a*).

Materials and Methods

Antibodies

To raise antibodies against a myosin-I consensus sequence, a 19 amino acid peptide was synthesized which duplicates the amino acid sequence YLGLLENVRVRRAGYAYRQ (611–629) of bovine brush border myosin-I (28). A cysteine residue was added at the carboxy-terminus of the peptide for the selective coupling to a carrier protein. The peptide was cross-linked with m-maleimidobenzoyl sulfosuccinimide ester (MBS; Pierce Chemical Co., Rockford, IL) to keyhole limpet hemocyanin (Calbiochem-Behring Co., San Diego, CA) as described by Green et al. (22). Rabbits were injected intradermally or subcutaneously with 300 μg coupled peptide emulsified in complete Freunds adjuvant (Gibco Laboratories, Eggenstein, FRG, and Sigma Co., Deisenhofen, FRG). For booster injections, 200 μg coupled peptide was emulsified in incomplete Freunds adjuvant. After two booster injections at 2–4 wk intervals, the rabbits were bled regularly. Peptide affinity columns were prepared by coupling the peptide to CNBr-activated Sepharose 4B (Pharmacia, Freiburg, FRG) according to the instructions of the manufacturer. Antibodies were affinity purified by passing the sera over the peptide column and elution with 0.2 M glycine, pH 2.8. An identical protocol was used for the generation and affinity purification of antibodies directed against the peptide derived myr 1 tail amino acid sequence (see Results) using the synthetic peptide NKNPKYKKLKDAIEEKIIIAEVVNKINC (antisera Tü 2) and the myr 1 NH_2-terminus using the synthetic peptide MAKKEVKSSLLDNMC (antisera Tü 30).

A monoclonal antibody (2.17) raised against cytoplasmic myosin-II from *A. castellanii* recognizing the motor domain (33) and a polyclonal antibody raised in sheep against rat GAP-43 were generous gifts of Drs. T. D. Pollard (Johns Hopkins Medical School, Baltimore, MD) and L. I. Benowitz (Harvard Medical School, Boston, MA), respectively.

Extraction and Partial Purification of myr 1

Extraction of myr 1. To test the extractability of myr 1a, two frozen brains (−70°C) from 2-d old rats were thawed and homogenized in 5 ml of 0.32 M sucrose, 5 mM Hepes pH 7.4 with a Dounce homogenizer. The homogenate was centrifuged in a TL-100 ultracentrifuge (Beckman Instruments, Palo Alto, CA) at 75,000 rpm for 15 min (rotor TLA 100.2). Pellets were resuspended in 10 mM Hepes pH 7.4, 10 mM $MgCl_2$, and 100, 200, or 400 mM NaCl, respectively. To these samples was added either 5 mM ATP, 15 mM CHAPS or nothing. One sample was extracted with 10 mM Hepes pH 7.4, 400 mM NaCl and 10 mM Na-pyrophosphate. After centrifugation (75,000 rpm, 15 min) supernatants were analyzed for extracted myr 1a by immunoblotting.

To test for ATP-dependent extractability of myr 1b and 1c, adult rat lung tissue was homogenized in a Dounce homogenizer in 0.32 M sucrose, 5 mM Hepes pH 7.4 and centrifuged at 16,000 rpm in a Sorvall SS34 rotor (DuPont-Nemours, Bad Homburg, FRG) for 20 min. Pellets were resuspended in 0.1 M NaCl, 20 mM Hepes pH 7.4, 10 mM $MgCl_2$, 1 mM 2-mercaptoethanol, 0.1 mM EGTA and 0.1% Triton X-100 (buffer A) and centrifuged in a TLA-100.4 rotor at 75,000 rpm for 20 min. Pellets were extracted in buffer A with or without the addition of ATP (5 mM final concentration) and centrifuged as above. Supernatants were analyzed for the presence of myr 1b and 1c by immunoblotting.

Partial Purification of myr 1a. Approximately 340 frozen rat brains (postnatal days 1–7) were homogenized in 500 ml of 0.32 M sucrose, 5 mM Hepes pH 7.4, and centrifuged in a Ti60 rotor at 50,000 rpm for 1 h. The pellet was extracted with 100 mM NaCl, 10 mM $MgCl_2$, 10 mM Hepes pH 7.4 and centrifuged again. The resulting pellet was extracted two more times with the same buffer containing 5 mM ATP. The ATP-extracts were loaded onto a Mono Q (HR 5/5) column (Pharmacia) preequilibrated in 20 mM Hepes pH 7.4, 20 mM NaCl, 2 mM $MgCl_2$, 0.2 mM EDTA and 0.5 mM 2-mercaptoethanol. Applying a linear NaCl gradient (20 mM–1 M) myr 1a eluted as a broad peak starting at 550 mM NaCl. Fractions containing myr 1a were pooled and dialyzed against 50 mM KCl, 10 mM Hepes pH 7.4 and 1 mM dithiothreitol. After dialysis, the sample was loaded onto a 5 ml ADP-agarose column (GIBCO BRL, Eggenstein, FRG) which had been preequilibrated in the dialysis buffer. The column was developed with a KCl gradient (50 mM–1 M) in a total volume of 40 ml, followed by a wash with a buffer containing 0.45 M NaCl, 20 mM Hepes pH 7.4, 50 mM KCl and 1 mM dithiothreitol. Finally, myr 1a was eluted with the wash buffer supplemented with 20 mM $MgCl_2$ and 10 mM ATP. Throughout the purification the protease inhibitors PMSF (0.1 mM) and pepstatin (2 μg/ml) were included.

Partial Purification of myr 1b and 1c from Rat Liver. 50 g of frozen adult rat liver was homogenized in 200 ml of 0.32 M sucrose, 5 mM Hepes pH 7.4 and centrifuged in a SS34 rotor at 18,000 rpm for 30 min. The resulting pellet was extracted with buffer B (320 mM NaCl, 20 mM Hepes pH 7.4, 10 mM $MgCl_2$, 1 mM 2-mercaptoethanol, 0.1 mM EGTA, and 2 mM NaN_3) containing 0.1% Triton X-100 and was centrifuged in a Ti 50.2 rotor at 45,000 rpm for 1 h. The pellet was reextracted with buffer B before being extracted with buffer B supplemented with 5 mM ATP. The ATP-extract was brought to 50% ammonium sulfate and the precipitate was collected, dissolved in a buffer (10 ml) of 50 mM KCl, 20 mM Hepes pH 7.4, 10 mM $MgCl_2$, 0.1 mM EGTA, 0.5 mM 2-mercaptoethanol and 2 mM NaN_3 and dialyzed against the same buffer to yield an actomyosin pellet. The dialysate was centrifuged at 75,000 rpm for 30 min (rotor TLA 100.4). The pellet was extracted twice in buffer C (0.5 M NaCl, 20 mM Hepes pH 7.4, 10 mM $MgCl_2$, 0.1 mM EGTA, 0.5 mM 2-mercaptoethanol, and 2 mM NaN_3) supplemented with 10 mM ATP and centrifuged. The supernatants were combined and concentrated in a Centricon 30 (Amicon, Beverly, MA) before loading onto a Superdex 200 HiLoad (16/60) gel filtration column (Pharmacia) equilibrated in buffer C supplemented with 2 mM ATP. Fractions containing myr 1b and 1c were pooled and dialyzed against low salt buffer (50 mM NaCl, 20 mM Hepes pH 7.4, 5 mM $MgCl_2$, 0.1 mM EGTA, 0.5 mM 2-mercaptoethanol and 2 mM NaN_3) supplemented with 1 mM ATP followed by chromatography over a Mono Q (HR5/5) ion-exchange column (Pharmacia) equilibrated in the low salt buffer. Myr 1b and 1c were eluted with a NaCl gradient and tested for nucleotide-dependent binding to F-actin. All protein purification procedures were performed at 4°C.

Amino Acid Sequence Determination

Partially purified myr 1a was separated by SDS-PAGE and stained with Coomassie blue. The myr 1a protein band was excised, loaded onto a 10–20% gradient SDS-polyacrylamide gel and re-electrophoresed in the presence of *S. aureus* V8 protease (6 μg) as described by Cleveland et al. (9). Digested proteins were electrophoretically transferred to Polyvinylidene difluoride immobilon membrane (Millipore, Eschborn, FRG), stained and sequenced as described by Matsudaira (42), using an Applied Biosystems AB 470A gas-phase sequencer.

Actin Binding

Actin was purified from rabbit skeletal muscle as described by Pardee and Spudich (51). Actin was polymerized at 1 mg/ml in a buffer of 100 mM KCl, 2 mM $MgCl_2$, 20 mM Hepes pH 7.4, 0.5 mM 2-mercaptoethanol and 2 mM NaN_3. Actin binding assays were performed in total volumes of 100 μl containing 5 μM actin. Fractions eluted from the Mono Q column enriched for myr 1a or myr 1b and 1c (see above) were added to the assay mixture. The assay buffer consisted of 20 mM Hepes pH 7.4, 2 mM $MgCl_2$, 0.5 mM 2-mercaptoethanol, 0.4 mM NaN_3, 20 mM KCl and ~300–350 mM NaCl. Samples were incubated on ice for 20–30 min. ATP (final concentration 2 mM) was added from a concentrated stock solution (0.1 M) 5 min before centrifugation. Supernatants and pellets were obtained after centrifugation (rotor TLA 100.2) at 75,000 rpm for 20 min and analyzed for myr 1 protein by immunoblotting and actin by Coomassie blue staining of SDS-gels, respectively.

Tissue Preparation and Subcellular Fractionation

Tissues were homogenized in 1% SDS, 1 mM 2-mercaptoethanol and protein concentrations were determined by a modification (41) of the method of Lowry et al. (38). Alternatively, they were homogenized in 0.32 M sucrose, 5 mM Hepes pH 7.4 and protein concentrations were determined by the method of Bradford (5). Aliquots of the homogenates were immediately placed in SDS gel sample buffer (37) and boiled.

Growth cone fractions were very generous gifts of Drs. S. Helmke and K. Pfenninger (University of Colorado Health Sciences Center, Denver, CO) and were prepared as described (53). A crude preparation of neuronal and glial cell bodies was prepared according to Sbaschnig-Agler et al. (58) and was also a gift of Drs. Helmke and Pfenninger.

Isolation of a myr 1 cDNA-Probe by PCR Amplification

First strand cDNA was synthesized at 42°C using 1 μg poly(A)$^+$ RNA purified from E19 embryonic rat brain tissue, superscript reverse transcriptase (GIBCO-BRL, Gaithersburg, MD) and random hexamer primers. After phenol/chloroform extraction and ethanol precipitation the cDNA was resuspended in 20 μl of H_2O and stored at 4°C. PCR reactions in 50 μl total volume included 2 μl cDNA, PCR buffer (50 mM KCl, 3 mM $MgCl_2$, 100 μg/ml gelatine and 10 mM Tris-HCl pH 8.3), 200 μM each of dATP, dGTP, dCTP, and dTTP, 25 U/ml Taq polymerase (Boehringer, Mannheim, FRG) and 100 pmol of the 32-fold degenerate primer MB16 (sense) 5'CAGAATTCCCICA(C/T)TA(C/T)ATI(C/T)GITG(C/T)ATIAA(A/G)CC 3', containing an EcoRI site and the 16-fold degenerate primer MB22 (antisense) 5' TCGGATCCGGCATI(C/T)(G/A)GAT(G/A)AT(C/T)TT 3' containing a BamHI restriction site. MB16 was derived from the amino acid sequence PHYIRCIKP and MB22 from the amino acid sequence KIFIRMP. The reaction was cycled 40 times (94°C for 1 min, 50°C for 1 min and 72°C for 1 min). The amplified PCR products were separated on a 4% agarose gel (FMC Bioproducts, Rockland, ME; NuSieve GTG 3:1) and fragments of the expected size (318 bp) were isolated and purified using Qiaex Glassmilk (Diagen, Hilden, FRG). The purified fragments were cloned into a dT tailed pBluescript SK+ vector (Stratagene Corporation, La Jolla, CA) as described by Marchuk et al. (40). Selected clones containing a 318 bp fragment were subjected to DNA sequencing. The oligonucleotides were synthesized using a Gene Assembler Plus DNA Synthesizer (Pharmacia, Milwaukee, WI).

PCR Amplification of the myr 1c Isoform

Two PCR-primers corresponding to nucleotide 2237-2254 and nucleotide 2843-2863 of myr 1a which flank the region of divergence between myr 1a and myr 1b were synthesized. Reactions were cycled 30 times (92°C for 1 min, 62°C for 1 min and 72°C for 1 min) using first-strand cDNA from rat kidney as template. The obtained PCR-fragments were cloned and sequenced as described above.

Library Screening

A 1.2 kb cDNA clone was isolated from an embryonic (E18 rat brain) lambda gt10 cDNA library (Clonetech, Palo Alto, CA) after screening with the myr 1 PCR fragment. This clone was used to screen a lambda Zap II cDNA library from adult rat brainstem/spinal cord tissue (Stratagene) and 12 positive clones were isolated out of 2×10^6 recombinants. pBluescript SK plasmids were excised from lambda ZAP II clones according to the instructions of the supplier. The clones Z9A, Z7A, Z30A, Z39A, and Z34A were subjected to DNA sequencing.

DNA Sequencing

Nucleotide sequences were determined by the method of Sanger et al. (57) using double-stranded DNA templates and the USB Sequenase version 2.0 kit (US Biochemicals, Cleveland, OH). For sequencing, a series of nested deletions were constructed by the method of Henikoff (27) with an Erase-a-base kit (Promega Corp., Madison, WI). All sequences were determined for both strands. Nucleotide and amino acid sequences were analyzed with the PC/Gene (IntelliGenetics, Mountain View, CA) and the University of Wisconsin Genetics Computer Group (UWGCG) (14; version 7) software packages. Sequence identities and similarities were determined using the UWGCG program GAP.

RNA Purification and Northern Blot Analysis

Total RNA was prepared according to the Method of Chirgwin et al. (8) and poly(A)$^+$ RNA was isolated using the PolyATract mRNA Isolation System (Promega Corp.). For northern blot analysis, poly(A)$^+$ RNA was electrophoresed in 1% agarose gels containing 2.2 M formaldehyde and transferred onto Hybond-N nylon membranes (Amersham, Braunschweig, FRG). The filters were prehybridized and hybridized at 42°C in 50% formamide, 5× SSC, 50 mM Na-phosphate pH 7, 0.5% SDS, 0.1 mg/ml poly(A) and 0.25 mg/ml denatured salmon sperm DNA. DNA probes were labeled using a random primer labeling kit (Boehringer). Filters were washed (2× SSC, 0.1% SDS at RT, 2 × 30 min, 0.1× SSC, 0.1% SDS at 65°C, 10 min) and exposed to X-Omat AR film (Eastman Kodak Co., Rochester, NY) at −70°C with a Cronex Quanta III intensifying screen (Du Pont, Wilmington, DE).

Miscellaneous Techniques

Gel electrophoresis was performed according to Laemmli (37) and immunoblotting according to Towbin et al. (63). For immunoblotting, primary antibodies were appropriately diluted in 5% nonfat dry milk and 0.1% Triton X-100 as blocking agents followed by ^{125}I-protein A (Amersham) or alkaline phosphatase conjugated secondary antibodies. Bound ^{125}I-protein A was visualized by autoradiography using Kodak XAR-5 x-ray film and intensifying screens. Alkaline phosphatase was visualized by the ProtoBlot system (Promega Corp.). Calmodulin gel and blot overlays were performed as described by Slaughter and Means (59). ^{125}I-calmodulin was purchased from New England Nuclear (Dreieich, FRG).

Results

Identification of myr 1a with Antibodies Directed Against a Conserved Myosin-I Sequence

The rabbit polyclonal antiserum G-371 raised against the 18 amino acid synthetic peptide corresponding to amino acids 611–629 of bovine brush border myosin-I, a well conserved region among the sequenced myosin-Is, reacted with three major proteins of neonatal rat brain on immunoblots (Fig. 1 *a*). Their apparent molecular masses ranged from ~90–130 kD. These molecular weights are well within the known molecular weight range of myosin-Is and above the minimal expected size of ~80 kD corresponding to the size of the myosin motor domain. The protein with an apparent molecular mass of 130 kD was named myr 1a and further investigated.

Myr 1a is Selectively Released by ATP

Characteristic of myosin-I molecules is their selective extractability from tissue homogenates with ATP. Upon homogenization of neonatal rat brain tissues, myr 1a protein was present in the soluble and in the particulate fraction and was only released from the particulate fraction by the addition of ATP (Fig. 1 *b*). High salt concentrations (400 mM NaCl) or detergent (15 mM CHAPS) were not sufficient to solubilize the protein. The addition of pyrophosphate, a compound which solubilizes protozoan myosin-Is efficiently (55), yielded a less efficient extraction of myr 1a. The selective extraction of myr 1a with ATP indicated that myr 1a could indeed represent a myosin-I molecule.

Myr 1a Binds in a Nucleotide-dependent Manner to F-Actin

A further characteristic of actin-based motor molecules is their nucleotide-dependent binding to F-actin. To test for nucleotide-dependent F-actin binding of the myr 1a protein, we partially purified the protein by ATP-extraction and Mono Q ion exchange chromatography (see Materials and Methods). The partially purified myr 1a sedimented together with F-actin in the absence of the nucleotide ATP, but not in the presence of ATP (Fig. 1 *c*), as predicted for a myosin-I motor molecule.

Peptide Sequences Classify myr 1a as a Myosin-I

To obtain peptide sequence information for the myr 1a pro-

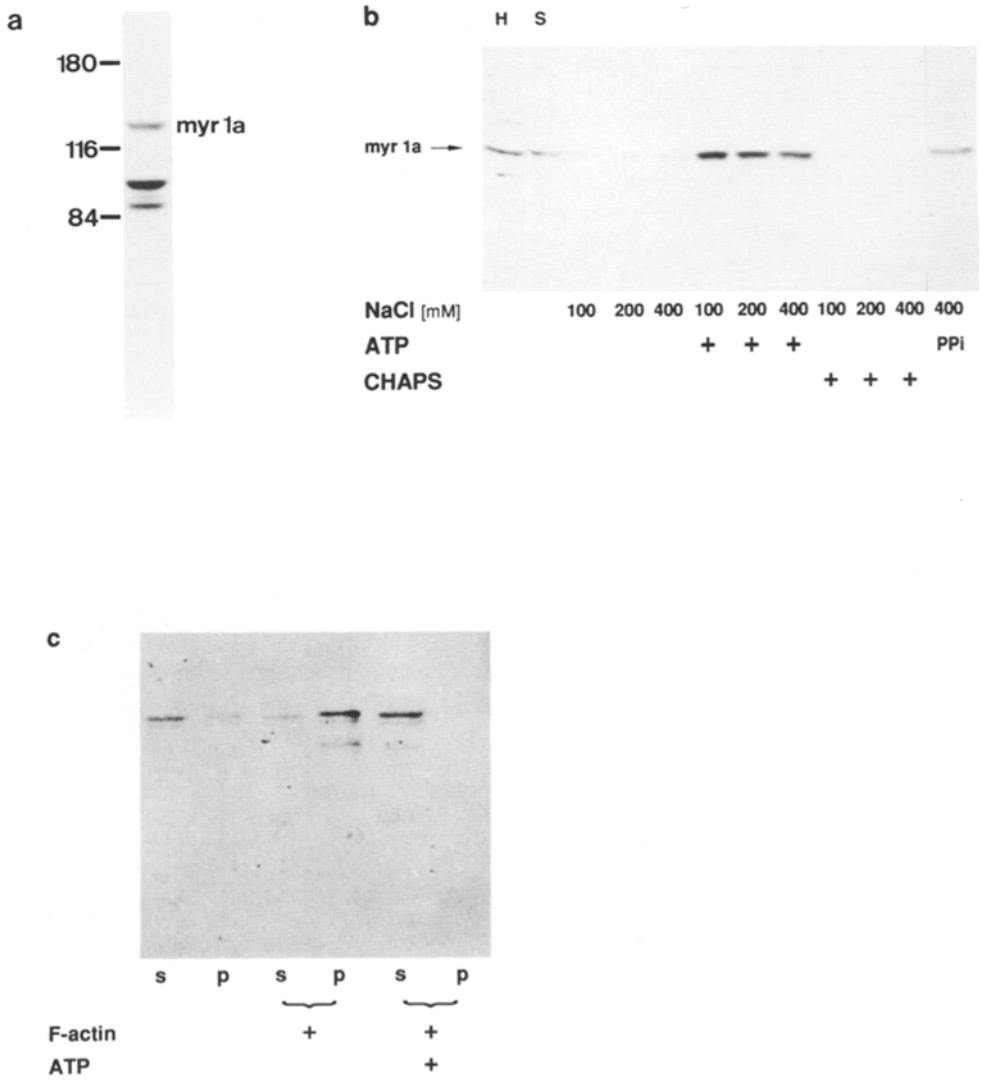

Figure 1. Identification and characterization of myr 1a. (*a*) Immunoblot of neonatal rat brain homogenate with antibody G-371 directed against the myosin-I consensus peptide. The myr 1a band and molecular weight standards (in kD) are indicated. (*b*) ATP-dependent extraction of myr 1a from neonatal rat brain tissue. Neonatal rat brain was homogenized in 0.32 M sucrose (*H*) and centrifuged to obtain supernatant (*S*) and pellet. The pellet was extracted with a buffer containing 10 mM Hepes, pH 7.4, 10 mM $MgCl_2$ and the indicated concentrations of NaCl, either in the absence or presence (+) of ATP (5 mM), CHAPS (15 mM) or pyrophosphate (PP_i, 10 mM). Proteins released into the supernatant were separated on SDS-PAGE, transferred to nitrocellulose and probed with the Tü 30 antibody. The myr 1a band is indicated on the left. (*c*) Nucleotide-dependent binding of myr 1a to F-actin. Partially purified myr 1a from rat brain (see Materials and Methods) was incubated in the absence and presence (+) of actin (5 μM) and ATP (2 mM), respectively. The F-actin binding was assayed by cosedimentation with F-actin. Supernatants (*s*) and pellets (*p*) were analyzed for myr 1a protein by immunoblotting with the G-371 antibody.

tein, further purification of the protein was necessary. ADP-agarose affinity purification and elution with ATP yielded a visible protein band on Coomassie blue stained SDS-gels (Fig. 2). The major contaminants still present in the preparation were conventional myosin, actin and tubulin. After gel purification of myr 1a and cleavage with *S. aureus* protease V8, four internal peptide sequences were derived (see Fig. 5). The peptide sequences AFGNAKTVRNDNSSRFGK-YMDIEFDFKGDPL, FVDKNNDLLYRDLS, and FGR-SKIFIRNPRTLFQLE revealed a high homology to well conserved regions in the myosin-I motor domains. Furthermore, the peptide sequence NKNPKYKKLKDAIEEKII-IAEVVNKIN exhibited a significant homology to sequences in the brush border myosin-I tail domain (see Fig. 7). Taken together, these data strongly suggested that myr 1a is a member of the myosin-I superfamily.

Myr 1a Binds Calmodulin

Brush border myosin-I has been shown to bind calmodulin

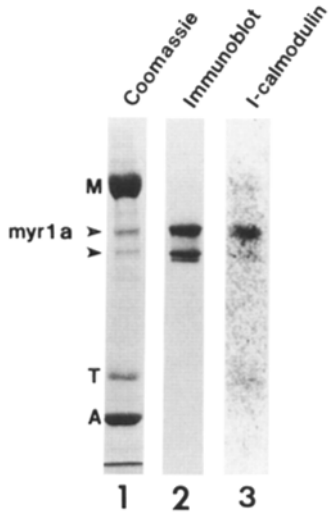

Figure 2. Partial purification of myr 1a and calmodulin binding. Partially purified myr 1a from rat brain (see Materials and Methods) was separated on SDS-PAGE and stained for protein with Coomassie blue (lane *1*); for myr 1a by immunoblotting with G-371 antibody (lane *2*); and probed for calmodulin binding by ^{125}I-calmodulin overlay using 0.1 mM $CaCl_2$ (lane *3*). Major contaminants still present in the preparation were conventional myosin (*M*), tubulin (*T*), and actin (*A*). Myr 1a and a presumed breakdown product are indicated by arrowheads.

in gel overlay assays (20). We therefore tested whether myr 1a was also able to bind calmodulin. Indeed, myr 1a did bind calmodulin both in the presence and absence of calcium ions (Fig. 2, lane *3*, and data not shown).

Myr 1a Expression is Regulated during Neuronal Development

We determined the developmental expression pattern of myr 1a in rat forebrain. As shown in Fig. 3, myr 1a protein levels are regulated during rat forebrain development. The highest levels were found between embryonic day 17 and postnatal day 4 with a marked downregulation in the second week after birth. From there on, myr 1a levels stayed more or less constant during brain ontogeny.

Myr 1a Is Enriched in Purified Growth Cone Particles

To analyze the subcellular distribution of myr 1a in fetal rat brain, we probed subcellular fractions, specifically purified growth cone particles, for the presence of myr 1a (Fig. 4). Myr 1a was significantly enriched in purified growth cone particles as compared to homogenate. In contrast, conventional myosin was clearly not enriched in purified growth cone particles (Fig. 4, lanes *1* and *5*). However, GAP-43, a protein known to be preferentially localized in growth cones (21, 44) was enriched to a greater extent than myr 1a in purified growth cone particles. When purified growth cone particles were further fractionated into a cytoskeletal fraction (detergent insoluble) and a detergent extractable fraction, myr 1a partitioned mainly into the cytoskeletal fraction, like myosin and to a lesser extent also GAP-43 (Fig. 4, lanes *6* and *7*). Myr 1a and GAP-43 both preferentially partitioned into fractions enriched in membranes (perykarya membrane fraction, low speed supernatant, growth cone particles) and they both were less enriched in a fraction rich in soluble proteins (A-fraction). This distribution was in marked contrast to the distribution of conventional myosin. These subcellular fractionation data showed that myr 1a was present in purified growth cone particles and was possibly associated with membranes.

Isolation and Analysis of myr 1 cDNA Clones Reveals Three Splice Forms

We identified cDNA clones corresponding to three alternative myr 1 splice forms, called myr 1a, myr 1b, and myr 1c. Clones for myr 1a (Z7A) and myr 1b (Z9A) were isolated after screening a cDNA library from adult rat brainstem and spinal cord with a specific cDNA probe (see Materials and Methods). Sequencing of the isolated clones revealed that clone Z9A contained the complete open reading frame of myr 1b with part of the 5′ and 3′ noncoding sequence, and clone Z7A contained part (amino acids 683–1,136) of the open reading frame of myr 1a. The complete nucleotide and deduced amino acid sequences of myr 1a are shown in Fig. 5. The nucleotide sequence of the PCR fragment 22/16-1 (see Materials and Methods), that was used for the initial library screening, was identical to the corresponding sequence in these clones (Fig. 5, *arrowheads*). Sequence information of the third splice form, myr 1c, was obtained after PCR amplification of rat kidney cDNA with myr 1 specific primers. The open reading frame of myr 1a–c started with an initiation sequence that was similar to the consensus initiation sequence described by Kozak (35). Nucleotide sequences of the isolated myr 1 clones were identical, except for a short region of diversity between myr 1a–c. The deduced amino acid sequences of the myr 1 splice forms differed at amino acid 795 in that myr 1a contained three imperfect repeats of 29 amino acids, myr 1b two imperfect repeats of 29 amino acids and myr 1c a single copy of the 29 amino acid motif (Fig. 6). In good agreement with the molecular masses determined by SDS-gels (130 kD for myr 1a), the calculated masses from the deduced amino acid sequences were 131,917 D for myr 1a (1,136 amino acids), 128,428 D for myr 1b (1,107 amino acids) and 124,870 D for myr 1c (1,078 amino acids), respectively. The isolated cDNA clones were encoding the four peptide sequences determined by direct amino acid sequencing of purified myr 1 protein (underlined amino acids in Fig. 5). These data demonstrated that the isolated cDNA clones were indeed encoding myr 1. Furthermore, the peptide sequence corresponding to the region recognized by the polyclonal antiserum G-371 was identified at position 618-636 of myr 1a–c (*double underlined*, Fig. 5).

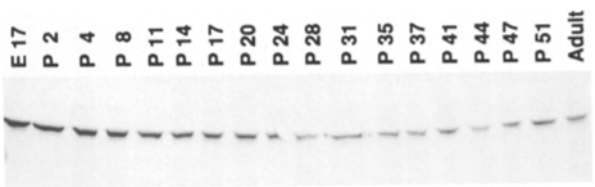

Figure 3. Developmental expression of myr 1a in rat forebrain. Equal amounts of protein from embryonic day 17 (E17), postnatal days 2–51 (P2-51) and adult rat forebrain tissue homogenates were separated on SDS-PAGE and transferred to nitrocellulose. The nitrocellulose was incubated with antibody Tü 30 followed by a secondary antibody coupled to alkaline phosphatase.

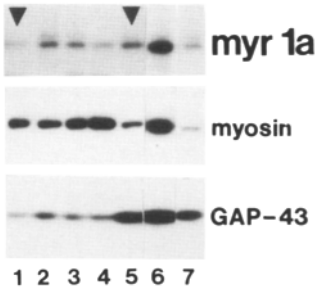

Figure 4. Subcellular distribution of myr 1a in fetal rat brain. Identical immunoblots were reacted with G-371 antibody recognizing myr 1, antibodies against conventional myosin heavy chain (2.17; myosin) and GAP-43. Lanes: (*1*) homogenate; (*2*) perikarya; (*3*) low speed supernatant; (*4*) A-fraction; (*5*) purified growth cone particles; (*6*) pellet; and (*7*) supernatant of purified growth cone particles extracted with 0.5% Nonidet P-40. Equal amounts of protein from each subcellular fraction were separated on SDS-PAGE. The arrowheads highlight the fractions of homogenate and purified growth cone particles for comparison. For the method of subcellular fractionation and nomenclature of subcellular fractions see Pfenninger et al. (53) and Sbaschnig-Agler et al. (58).

Sequence Analysis and Structural Organization of myr 1

Analysis of the deduced myr 1c amino acid sequence revealed an amino-terminal motor domain (amino acids 1–705), a regulatory domain with putative calmodulin/light chain binding

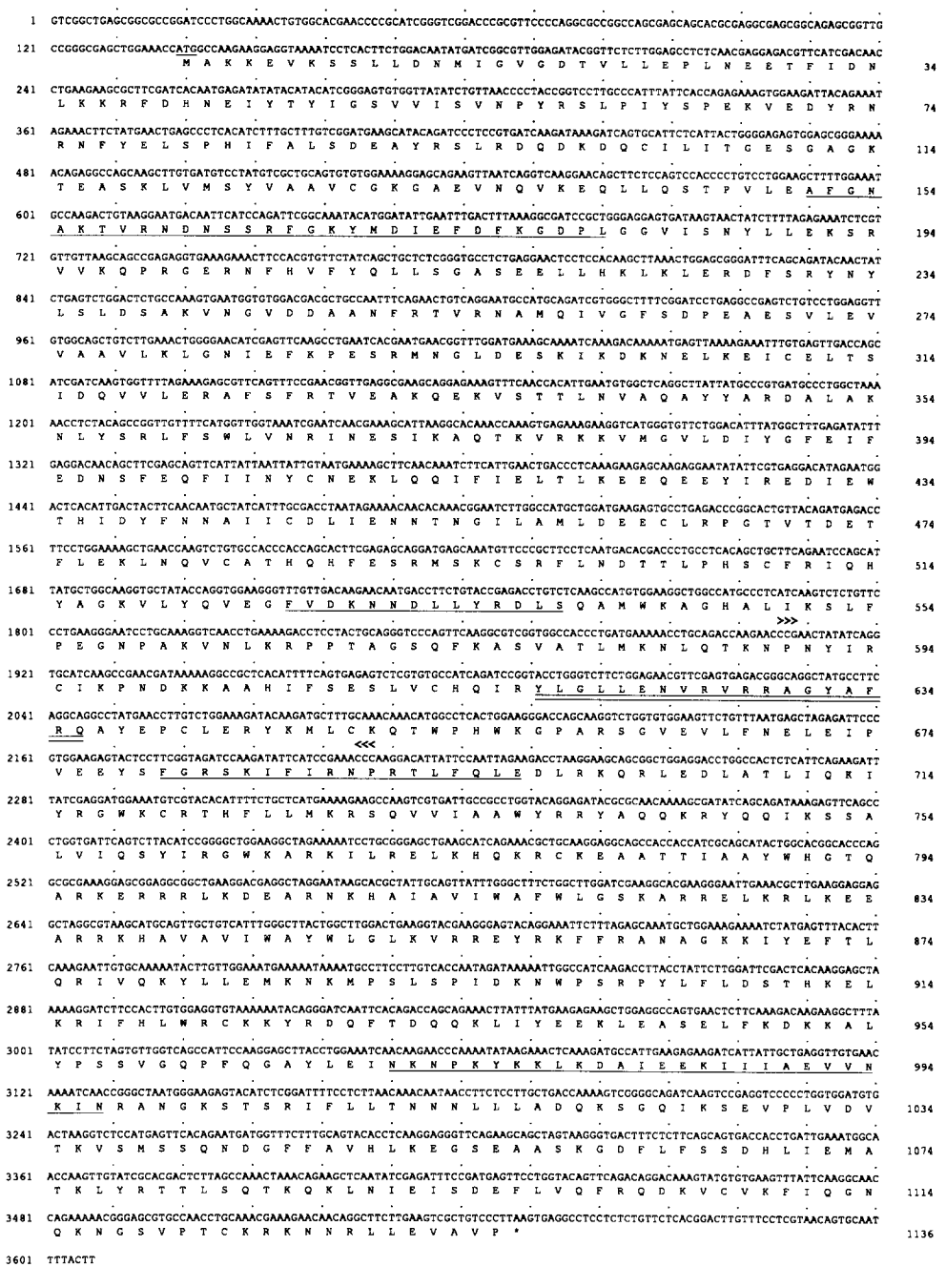

Figure 5. Nucleotide and deduced amino acid sequences of the myr 1a cDNA. The amino acid sequence of myr 1a is shown below the nucleotide sequence. The start codon at positions 139–141 is underlined. Peptide sequences determined by direct amino acid sequencing after cleavage of purified myr 1 protein with *S. aureus* protease as described in Materials and Methods are underlined. The double underlined peptide sequence indicates the corresponding region recognized by the polyclonal antiserum G-371. Arrowheads above the nucleotide sequence mark the ends of the PCR-generated fragment used for the initial library screening. The nucleotide sequence data are accessible through EMBL, GenBank, and DDBJ nucleotide sequence databases under the accession number X68199.

sites (amino acids 706–794) and a carboxy-terminal tail domain (amino acids 795–1078).

The Motor Domain. The amino-terminal motor domain exhibited a consensus ATP-binding site and sequences which are thought to be important for actin binding (Fig. 7). A comparison of the myr 1 motor domain sequence with that of bovine brush border myosin-I indicated that these two domains are highly homologous (66% identity, 78% similarity). With the exception of seven additional amino acids at the very amino terminus of myr 1, the two sequences were aligned without the introduction of gaps.

The Regulatory Domain. The regulatory domain consisted of multiple repeats of putative calmodulin/light chain binding motifs (Fig. 8). Myr 1a–c contained up to six con-

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myr 1a  791 HGTQARKERR RLKDEARNKH AIAVIWAFWL GSKARRELKR LKEEARRKHA VAVIWAYWLG LKVRREYRKF 860
myr 1b  791 HGTQ..... ..........  ..........  ...ARRELKR LKEEARRKHA VAVIWAYWLG LKVRREYRKF 831
myr 1c  791 HGTQ..... ..........  ..........  ..........  ..........  ..........  ..VRREYRKF 802
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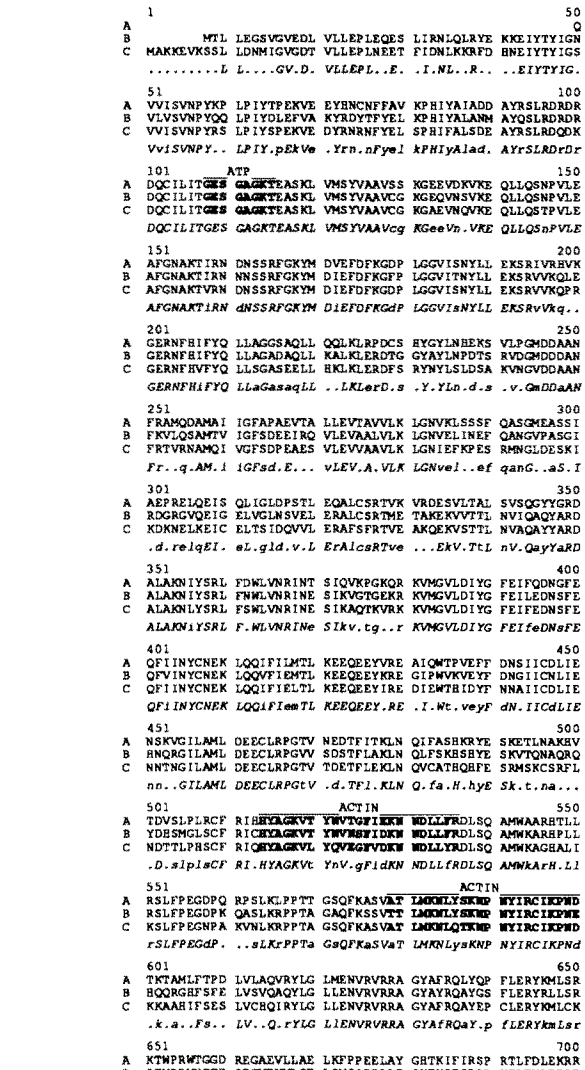
Figure 6. Identification of alternative spliceforms of myr 1. Three alternative splice forms of myr 1, myr 1a–c were identified. Alignment of the deduced myr 1 amino acid sequences revealed that myr 1b exhibits a deletion of 29 amino acids and myr 1c a deletion of 2 × 29 amino acids as compared to myr 1a.



secutive repeats (amino acids 706–867) of the IQ-motif as defined by Cheney and Mooseker (7) (Fig. 8 *A*). This motif has been shown to be responsible for the Ca^{2+}-independent calmodulin binding of the nonmyosin protein neuromodulin (6). Three consecutive repeats of this IQ-motif had previously been identified in brush border myosin-I (7, 61). These three brush border myosin-I repeats shared an identity of 60% (80% similarity) with the first three repeats in myr 1. As in brush border myosin-I, the second repeat in myr 1 was the least similar to the IQ-consensus sequence.

Partially overlapping with the sequence (amino acids 766–774) of the third IQ-motif repeat, the myr 1 regulatory domain contained variable numbers of imperfect 29 residue repeats. These repeats were also fitted into the IQ-motif (Fig. 8 *A*). However, the relation of these repeats to the IQ-motif seemed uncertain, because highly conserved residues of the IQ-motif, like the glutamine (Q) residue and the arginine (R) residue following three residues after the conserved glutamine residue were not conserved in these repeats. These 29 residue repeats were present in one constitutive and two alternatively spliced variants. Individual pairwise comparison of these amino acid repeats revealed 72% identity between the two facultative 29 amino acid repeats (amino acids 795–823 and 824–852) and ~41% between the constitutive (amino acids 766–794) and each of the two facultative 29 amino acid repeats. A 29 amino acid alternative splice insert with a similar sequence (55% identity to the constitutive myr 1 repeat) has been reported for a low abundance isoform of chicken brush border myosin-I (23) (Fig. 8 *B*). All four of these 29 amino acid repeats fit the motif with the consensus sequence XRBXXBXLKXXXRXBXA#XXIXA*WXGXX (*B*, basic residue; *#*, hydrophobic residue; *, aromatic residue) (Fig. 8 *B*). A synthetic peptide corresponding to the chicken brush border myosin-I alternative splice insert sequence has been demonstrated to bind calmodulin (23). Therefore, the 29 amino acid repeats found in myr 1 are likely to be involved in calmodulin/light chain binding. The three alternative splice forms of myr 1 differed in their numbers of 29 amino acid repeats. In total, myr 1a contained six, myr 1b five and myr 1c four putative calmodulin/light chain binding sites.

The Tail Domain. The carboxy-terminal tail domain of myr 1a (~33 kD) which followed the variable regulatory domain was identical in all three splice forms. Like the tail domains of other myosin-I molecules, the myr 1 tail domain exhibited a high number of basic residues (~20%). Specific functions of myosin-I molecules were thought to depend on their individual tail domains. Therefore, it was of particular interest that the tail domain of myr 1 shared significant homology with the tail domain of bovine brush border myosin-I. In this carboxy-terminal domain, 46% of the amino acids

Figure 7. Alignment of avian brush border myosin-I (19) (*A*), bovine brush border myosin-I (28) (*B*) and myr 1c (*C*) heavy chains. The derived consensus sequence is shown below the aligned sequences. Amino acids that are identical in all three sequences are indicated in capital letters and amino acids identical in two of the three sequences in lower case letters. ATP and putative actin binding sites in the motor domain are indicated.

A

Neuromodulin	33	KAATKIQASF RGHITRKKLK GEK	55
myr 1a	706	DLATLIQKIY RGWKCRTHFL LMK	
		RSQVVIAAWY RRYAQQKRYQ QIK	
		SSALVIQSYI RGWKARKILR ELKHQKRCK	
		EAATTIAAYW HGTQARKERR RLKDEARNK	
		HAIAVIWAFW LGSKARRELK RLKEEARRK	
		HAVAVIWAYW LGLKVRREYR KFFRANAGK	867

B

CBBMI (splice insert)		SRRLLRELKV QRRRHLAAST ISAYWKGYQ	
myr 1a	766	ARKILRELKH QKRCKEAATT IAAYWHGTQ	
		ARKERRRLKD EARNKHAIAV IWAFWLGSK	
		ARRELKRLKE EARRKHAVAV IWAYWLGLK	852
		.R+..+.LK. ..R.+.A#.. I.A*W.G..	

Figure 8. Putative calmodulin/light chain binding sites in the regulatory domain of myr 1. (*A*) Alignment of the IQ-motifs present in myr 1a (amino acids 706–867) with the calmodulin binding sequence of neuromodulin (*bold*) (6). The three imperfect 29 residue repeats with homology to the chicken brush border splice insert are underlined. Conserved amino acids are printed in bold type. (*B*) Alignment of the three myr 1a 29 residue repeats (amino acids 766–852) with the 29 residue chicken brush border myosin-I alternative splice insert (23). The derived 29 residue motif consensus sequence is shown below the alignment. Capital letters indicate conserved residues, + indicate conserved basic residues, * conserved aromatic residues, and # conserved hydrophobic residues. Conserved amino acids are printed in bold type.

were identical (67% were similar with the introduction of a single gap of one residue) with the analogous domain in bovine brush border myosin-I. This homology was lower as compared to the homologies found in the motor domain and the overlapping regions of the regulatory domain, but clearly indicated that myr 1 and brush border myosin-I are related proteins.

Expression of myr 1 mRNA in Various Tissues

Northern blot analysis of poly(A)$^+$ RNA from adult (Fig. 9 *A*) and neonatal (Fig. 9 *B*) rat tissues revealed that myr 1 was expressed in most of the investigated tissues. A broad band with an apparent size range of 5.7–6.2 kb was strongly labeled in adult lung, but was barely detectable in adult intestine. Expression of myr 1 was detected in all of the investigated neonatal tissues. Remarkably high levels of transcripts were observed in neonatal brain and lung tissues. In striking contrast to the preferential expression of brush border myosin-I in intestine (19, 32), northern blot data indicated a widespread tissue distribution of myr 1. A faint band of unknown origin below the main band was also detected.

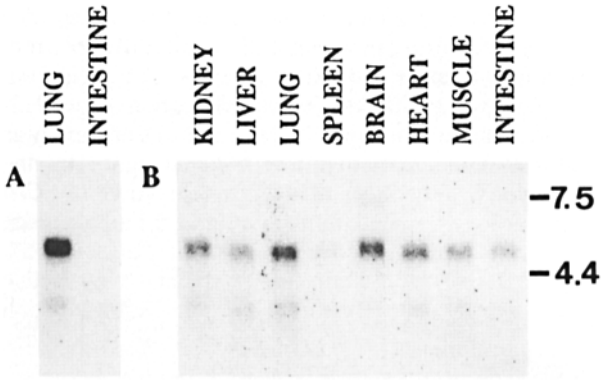


Figure 9. Northern blot analysis of myr 1 mRNA in adult and neonatal rat tissues. Poly(*A*)$^+$ RNA was purified from adult (*A*) and neonatal rat tissues (*B*). Northern blots (1 μg Poly(*A*)$^+$ RNA/lane) were hybridized with the PCR fragment 22/16-1 as described in Materials and Methods. The respective tissues are indicated above each lane. Size standards are in kilobases.

Differential Expression of myr 1 a–c

In agreement with the identification of three myr 1 splice forms by cDNA cloning, we detected three closely spaced protein bands upon immunoblotting with myr 1 specific antibodies (Fig. 10). Several antisera specific for myr 1 were raised (see Materials and Methods and unpublished results). All of these antisera recognized strongly the identical 130-kD protein band in embryonic rat brain originally identified by the consensus myosin-I antibody G-371. The 130-kD protein band was the slowest migrating band observed in any tissue. Because the apparent molecular mass coincided with the deduced molecular weight of myr 1a, we refer to this band as myr 1a. In adult brain regions we observed an additional band running slightly ahead of myr 1a with an apparent molecular mass expected for myr 1b. The detection of myr 1a and myr 1b in brainstem (Fig. 10) is in excellent agreement with the cloning of myr 1a and myr 1b out of a brainstem cDNA library. A third, again slightly faster migrating immunostained protein band was apparent in many tissues but was most prominent in lung tissue (Fig. 10). Because the

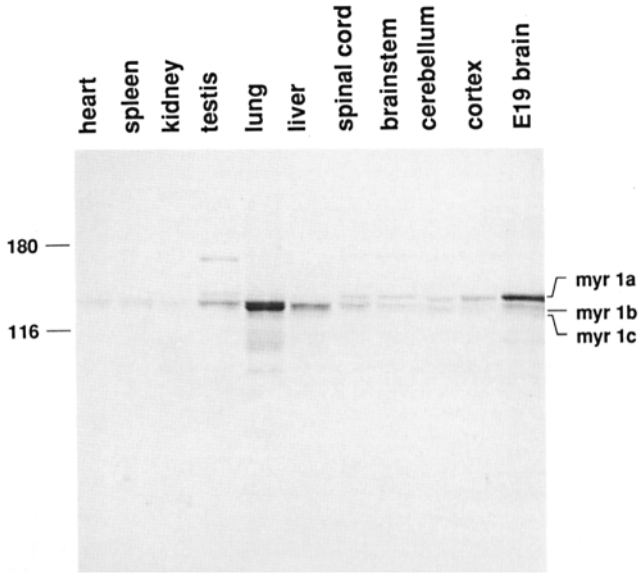


Figure 10. Tissue distribution and differential expression of myr 1 *a–c*. Equal amounts of protein from different tissues were separated on SDS-PAGE and immunoblotted with the antibody Tü 30 followed by a secondary antibody coupled to alkaline phosphatase. The respective positions for myr 1a, myr 1b, and myr 1c are indicated. Molecular weight standards in kDa are shown on the left. Tissues are indicated on top of each lane, with E19 brain denoting embryonic day 19 rat brain.

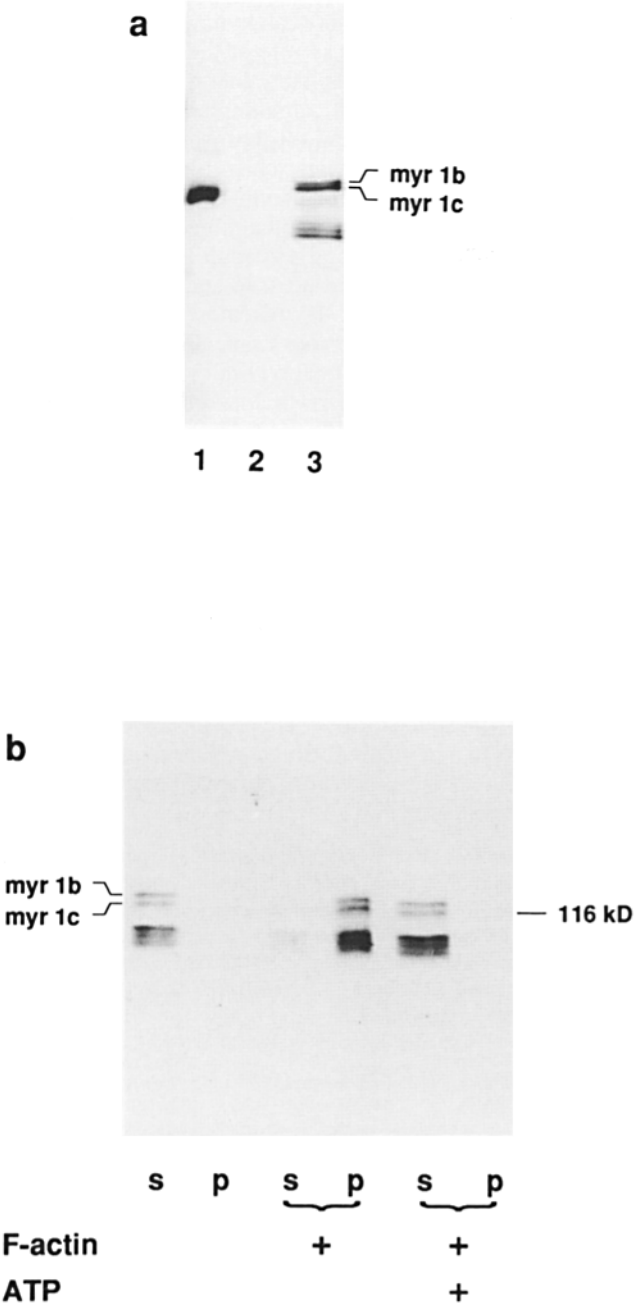


Figure 11. (*a*) ATP-dependent extraction of myr 1b and 1c. Rat lung tissue was homogenized (lane *1*) and extracted in the absence (lane *2*), or presence (lane *3*) of ATP as described under Materials and Methods. Proteins were separated on SDS-PAGE and immunoblotted with the Tü 30 antibody. Myr 1b and 1c recognized by the Tü 30 antibody are selectively extracted by ATP and are marked. (*b*) Nucleotide-dependent binding of myr 1b and 1c from rat liver to F-actin. The myr 1b, 1c protein doublet recognized by the Tü 2 antibody in rat liver was partially purified as described in Materials and Methods and incubated in the absence or presence (+) of actin (5 μM) and ATP (2 mM). The F-actin binding was assayed by cosedimentation with F-actin. Supernatants (*s*) and pellets (*p*) were analyzed for myr 1b and 1c proteins by immunoblotting with the Tü 2 antibody. Molecular weight standard in kD is shown on the right. Breakdown products of myr 1b and 1c are also apparent. Myr 1b and 1c are marked on the left.

molecular mass of this band coincided with the predicted molecular weight for myr 1c and because we detected no additional bands by immunoblotting, we refer to it as myr 1c. Myr 1 a–c were differentially expressed in the various tested tissues. Myr 1a was most prominently expressed in embryonic brain tissue (see for comparison also Fig. 3) and in adult tissues of the various brain regions and in testis. Myr 1b and 1c were most prominently expressed in lung and liver. Lower levels of myr 1b and 1c were observed in many other tissues including intestine (Fig. 10, and data not shown).

The identical three protein bands were also recognized by the myosin-I consensus antibody G-371 as demonstrated by probing proteins specifically immunoprecipitated with the myr 1 specific antibody Tü 2.

Great care was taken to avoid any proteolysis by directly placing the excised tissues in 1% SDS and boiling of the tissues. Furthermore, after longer storage protein degradation could indeed be observed, but always yielded breakdown products with substantially lower apparent molecular masses (see, e.g., Figs. 2 and 11). A tissue dependent differential myr 1 breakdown also seemed extremely unlikely. And finally, the identification of three closely spaced bands was in excellent agreement with the identification of three myr 1 splice forms by cDNA cloning techniques. However, to even further verify that the bands we refer to as myr 1b and myr 1c behaved like myr 1a, we tested whether these proteins are selectively released by ATP and whether they bind in an ATP-dependent manner to F-actin. As shown in Fig. 11 *a*, myr 1b and 1c were selectively released by ATP from lung tissue particulate fractions. We partially purified myr 1b and 1c from rat liver as described in Materials and Methods and tested the two proteins for nucleotide-dependent F-actin binding. Both myr 1b and myr 1c cosedimented with F-actin in the absence of ATP, but not in the presence of ATP (Fig. 11 *b*), demonstrating nucleotide-dependent binding to F-actin.

Discussion

In the present study we describe the identification, characterization and cloning of a mammalian myosin-I, myr 1a–c, which exhibits a remarkable similarity to the brush border myosin-I. The identification was based on an antibody raised against a synthetic peptide with a highly conserved sequence specific for myosin-Is, however, sufficiently different from conventional myosins. The sequence spans the myosin-I sequence analogous to the flexible, exposed region of the SH2-SH1 reactive thiols of conventional myosins. The antibody as described in this report might be of general use for the identification of novel myosin-Is (Bähler, M., 1990. *J. Cell Biol.* 111:167*a*; Behrmann, T., C. Ruppert, R. Kroschewski, S. Kieferle and M. Bähler. 1992. *Eur. J. Cell Biol.* 57: 8 (Suppl. 36). Because modification of SH2 and SH1 reactive thiols in conventional myosins interferes with motor function (for review 49, 65), the antibody might also be of value for studying myosin-I motor function.

Amino acid sequence comparison revealed that rat myr 1 shares a remarkable homology with both the head and tail domains of bovine and chicken brush border myosin-Is. However, as opposed to brush border myosin-I which is expressed predominantly in intestine (19, 32), myr 1 was expressed in a variety of neonatal and adult tissues and was

barely detectable in adult intestine. Myr 1 was most abundant in lung, liver and brain and the expression pattern was not correlated with the abundance of microvilli. Furthermore, myr 1 was found to exist in at least three isoforms which most likely arise by differential splicing.

The existence of a brush border myosin-I multigene family had been suggested based on Southern blot data obtained with a bovine brush border myosin-I probe (28). In addition, antibodies raised against brush border myosin-I were found to react with a 135-kD calmodulin-binding protein from rat fetal brush borders (56), a 130-kD protein from mouse F9 teratocarcinoma cells (15), a 130- and a 140-kD protein from human intestinal cell lines (52) and a 150-kD protein from mouse brain (45). The relationships of these immunogens to myr 1 remain to be determined. Very recently, the purification of a 116-kD myosin-I from bovine adrenal gland and brain with similarities to brush border myosin-I has been described (4). However, the reported bovine 116-kD myosin-I peptide sequences make it very unlikely that this protein is identical to rat myr 1. The mentioned observations raise the possibility that there exists a larger family of brush border type myosin-Is.

Motor activity, calmodulin-binding and lipid-binding have been analyzed in some detail for brush border myosin-I (11, 26, 48, 61). Based on the sequence similarity of myr 1 with brush border myosin-I, it can be postulated that myr 1 shares many properties with brush border myosin-I. We have demonstrated that myr 1 is extracted from tissue homogenates by ATP, that it binds in a nucleotide-dependent manner to F-actin and that it binds calmodulin. It remains to be shown whether myr 1 exhibits actin-activated ATPase activity which is regulated by Ca^{2+}/calmodulin and whether myr 1 binds to negatively charged phospholipids and membranes.

To address the functions of brush border myosin-I, Coudrier et al. (13) expressed the tail domain of chicken brush border myosin-I in cell lines. Interestingly, the brush border myosin-I tail localized in cells without microvilli to organelles and disrupted the Golgi-complex suggesting a role in membrane trafficking. It will be interesting to determine whether overexpression of the brush border myosin-I tail interferes with the function of myr 1.

We have demonstrated that myr 1a can bind calmodulin and therefore myr 1 function is likely to be regulated by Ca^{2+}/calmodulin. Three repeats of a Ca^{2+}-independent calmodulin binding motif (IQ-motif, 7) were detected in myr 1. Overlapping with the third repeat was a second putative calmodulin/light chain binding motif, the 29 residue motif. Myr 1 splice forms differ in their numbers of the 29 residue motif in the regulatory domain. This motif shares a considerable homology with a 29 residue insert at the analogous position in chicken brush border myosin-I (23). Interestingly, a synthetic peptide of the brush border myosin-I insert sequence has been demonstrated to bind calmodulin (23). Therefore, the variable numbers of 29 residue repeats in myr 1 might lead to different numbers of calmodulin/light chain binding sites. The 29 residue motif does not fit well into the IQ-motif which binds calmodulin in the absence of Ca^{2+}. However, the 29 residue motif does also not conform to the highly basic amphipathic α-helix motif for calcium-dependent calmodulin binding sites (50). It contains a high number of basic and hydrophobic residues which are predicted to form a helical secondary structure. Further studies are needed to clarify whether this motif is a calmodulin/light chain binding site. Whatever the precise role of the 29 residue motif will turn out to be, it seems likely that this motif serves a regulatory function. Several calmodulin-binding sequences have also been shown to bind to phospholipid vesicles (16, 61) making the calmodulin/light chain binding motifs possible mediators between motor activity and cargo interaction.

Myr 1 is the first myosin-I motor molecule with a widespread distribution in neonatal and adult tissues. Individual members of myr 1 exhibited a tissue and developmentally regulated expression. Myr 1a was most prominently expressed in the developing nervous tissue. Furthermore, upon subcellular fractionation of fetal rat brain, myr 1a was found to be enriched in purified growth cone particles. Although the relative enrichment was lower than for GAP-43, an axonal growth cone marker protein (21, 44), this result does provide evidence for the presence of myr 1a in growth cones. Myr 1a might possibly be associated with a membranous structure since purified growth cone particles are enriched in membranous structures (53). However, in situ immunolocalization studies are required to determine its exact localization.

Myr 1b and 1c were predominantly expressed in adult non-neuronal tissues, e.g., lung and liver. The differential expression of the myr 1 splice forms could provide a specific means of adaptation to a particular cellular environment since they differ only in their numbers of putative calmodulin/light chain binding sites.

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