

# Rat myr 4 Defines a Novel Subclass of Myosin I: Identification, Distribution, Localization, and Mapping of Calmodulin-binding Sites with Differential Calcium Sensitivity

Martin Bähler, Ruth Kroschewski, Hanns-Eugen Stöffler, and Thomas Behrmann

Friedrich-Miescher Laboratorium der Max-Planck Gesellschaft, D-72076 Tübingen, Germany

**Abstract.** We report the identification and characterization of myr 4 (myosin from rat), the first mammalian myosin I that is not closely related to brush border myosin I. Myr 4 contains a myosin head (motor) domain, a regulatory domain with light chain binding sites and a tail domain. Sequence analysis of myosin I head (motor) domains suggested that myr 4 defines a novel subclass of myosin I's. This subclass is clearly different from the vertebrate brush border myosin I subclass (which includes myr 1) and the myosin I subclass(es) identified from *Acanthamoeba castellanii* and *Dictyostelium discoideum*. In accordance with this notion, a detailed sequence analysis of all myosin I tail domains revealed that the myr 4 tail is unique, except for a newly identified myosin I tail homology motif detected in all myosin I tail sequences.

The Ca<sup>2+</sup>-binding protein calmodulin was demonstrated to be associated with myr 4. Calmodulin binding activity of myr 4 was mapped by gel overlay assays to the two consecutive light chain binding motifs (IQ motifs) present in the regulatory domain. These two binding sites differed in their Ca<sup>2+</sup> requirements

for optimal calmodulin binding. The NH<sub>2</sub>-terminal IQ motif bound calmodulin in the absence of free Ca<sup>2+</sup>, whereas the COOH-terminal IQ motif bound calmodulin in the presence of free Ca<sup>2+</sup>. A further Ca<sup>2+</sup>-dependent calmodulin binding site was mapped to amino acids 776–874 in the myr 4 tail domain. These results demonstrate a differential Ca<sup>2+</sup> sensitivity for calmodulin binding by IQ motifs, and they suggest that myr 4 activity might be regulated by Ca<sup>2+</sup>/calmodulin.

Myr 4 was demonstrated to be expressed in many cell lines and rat tissues with the highest level of expression in adult brain tissue. Its expression was developmentally regulated during rat brain ontogeny, rising 2–3 wk postnatally, and being maximal in adult brain. Immunofluorescence localization demonstrated that myr 4 is expressed in subpopulations of neurons. In these neurons, prominent punctate staining was detected in cell bodies and apical dendrites. A punctate staining that did not obviously colocalize with the bulk of F-actin was also observed in C6 rat glioma cells. The observed punctate staining for myr 4 is reminiscent of a membranous localization.

**D**IRECTED movements along actin filaments are thought to be important for intracellular trafficking and cell shape changes. The motor molecule myosin converts chemical energy into directed mechanical force along actin filaments. Myosin (conventional myosin or myosin II) has been demonstrated to function in muscle contraction, cytokinesis, and capping of surface receptors (15, 33, 38, 49). However, it is difficult to envision one motor molecule being responsible for all actin-dependent motility phenomena. Recent evidence suggested that there exists a superfamily of myosin molecules (7, 26). All members of the superfamily share a characteristic motor domain with ATP and F-actin binding sites that defines them as myosins. Further, all known myosins contain variable numbers of light chains. In

addition, they exhibit different tail domains that are postulated to specify the respective function(s) of each myosin molecule (for review see references 6, 28, 50). Myosin I molecules (class I myosins) were first identified in *Acanthamoeba castellanii* and close relatives have been cloned from *Dictyostelium discoideum* (34–37, 44, 51, 59, 60). Until very recently, only a single myosin I from vertebrate tissue, known as brush border myosin I, had been characterized and cloned (for review see reference 6). However, biochemical data from various vertebrate tissues pointed to the presence of a family of myosin I molecules (4, 12, 42). Our laboratory has reported the characterization and cloning of a ubiquitous myosin I from rat that is related to brush border myosin I. This myosin I molecule exists in three splice forms, named myr 1 a-c (53). Partial sequences of two additional vertebrate myosin I molecules have also been presented (39, 56).

Address all correspondence to Dr. Martin Bähler, Friedrich-Miescher-Laboratorium, Spemannstr. 37-39, D-72076 Tübingen, Germany.

The myosin I molecules from vertebrate tissues identified so far have associated as light chains several molecules of the calcium-binding protein calmodulin (4, 46). The calmodulin molecules are thought to bind at the motor/tail domain junction (24, 32). In this region, all myosins contain one or more copies of an imperfect repeat of  $\sim 23$  amino acids, termed the IQ motif (6). It has been demonstrated for brush border myosin I and a myosin I isolated from adrenal gland that the actin-activated ATPase activity is modulated by the second messenger calcium (4, 9, 14, 58). Moreover, elevation of free calcium ion concentrations in vitro lead to the partial dissociation of calmodulin molecules from brush border myosin I and to the inactivation of its motor activity (9, 10). Therefore, the analysis of light chain/calmodulin binding could provide insight into the regulation of motor activity and more generally into the molecular mechanisms involved in regulating myosin function. Alternatively, unconventional myosins may be responsible for proper subcellular localization of calmodulin, as recently demonstrated for the *Drosophila* ninaC protein (52).

Several myosin I molecules have been localized to membranes (2, 3, 23, 46, 62), suggesting a role for myosin I molecules in membrane traffic. *Acanthamoeba* myosin IA, IB, and IC were localized differentially to specific membrane domains. Myosin IA was found in the cell cortex, beneath phagocytotic cups, and in association with small cytoplasmic vesicles. Myosin IB was associated with the plasma membrane, large vacuole membranes, and phagocytotic membranes (2). Myosin IC localized to the plasma membrane, large vacuole membranes, and the contractile vacuole membrane (3). All three myosin I's were also detected to varying degrees in the cytoplasm, suggesting the possibility that the association with membranes is under physiological regulation. In Dictyostelium, myosin I has been localized at the leading edge of migrating cells and in phagocytotic cups (22). Vertebrate brush border myosin I forms lateral links between the plasma membrane and the actin bundle of the microvilli (11, 46), and may also be associated with small vesicles (18, 20). A mammalian myosin I has been localized to filopodia, lamellipodia, and fine punctae in the perinuclear region (61). In accordance with the in situ localization at membranes, in vitro biochemical experiments demonstrated a high affinity interaction of *Acanthamoeba* myosin I's and brush border myosin I with anionic phospholipids and membrane preparations (1, 2, 29, 47).

To explore a potential involvement of myosin I molecules in membrane trafficking in nervous tissue, we attempted to identify myosin I molecules expressed in this tissue. In this report, we describe the identification and characterization of a novel mammalian myosin I called myr 4. It was identified with a previously described antibody raised against a conserved sequenced in myosin I molecules (53). Cloning and sequencing of myr 4 revealed that it does not belong to the brush border myosin I subclass, but it defines a new subclass of myosin I molecules. Myr 4 was demonstrated to bind calmodulin, and two calmodulin-binding sequence motifs with differential  $\text{Ca}^{2+}$ -sensitivity were identified. Analysis of all known myosin I tail sequences led to the identification of a conserved myosin I tail homology motif. Myr 4 was found to be expressed in many cell lines and tissues. Its expression in the brain was developmentally regulated. Localization studies suggested an organellar localization of myr 4.

## Materials and Methods

### Purification of myr 4

60–65 freshly excised rat brains were homogenized in 600 ml of 0.32 M sucrose, 5 mM Hepes, pH 7.4, using a motor driven Dounce homogenizer. The homogenate was centrifuged in a Sorvall SS34 rotor (Du Pont-Nemours, Bad Homburg, Germany) at 16,000 rpm for 30 min. The pellets were resuspended in  $\text{H}_2\text{O}$  to a volume of 400 ml and rehomogenized in a tight-fitting Dounce homogenizer. After centrifugation at 50,000 rpm for 1 h (Ti 60; Beckman Instruments, Inc., Fullerton, CA), the obtained pellets were resuspended in 0.2 M NaCl, 15 mM CHAPS, 20 mM Hepes, pH 7.4, 1 mM EDTA, and 0.1 mM EGTA. The suspension was centrifuged as above and the pellets were extracted two more times with the identical buffer. The final pellets were resuspended in either 0.2 M NaCl, 15 mM CHAPS, 20 mM Hepes, pH 7.4, 10 mM  $\text{MgCl}_2$ , or 0.4 M NaCl, 20 mM Hepes, pH 7.4, and 20 mM  $\text{MgCl}_2$  (the latter buffer yielded a better extraction efficiency for myr 4, but caused a higher level of contamination with conventional brain myosin). To this suspension we added 0.1 M ATP, pH 7.2, to a final concentration of 5 mM. After centrifugation, the pellets were reextracted. To the pooled supernatants we added a saturated solution of ammonium sulfate (pH 7.2, 0.1 mM EGTA) to a final concentration of 55%. The suspension was centrifuged in a GSA rotor (Sorvall) at 13,000 rpm for 30 min. The pellets were resuspended in and dialyzed against a buffer of 50 mM NaCl, 20 mM Hepes, pH 7.4, 1 mM  $\text{MgCl}_2$ , 1 mM dithiothreitol, 0.1 mM EGTA, and 2 mM  $\text{NaN}_3$ . During dialysis, a precipitate formed. The precipitate was collected by centrifugation for 1 h in a Ti 60 rotor at 50,000 rpm. The pellets were resuspended in dialysis buffer supplemented with 20 mM  $\text{MgCl}_2$ , and myr 4 was extracted twice with ATP (final concentration = 10 mM). The supernatants were loaded onto a column (FPLC Mono Q; Pharmacia, Freiburg, Germany) pre-equilibrated in 20 mM Hepes, pH 7.4, 20 mM NaCl, 2 mM  $\text{MgCl}_2$ , 0.2 mM EDTA, and 0.5 mM 2-mercaptoethanol. The Mono Q column was developed with a linear salt gradient and myr 4 eluted at an approximate ionic strength of 200–250 mM NaCl, just ahead of the bulk of conventional myosin. Fractions containing myr 4 were pooled, diluted 1:1 with  $\text{H}_2\text{O}$ , and loaded onto a 5-ml ADP-agarose column (GIBCO BRL, Gaithersburg, MD), pre-equilibrated in a buffer of 50 mM KCl, 20 mM Hepes, pH 7.4, and 1 mM dithiothreitol. The column was developed with a linear salt gradient (0.05–1 M KCl) in a total volume of 40 ml, followed by a wash with buffer containing 0.45 M NaCl, 20 mM Hepes, pH 7.4, 50 mM KCl, and 1 mM dithiothreitol. Finally, myr 4 was eluted with the same buffer supplemented with 20 mM  $\text{MgCl}_2$  and 10 mM ATP.

When the initial extraction from the tissue was performed in high salt buffer (0.4 M NaCl), conventional myosin was a prominent contaminant of the ADP-agarose eluate. Conventional myosin and myr 4 were separated by gel filtration chromatography on a Superose 6 column (Pharmacia) run in a buffer of 0.5 M NaCl, 20 mM Hepes, pH 7.4, 10 mM  $\text{MgCl}_2$ , 5 mM ATP, 0.1 mM EGTA, and 2 mM  $\text{NaN}_3$ .

### Amino Acid Sequence Determination

Purified myr 4 was separated by SDS-PAGE, stained with Coomassie blue, and excised from the gel. It was reelectrophoresed on a 10–20% gradient SDS-polyacrylamide gel in the presence of *Staphylococcus aureus* V8 protease (6  $\mu\text{g}$ ) as described by Cleveland et al. (8). Digested peptides were electrophoretically transferred to a polyvinylidene difluoride immobilon membrane (Millipore Corp., Bedford, MA), stained, and sequenced as described by Matsudaira (45) using a gas phase sequencer (Applied Biosystems, Inc., Foster City, CA).

### Actin Binding

Actin was purified from rabbit skeletal muscle as described by Pardee and Spudich (48) and stored as G-actin at  $-70^\circ\text{C}$ . Actin (1 mg/ml) was polymerized in a buffer of 100 mM KCl, 2 mM  $\text{MgCl}_2$ , 20 mM Hepes, pH 7.4, 0.5 mM 2-mercaptoethanol, and 2 mM  $\text{NaN}_3$ . F-actin-binding assays were performed in total volumes of 50 or 100  $\mu\text{l}$  containing 5  $\mu\text{M}$  actin. Fractions eluting from the Mono Q column (see above) enriched in myr 4 were added to the assay mixture. The assay buffer consisted of 20 mM Hepes, pH 7.4, 2 mM  $\text{MgCl}_2$ , 0.5 mM 2-mercaptoethanol, 0.4 mM  $\text{NaN}_3$ , 20 mM KCl, and  $\sim 175$  mM NaCl contributed by the myr 4 column fractions. Samples were incubated for 20–30 min at  $4^\circ\text{C}$ . ATP (final concentration = 2 mM) was added from a concentrated stock solution (100 mM) 5 min before centrifugation. Supernatants and pellets were obtained after centrifugation in

a TL-100 ultracentrifuge (rotor TLA 100.2; Beckman Instruments, Inc., Fullerton, CA) at 75,000 rpm for 20 min, and were analyzed for myr 4 content by immunoblotting.

### Isolation, Sequencing, and Analysis of myr 4 cDNAs

Degenerate sense and antisense primers were deduced from the determined peptide sequences FHGVKNM (primer 1) and KDKYMNV (primer 2), and EcoRI restriction sites were added at their 5'-ends. A cDNA fragment was amplified by the polymerase chain reaction using these primers (10  $\mu$ M primer 1, 3  $\mu$ M primer 2) and first-strand cDNA from rat brain poly (A)<sup>+</sup> RNA (0.2  $\mu$ g) reverse transcribed with a cDNA cycle kit (Invitrogen, San Diego, CA). 30 cycles (1 min at 94°C, 1 min at 50°C, and 3 min at 72°C) were run for amplification. The amplified PCR product was purified from low-melt agarose, digested with EcoRI, and subcloned in pBluescript KS vector (Stratagene, La Jolla, CA). The subcloned PCR fragment (nucleotides [nt]<sup>1</sup> 2443–2775) was labeled with [ $\alpha$ -<sup>32</sup>P]dCTP (Amersham Corp., Arlington Heights, IL) by a random primed labeling method (Boehringer Mannheim, Mannheim, Germany). With this probe,  $2 \times 10^6$  recombinants of an adult rat brain  $\lambda$ -ZAP-II cDNA library (generous gift of Dr. C. Garner, Zentrum für Molekulare Neurobiologie, Hamburg, Germany) were screened, and two independent clones (called 6 and 21a) were isolated. Hybridization and washing were performed according to standard procedures (54). The pBluescript plasmids were excised from the  $\lambda$ -ZAP-II phages according to the in vivo excision protocol of the supplier (Stratagene). The clones 6 and 21a were subjected to DNA sequencing. Since clones 6 (nt 256–3761) and 21a (nt 1741–3480) did not contain the entire coding region of myr 4, a rat brainstem/spinal cord  $\lambda$ -ZAP-II library (Stratagene) was screened with a fragment (nt 256–642) derived from clone 6. This screen yielded eight independent clones. Clone 34b (nt 1–530) derived from this screen was completely sequenced.

### DNA Sequencing

Nucleotide sequences were determined by the method of Sanger et al. (55) using double-stranded DNA templates and the Sequenase version 2.0 kit (U.S. Biochemical Corp., Cleveland, OH). For sequencing, a series of nested deletions were carried out according to the method of Henikoff (30) with an Erase-a-Base kit (Promega Corp., Madison, WI). All sequences were determined from both strands of the cDNA. Nucleotide and amino acid sequences were analyzed with the PC-Gene software package (Intelligenetics, Mountain View, CA) and the University of Wisconsin Genetics Computer Group (reference 16, version 7.0) software package.

### Cloning and Expression of Fusion Proteins

Myr 4-specific primers with added recognition sequences of restriction endonucleases were used to amplify defined myr 4 fragments by PCR. PCR reactions were run through 30 cycles (1 min at 92°C, 1 min at 60°C, and 1 min at 74°C). PCR fragments were separated on 1% agarose gels and purified with glassmilk (Qia-Ex; Diagen, Hilden, Germany). The isolated fragments were cloned into pUC18 using the sure clone ligation kit (Pharmacia) and subcloned into the pIH-902 expression vector (New England Biolabs, Schwalbach, Germany). The three fragments encompassing amino acids 500–699, 500–721, and 500–743 were cloned into the BamHI and HindIII cloning sites of pIH-902. Fragments encompassing amino acids 722–777, 743–777, and 810–876 were cloned into the EcoRI and PstI cloning sites, whereas the fragments encoding amino acids 743–898 and 810–898 were cloned into the EcoRI and XbaI cloning sites. The fragment encompassing amino acids 747–858 was cloned into the EcoRI site of the expression vector pMalCRI (New England Biolabs). The fusion protein encoding amino acids 776–1006 was constructed by ligating a PstI/HindIII fragment of clone 21a into the corresponding restriction sites of pIH-902. Nested deletions starting from the 3'-end of the subcloned 21a cDNA were generated to yield eight successively more truncated fusion proteins. Their 3'-sequences were determined by DNA sequencing.

The plasmids coding for the various fusion proteins were transformed into *Escherichia coli*, DH5 $\alpha$  strain and cells were grown in Terrific Broth medium to an OD<sub>600</sub> = 0.4. Expression of fusion proteins was induced by addition of isopropyl- $\beta$ -D-thiogalactopyranosid (0.3 mM). After 3 h of incubation, cells were harvested and dissolved in SDS gel sample buffer and boiled for 3–5 min.

1. Abbreviation used in this paper: nt, nucleotides.

### Calmodulin-binding Assay

Approximately equal amounts of fusion proteins were separated by 10% SDS-PAGE. Gels were overlaid with <sup>125</sup>I-calmodulin (purchased from New England Nuclear, Dreieich, Germany) in the presence or absence of free calcium (0.1 mM CaCl<sub>2</sub> and 3 mM EGTA, respectively) as described by Slaughter and Means (57). The dried gels were exposed to Kodak X-Omat AR film at -70°C with an intensifying screen (Cronex Quanta III; Du Pont, Wilmington, DE).

### Generation of myr 4-specific Antibodies

The synthetic peptide GTFVVPVANELKRKDKYMNC (amino acids 839–856) was coupled to keyhole limpet hemocyanin with *m*-maleimidobenzoyl sulfosuccinimide ester (Pierce Chemical Co., Rockford, IL) and injected into rabbits (Tü 12 and Tü 13) were affinity purified over the myr 4 fusion protein MBP/21a tail (amino acids 776–1006) coupled to resin (AminoLink; Pierce Chemical Co.). The fusion protein MBP/21a tail was expressed in *E. coli* and purified from washed inclusion bodies by preparative SDS-PAGE. The MBP/21a tail fusion protein band was made visible by copper staining (41), cut out of the gel, destained, frozen in dry ice, and minced in a mortar. Protein was eluted from the minced gel (1% SDS, 0.1 mM EGTA, and 50 mM EDTA), concentrated (Centriprep-30; Amicon, Beverly, MA), and finally pelleted with an equal volume of cold acetone in a dry ice/ethanol bath and washed once with 50% acetone. The dried protein pellet was resuspended in 5 M urea, 0.1 M NaHCO<sub>3</sub>, pH 8, and coupled to AminoLink resin (Pierce Chemical Co.) according to the instructions of the manufacturer. Antibodies were affinity purified as described (53). Antibody Tü 14 was raised against fusion protein QE8/21a, which was constructed by cloning cDNA clone myr 4/21a (amino acids 513–1006) over BamHI and HindIII sites into the His-tag pQE8 vector (Qiagen, Chatsworth, CA). The bacterial fusion protein was affinity purified under denaturing conditions over Ni-NTA resin (Qiagen), as described by the manufacturer. Antisera Tü 14 was affinity purified on the MBP/21a tail resin as described above.

### Cell Culture

Madin-Darby canine kidney cells and HepG2 cells (obtained from Dr. Iris Geffen, Biozentrum, Basel, Switzerland), as well as NMU cells (American Type Culture Collection [ATCC], Rockville, MD), were grown in MEM medium, 10% fetal calf serum. C6 cells (obtained from Dr. B. Hamprecht, Tübingen, Germany), Neuro-2A (Dr. Elisabeth Pollerberg, Physiologische Chemie, Max-Planck Institut, Tübingen) and COS cells (Dr. Christine Dreyer, Max-Planck Institut, Tübingen) were grown in DME, 10% fetal calf serum. NIH 3T3 cells (Dr. Iris Geffen) were cultured in DME and 7.5% fetal calf serum; A1T20 cells (ATCC) in Ham's F10 with 10% horse serum and 2.5% fetal calf serum; PC12 cells (ATCC) in RPMI 1640, 10% horse serum, and 5% fetal calf serum; IC-21 cells (ATCC) in RPMI 1640 and 10% fetal calf serum; P388D<sub>1</sub> cells (ATCC) in RPMI 1640 and 15% fetal calf serum. All cells were grown at 37°C in a humidified 10% CO<sub>2</sub> atmosphere.

### Immunofluorescence

For indirect immunofluorescence labeling, cells were washed in PBS and fixed in either 3 or 4% paraformaldehyde in PBS. After quenching with 0.1 M glycine/PBS, cells were permeabilized for 10 min in 0.05% saponin. Primary and secondary antibodies were appropriately diluted in 10% normal goat serum/PBS. Secondary goat anti-rabbit antibodies coupled to rhodamine or Cy3 were purchased from Dianova (Hamburg, Germany), fluorescein-coupled phalloidin was from Molecular Probes (Eugene, OR), respectively. To remove unbound antibodies, cells were rinsed several times with PBS and then mounted (15% moviol and 30% glycerol dissolved in PBS, pH 8).

Immunostaining of frozen rat brain sections was performed as follows: rats were anesthetized with 7% chloralhydrate, transcardially perfused with PBS, followed by fixative consisting of 4% paraformaldehyde in PBS. The brain was excised, equilibrated in 30% sucrose/PBS, molded in Tissue Tek (Miles Inc., Elkhart, IN), and frozen in liquid nitrogen. Cryosections (10  $\mu$ m) cut at -20°C in a FrigoCut (model 2700; Reichert und Jung, Nussloch/Heidelberg, Germany) were adsorbed onto glass slides pretreated with 1% gelatin, 0.05% (NH<sub>4</sub>)CrO<sub>4</sub>. Sections were incubated overnight at 4°C with affinity-purified myr 4 antibodies appropriately diluted in PBS, 10% goat serum, 0.5% Triton X-100 followed by three washes with PBS. After incubation with goat anti-rabbit Cy3 (1:500) for 1–2 h at room temperature and three washes with PBS, sections were mounted as described above.

## Results

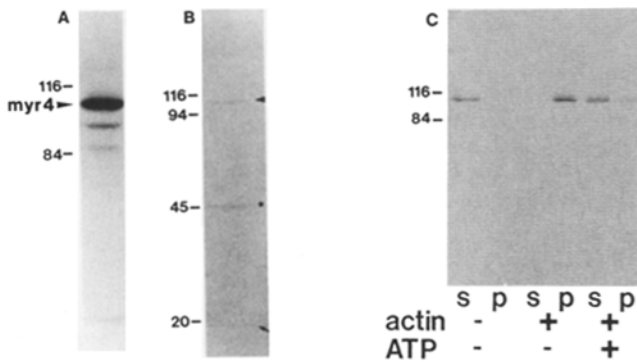
### Identification and Characterization of myr 4

We used our previously described myosin I consensus antibody (53) to probe adult rat brain tissue for novel myosin I(s). In this tissue, the consensus antibody reacted strongly with a protein of 110 kD (Fig. 1 A). In addition, it also recognized a faint band at 130 kD, previously identified as myr 1a (53), and two proteins of 95 and 85 kD, respectively. The 110-kD protein was termed myr 4 (myosin from rat) and further characterized.

A characteristic of myosin molecules is their nucleotide-dependent binding to F-actin. To test myr 4 for nucleotide-dependent F-actin binding activity, we partially purified myr 4. Myr 4 was extracted with MgATP and high ionic strength buffer, precipitated by ammonium sulfate, dialyzed against a low salt buffer to form an acto-myosin pellet, extracted with MgATP, and chromatographed over a MonoQ column. Fractions enriched in myr 4 protein were tested for F-actin binding of myr 4. As shown in Fig. 1 C, myr 4 did cosediment with F-actin in the absence of MgATP, but not in the presence of MgATP, demonstrating ATP-dependent binding.

### Primary Structure Determination of myr 4

To obtain peptide sequence information, we attempted to purify the protein (see Materials and Methods) (Fig. 1 B). The major contaminant still present in the purified preparation was actin. This preparation also contained a protein that comigrated on SDS-PAGE with calmodulin. The myr 4 protein band was cut out of SDS gels and reelectrophoresed in



**Figure 1.** (A) Identification of myr 4. Immunoblot of adult rat fore-brain homogenate with antibody G-371 directed against a myosin I consensus peptide. The myr 4 band and molecular mass standards (in kilodaltons) are indicated. (B) Purification of myr 4. Partially purified myr 4 was separated on 7.5–15% gradient SDS-PAGE and stained by Coomassie blue. Purification was monitored by immunoblotting with antibody G-371. Molecular mass standards (in kD) are indicated. Myr 4 is marked by an arrowhead, and actin, the major contaminant in the preparation, is denoted by an asterisk. A protein band comigrating with calmodulin is indicated by an arrow. (C) ATP-dependent binding of myr 4 to F-actin. Partially purified myr 4 (Mono Q fractions, see Materials and Methods) was incubated in the absence (–) or presence (+) of actin (5  $\mu$ M) and ATP (2 mM), respectively. The F-actin binding was assayed by cosedimentation with F-actin. Supernatants (s) and pellets (p) were analyzed for their myr 4 content by immunoblotting with the G-371 antibody. Molecular mass standards (in kilodaltons) are indicated on the left.

the presence of *S. aureus* V8 protease. Five of the obtained myr 4 fragments were subjected to peptide sequencing (underlined in Fig. 2). Degenerate oligo nucleotide primers corresponding to two myr 4 peptide sequences determined by direct protein sequencing were used to amplify a cDNA fragment by PCR. A rat brain cDNA library was screened with the amplified cDNA fragment and the cDNA clones 6 and 21a were isolated. Since these clones did not contain the entire coding region of myr 4, a brainstem/spinal cord cDNA library was screened with a 5' fragment of clone 6. Several clones were isolated, and clone 34b was further analyzed. The myr 4 cDNA exhibited an open reading frame of 3,018 bp coding for a protein of 1,006 amino acids (Fig. 2). The open reading frame of myr 4 started with an initiation sequence conforming to the consensus initiation sequence described by Kozak (40). The calculated molecular mass from the deduced amino acid sequence of 116,190 D was in good agreement with the apparent molecular mass of 110 kD determined by SDS-PAGE. The five peptide sequences obtained by direct amino acid sequencing were found to match exactly with the deduced amino acid sequence (Fig. 2). Furthermore, the peptide sequence recognized by the consensus antibody G-371 was identified at amino acids 612–630. These data provided unequivocal evidence that the cDNA clones were coding for myr 4.

### Sequence Analysis and Structural Organization of myr 4

Sequence analysis of the deduced amino acid sequence revealed that myr 4 exhibits an amino-terminal head (motor) domain (amino acids 1–699), a putative light chain binding domain (amino acids 700–762), and a carboxy-terminal tail domain (amino acids 763–1006). The motor domain of myr 4 was homologous to the motor domains of other myosins. It contained the characteristic sequence motifs such as ATP-binding site, actin binding site, and conserved sequence motifs of as yet unidentified functions. Based on its motor domain, sequence myr 4 could be classified as a myosin I. However, it could not be grouped with any of the known myosin I subclasses (Fig. 3). The motor domain amino acid sequence revealed an identity of 51.3% to rat myr 1c, 49.3% to chicken brush border myosin I, and 48.7% to bovine brush border myosin I. It exhibited 49.3% identity to *D. discoideum* myosin IA and 48.5% to *D. discoideum* myosin IE. Myr 4 was less similar to the other vertebrate myosin I's than they are to each other. Chicken brush border, bovine brush border, and myr 1 myosin I's exhibit 66–69% sequence identity. Myr 4 may therefore represent the first member of a novel subclass of myosin I's because the myosin I molecules identified in *A. castellanii* and *D. discoideum* are also more closely related to each other than to myr 4 (Fig. 3). This conclusion is also supported by the identification of a myosin I from the phylogenetically distant fruit fly *Drosophila melanogaster*, which groups together with myr 4 (Morgan, N. S., D. M. Skovronsky, S. Artavanis-Tsakonas, and M. Mooseker, personal communication) and by amino acid sequence analysis of the tail domain (see below).

A recently published partial myosin I motor domain sequence from rat origin, referred to as myosin I $\gamma$  (56), was virtually identical to amino acids 145–571 in myr 4. The partial myosin I $\gamma$  sequence differed from the myr 4 sequence in

1 CGAGCAGGACGAGCCGCGCGCTCTCCGCTCTCGCCACACGGCCCCACACCCCTCAGCCACCGGGAGGAGCAACGCGCGGCTCGCCGTTTCAGAGCTACGGGGCCGGAGT  
121 CTGAGGAGTGGCGGCTCTATCTCCGGCCGACCCCGCTCCGAGCACCCCCGCCAGCCGAGTCCACCTGAGCCGGCCCATGGCGGAGCAGGAGCCTTGAGTTTGGCAAGGG  
M A E Q E S L E F G K A 12

241 GACTTCGTGCTGATGGACACCGTCTCCATGCCCCAGTTCATGGCCAACTTAGGCTCAGATTTGAGAAAGGAGCGCATCTATACATTCATGGCGAGGTGGTGGTTCCGTGAACCCATAC  
D F V L M D T V S M P E F M A N L R L R F E K G R I Y T F I G E V V V S V N P Y 52

361 AAGTGCTGAACATCTATGGAAGGACAAATGAGCAGTACAAAGTCCGGAGCTGTACGAGAGACCTCTCTCTCGTATCTTGGACGCTGCTTACAAGGCTATGAAGGGCGGA  
K V L N I Y G R D T I E Q Y K G R E L Y E R P P H L F A I L D A A Y K A M K R R 92

481 TCAAAGACACCTGTATGATATCAGGGAAAGTGGAGCTGTAAACAGAGCCAGTAACTACATCATGAGTATATCGCCGCATCACCAACCAGCCAGAGCAGAGATAGAA  
S K D T C I M I S G E S G A G K T E A S K Y I M Q Y I A A I T N P S Q R A E I E 132

601 AGAGTGAAGAATATGTTGCTGAAGTCAAATGTTTTGGAAAGCTTTGGAAATGCCAAAACCAATCGCAATGACAATCCAGCAGGTTGGGAAATACATGGATATCAACTTTGACTTC  
R V K N M L L K S N C V L E A F G N A K T N R N D N S S R F G K Y M D I N F D F 172

721 AAGGGAGACCCCATGGGGACATATAATAACTACTTCTGGAAAAGTCTCGTGAATTTGTGCAACAGCCCGGAGAAAGCTTCCATTTCTTATCAGCTACTTCAGGGAGGTTCC  
K G D P I G G H I N N Y L L E K S R V I V Q Q P G E R S F H S F Y Q L L Q G G S 212

841 GAGCAGATGCTGATTCTCTACATCTGCAGAACTACTCTCATCTCAACTACATCCGCTGGGGCACAAGTCTTCCATCAATGATGCTGCAGAGTTCAAAGTTGTAGCTGAT  
E Q M L H S L H L Q K S L S S Y N Y I R V G A Q L K S S I N D A A E F K V V A D 252

961 GCCATGAAAGTAAATGGATCAAACCTGAGGAGATTCAAACAGTGTATAAATTCGGCTGCTTCTTCACTGGGAAATTTAAATTTATAGTAGAGTGGACACCCACTTATTGAG  
A M K V I G F K P E E I Q T V Y K I L A A I L H L G N L K F I V D G D T P L I E 292

1081 AATGGCAAAGTTGATCTGTGATAGCAAGTTCCTCAACCAAGGCAGACATGGTGGAGAAAGCCCTGCTTACCGGACGGTGGCCACAGGCCGTGACATCATGCAAAACAGCACAG  
N G K V V S V I A E L L S T K A D M V E K A L L Y R T V A T G R D I I D K Q H T 332

1201 GAACAGGAGGCTAGCTACGGCAGAGACCCCTTGGCAAGGCAATATYERGLCTFTTCGIVTRINDIIEVKNKDDTTV 372

1321 CATGGGAAAACACAGTTATGGCGCTTTGGATATCTATGGCTTGAATCTTTGACAACAACAGCTTCGAGCAGTCTGCATTAAGTACTGCAATGAGAAGTGCAGCAGCTTTCATT  
H G K N T V I G V L D I Y G F E I F D N N S F E Q F C I N Y C N E K L Q Q L F I 412

1441 CAGCTGGTGGTGAAGCAGGAGCAAGAGGATACCAGCGGGAAGGAATCCCTCGAAAACACATGATTACTTCAACAACAGATCATTGTGGACCTCGTGGAGCAGCAGCAAAAGGCATC  
Q L V L K Q E Q E E Y Q R E G I P W K H I D Y F N N Q I I V D L V E Q Q H K G I 452

1561 ATTGCAATCCTGGATGACCGGTGTATGAATGTGGCAAAGTCTGATGGAATTCCTCGAGGCCCTTAACAGTAAATGGGCAAAACATGGTCATTTTCCAGCCGAAAACCTGTGCC  
I A I L D D A C M N V G K V T D G M F L E A L N S K L G K H G H F S S R K T C A 492

1681 TCAGACAAAATCTGGAGTTTGTAGCGGACTTTCGAATCCGGCATTTATGAGGTGATGCTGTATTCCTGTCATCGGTTTCATTGACAAAACAAAGATCTTTATTTCAAGACTTCAAG  
S D K I L E F D R D F R I R H Y A G D V V Y S V I G F I D K N K D T L F Q D F K 532

1801 CGCCTCATGTATAACAGCTCAAATCCTGTGCTGAAGAATATGTGGCGGAAGCAAGTACGAGTACAGAGGTGACCAAGAGCCCTGACCGCCGCCACCTGTTTAAAGAAATCCATG  
R L M Y N S S N P V L K N M W P E R K L S I T E V T K R P L T A A T L F P K N S H 572

1921 ATTGCTTAGTAGACAACCTTGCTTCAAAGGAGCCATACTATGTACGTGTCATCAACCCCAACGACAAGTCCCCACAGATTTTGTATGATGAGCGTGGCCGCCATCAGGTAGAATAC  
I A L V D N L A S K E P Y Y V R C I K P N D K K S P Q I F D D E R G R H Q V E Y 612

2041 CTGGGACTCCTGAAAATGTGAGAGTGGCGGGGAGGGTTTGCCTTCCGCGCAGCTATGAGAAGTTTCTTACAGATACAAGATGATCTCTGAGTTCACCTGGCCTAACCCAGCCT  
L G L L E N V R V R R A G F A F R Q T Y E K F L H R Y K M I S E F T W N H D L 652

2161 CCTTCAGACAAGGAGGCTGCAAGAACTCATAGAGCGATGCTGGCTTTCAGGATGACGTAGCATATGGGAAGACAAAATTTTCATTGCAACACCCCGTACGCTGTTCACTTTGGAAGAA  
P S D K E A V A K K L I E R C G F Q D D V A Y G K T K I F I R T P R T L F T L E E 692

2281 CTCGCGCCAGCATGCTGCTCAGGGTGTCTCTTCTACAAAAGTGTGGCGTGGCACCTTGGCCGATCGGTCACAAGAGGACCAAGGCAGCTCTGACAATAATCAGGTACTACCGG  
L R A Q M L V R V V L F L Q K V W R G T L A R M R Y K R T K A A L T I I R Y Y R 732

2401 CGCTATAAGTGAAGTCTGATCCACGAGGTAGCCAGCGATTCACCGGGTCAAGAACATGAGAGACTACGGGAAGCACGTAAGTGGCCACCCCAAGCTTCTGGCCGTTTT  
R Y K V K S Y I H E V A R R F H G V K N M R D Y G K H V K W P T P P K V L R R F 772

2521 GAGGAGCCCTGCAGTCCATTTTAAATAGATGGAGACATCCCAACTCATCAAGACCATACCTGCATCAGACCTCCCAAGTTCAGGGCAAAGTTGACCCATGGAGATCTTGAAGGT  
E E A L Q S I F N R W R A S Q L I K T I P A S D L P Q V R A K V A A M E M L K G  
x 812

2641 CAAGGGCAGACCTTGGGCTCCAGAGGCCGCGGGAAGGCAACTATCTGCTCAAAGCCAGATACACCTCAGACCTCGGCACTTTCGTCAGTGTCAATGAGTGAACGCAAGGAC  
Q R A D L G L Q R A W E G N Y L A S K P D T P Q T S G T F V P V A N E L K R K D 852

2761 AAATATATGAATGTTCTCTTTCTGTCATGTCGGAAGTGAATCGATTTAGTAAGGTAGAAGACCGACCAATTTTGTCACTGACCGTCACTGTATAAAATGGACCCCACTAAGCAG  
K Y M N V L F S C H V R K V N R F S K V E D R A I F V T D R H L Y K M D P T K Q 892

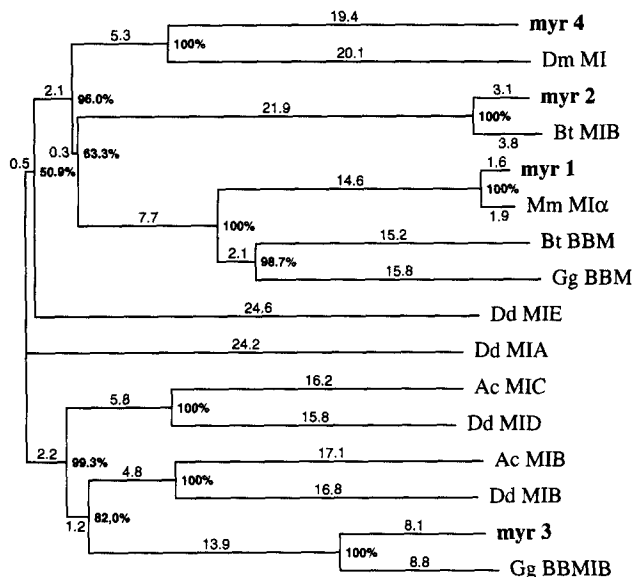
2881 TACAAGTGTGAAGACCATCCACTGTACAATTTGACTGGCCGAGTGTGTCGAATGGAAAGGACCGAGTGTAGTGTCCACACAAAAGACCAAAAGACCTCATCGTTCGCTCTTC  
Y K V M K T I P L Y N L T G L S V S N G K D Q L V V F H T K D N K D L I V C L F 932

3001 AGCAACAGCCGACCCATGAGAGCGGATGGGAACTTGTGGAGTCTGGTGAATCATTTCAAGAGTGAAGAGCCGACCTTCAAGTGAACGTCACCAACCCGGTGCAGTGCAGCCTG  
S K Q P T E R I G E L V G V L V N H F K S E K R H L Q V N V T N P V Q C S L 972

3121 CATGGGAAGAAATGCACCGTATCCGTGGAGACCCGGCTTAACAGCCAGACGCTGACTTCAACAAGACCGCTCCGGCTTCATCTCAGTGTGCCGGGAATGACTGCTGCACACCGGA  
H G K K C T V S V E T R L N Q P Q P D F T K N R S G F I L S V P G N 1006

3241 GGCCCTGCCCCAGGGCAAGAGCCTGGGCTGGCACACAGCAGGCCAGTCTCGCCTGCCATCCCCACATCTGTCCACCTCGCTCAACATCCCAGGAGCTCGGCTAATCGCTGTTT  
3361 GGCCCTAATGCCACTAGAAATCAGCTTGAAGGGCTCCGCTGGGCGAGACTCTCTGCCTTTCTGTGGCCCTGCTCATGACCTGTCTACCTGGCTTTCTGCCTCTGCCTCGTGCCT  
3481 CTGCTCTCAACTTTCATACTCCCTGCTCAGGAAACCAAGACCCCTCTTACGCTGCTGGGCTGACAGCTAGACCCTTCCACCCCAAGCGACTGGATGCTGTAGTGGCAGGAGT  
3601 GCAGTGGCCACTGCTGACCCAGCAGAGGATGAGAGGCCCGGAGCCAGGACCGAGTTCCTGGATCAAAGCCACATTATCTTAGAGGTTTGGATCCAGCATCCGTGGCAGAGGATT  
3721 GGCCCTGCTTTGTGACCCCTACTGATGCCTTCCACAACAT

Figure 2. Nucleotide and deduced amino acid sequence of myr 4 cDNA. Start codon (nt 206-207) and stop codon (nt 3223-3225) are indicated in bold letters. The nucleotide sequence of the initial PCR fragment is underlined. Peptide sequences obtained from purified myr 4 by V8-protease digestion and subsequent direct amino acid sequencing are underlined. X's denote unassigned amino acid residues. The peptide sequence in bold type represents the amino acid sequence recognized by the polyclonal antiserum G-371. The nucleotide sequence data are accessible through EMBL, GenBank, and DDBJ nucleotide sequence databases under the accession number X71997.



**Figure 3.** Unrooted phylogenetic tree of myosin I head domains. This tree was constructed using the CLUSTAL V program (31). The horizontal branch lengths are in units of percent sequence divergence (indicated above each line). The sequence divergence between any pair of sequences is equal to the sum of the horizontal branch lengths connecting the two sequences. Bootstrapping values for each node are given in percent; these values indicate the number of times out of 10,000 data resampling trials that the sequences below a given node were found to cluster together. *Dm MI*, *D. melanogaster* myosin I (Morgan, N. S., D. M. Skovronsky, S. Artavanis-Tsakonas, and M. Mooseker); *myr 2*, rat myosin I (accession number X74800); *Bt MIB*, bovine myosin IB (accession number Z22852); *myr 1*, rat myosin I (53); *Mm MI $\alpha$* , mouse (*mus musculus*) myosin I $\alpha$  (56); *Bt BBM*, bovine brush border myosin I (32); *Gg BBM*, chicken brush border myosin I (24); *Dd MIE*, *D. discoideum* myosin IE (60); *Dd MIA*, *D. discoideum* myosin IA (59); *Ac MIC*, *A. castellanii* myosin IC (35); *Dd MID*, *D. discoideum* myosin-ID (34); *Ac MIB*, *A. castellanii* myosin IB (37); *Dd MIB*, *D. discoideum* myosin IB (36); *myr 3*, rat myosin I (accession number X74815); *Gg BBMIB*, chicken brush border myosin IB (Knight, A., and J. Kendrick-Jones, accession number X70400).

four amino acid residues, namely residues 323, 353, 364, and 550.

The putative light chain binding domain of myr 4 (amino acids 700–762) contained two consecutive IQ motifs that are predicted to serve as calmodulin/light chain binding sites (see Fig. 6 and below). These two IQ motifs were followed by an additional 19 mostly basic and hydrophobic residues. Overlapping with the IQ motifs, we noted four BBXB motifs (B, basic residue; X, any residue), each spaced by exactly nine amino acid residues. The carboxy-terminal border of the regulatory domain was defined by a cluster of proline residues (PTPP, amino acids 763–766).

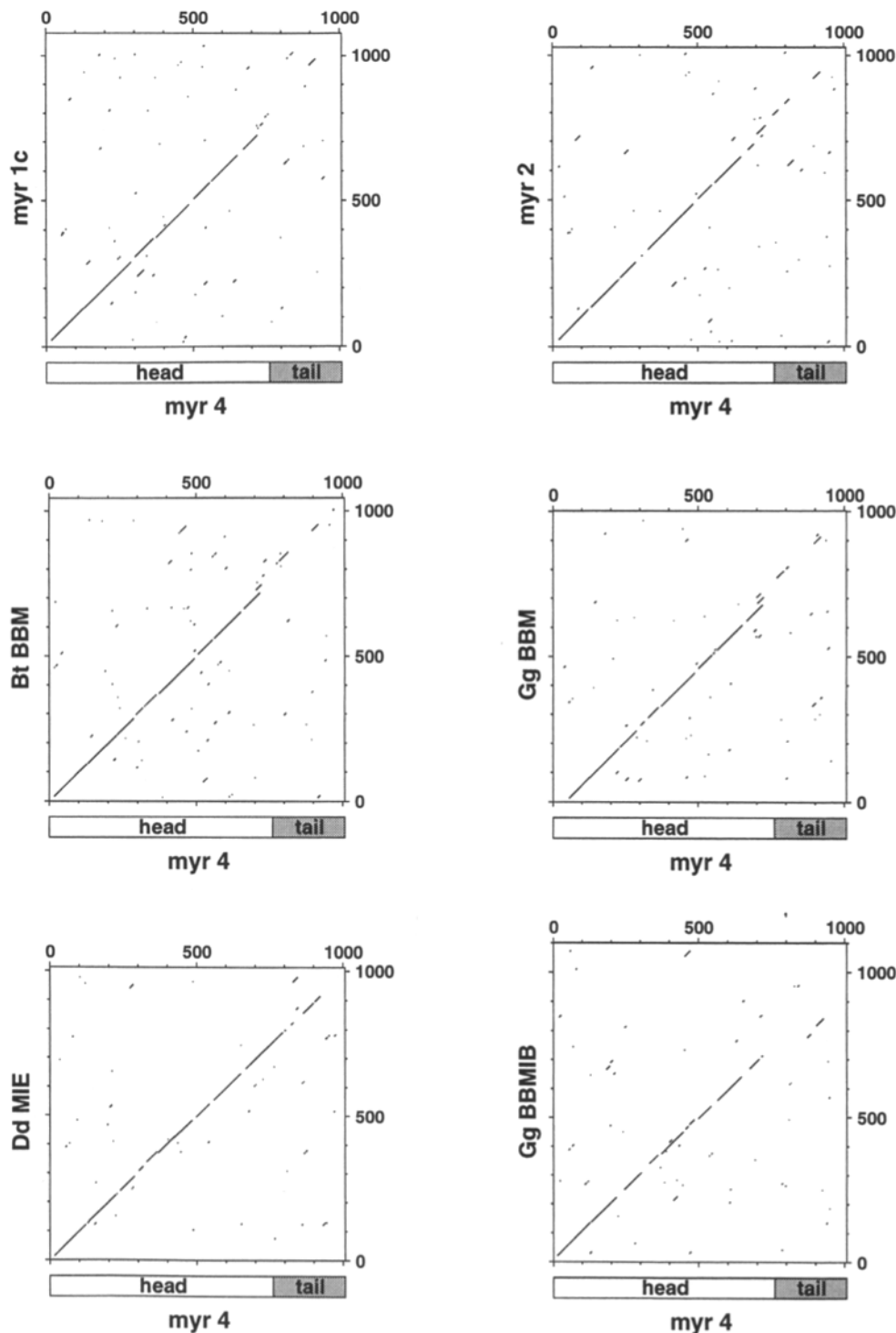
The tail domain of myr 4 (amino acid 763–1006) is enriched in basic ( $pI = 10.77$ ) and hydrophobic (31.8%) amino acid residues as is typical for the tail domains of other myosin I molecules. However, pairwise tail sequence comparisons (starting after the last IQ motif) with other vertebrate myosin I's revealed a sequence identity of only 20–27%

(43–49% similarity). Repeatedly shuffling the myr 4 tail sequence yielded values of 20% identity (40% similarity), demonstrating the limited sequence homology of the myr 4 tail with other myosin I tails. In comparison, chicken brush border myosin I, bovine brush border myosin I, and myr 1 exhibited 42–50% sequence identity (64–72.5% similarity). These differences in tail amino acid sequence conservation between myr 4 and the other vertebrate myosin I's, as well as the disparity between tail and head domain sequence conservation, are illustrated in Fig. 4. Examination of such tail amino acid sequence comparisons between currently known myosin I molecules revealed, however, that they share a sequence motif that was present in all of them, and hence represented a myosin I tail homology motif (Fig. 5). This motif was found only in the tail domains of class I myosin molecules, but not in tail domains of myosins belonging to other classes. The motif was characterized by three short blocks of conserved residues (26–40 amino acids in length) that were or were not interrupted by insertions specific for myosin I subclasses as defined already by comparison of head sequences (see Fig. 3). Myr 4 and *D. discoideum* myosin IE exhibited an insertion between the first and second block of conserved residues; the amoeboid myosin I's exhibited an insertion between the second and third block of conserved residues, whereas the brush border type myosin I's exhibited no insertion (Fig. 5). The degree of conservation in the homologous parts also fitted well with the classification deduced from head sequence alignments. Most highly conserved was the third block of residues encompassing 26 amino acids. This part of the motif is characterized by alternating hydrophobic and hydrophilic residues and two conserved charged residues, an aspartic acid and a histidine residue, respectively.

#### Analysis of Calmodulin Binding by myr 4

Several vertebrate myosin-I molecules have been demonstrated to bind the  $Ca^{2+}$ -binding protein calmodulin (CaM) (4, 25, 53). We therefore analyzed whether myr 4 exhibited any CaM-binding activity. CaM was detected in preparations of affinity-purified myr 4 by immunoblotting but not in preparations purified with control antibodies (data not shown), suggesting that indeed CaM is associated with myr 4. Evidence in support of this notion was also provided by immunoprecipitation experiments from  $^{35}S$ -labeled C6-glioma cells. A band comigrating with authentic CaM was specifically immunoprecipitated by myr 4 antibodies (data not shown). In addition, a band with the appropriate molecular weight for CaM was consistently observed in fractions enriched for myr 4 (see Fig. 1). Furthermore, myr 4 was able to bind CaM both in the presence and absence of free  $Ca^{2+}$  in CaM overlay assays (data not shown).

To identify the CaM binding sequences in myr 4, we constructed a series of fusion proteins encompassing various portions of the two IQ motifs present in the putative light chain binding domain of myr 4 (Fig. 6 D). It had previously been postulated that IQ motifs represent calmodulin/light chain-binding sites (6). CaM overlay assays demonstrated that both myr 4 IQ motifs were able to bind CaM albeit under different free  $Ca^{2+}$  concentrations (Fig. 6). The amino-terminal IQ motif (amino acids 700–721) bound CaM only weakly in the presence of free calcium but strongly in the ab-



**Figure 4.** Dot matrix comparison of myr 4 amino acid sequence with other vertebrate myosin-I sequences and the *Dictyostelium* myosin IE sequence. The amino acid sequences were compared using the GCG software package with a stringency of 14 amino acids in a window of 25 amino acids. The dot matrix representation demonstrates the high level of conservation in the head domains and the lack of conservation in the tail domains with exception of the tail homology motif. Abbreviations used for myosin I molecules are described in Fig. 3.

sence of free calcium ions. In contrast, the carboxy-terminal IQ motif (amino acids 722–743) bound calmodulin strongly in the presence of free calcium, but only weakly in the absence of free calcium. The difference in  $Ca^{2+}$ -concentrations required for optimal binding of CaM by the two IQ motifs is unlikely to be an artifact of fusion protein construction or the gel overlay assay because binding was strictly dependent on the presence of the respective 22 amino acids. Furthermore, the fusion protein that contained both IQ motifs

(amino acids 500–743) bound calmodulin both in the absence and presence of free calcium in a manner equivalent to the sum of the two separate IQ motifs. These results demonstrated that each of the two IQ motifs encoded a CaM-binding site. However, the two IQ motifs exhibited drastically different free calcium ion requirements for optimal binding of CaM.

Remarkably, a myr 4 tail fusion protein encoding amino acids 776–1,006 also exhibited CaM-binding activity (Fig.

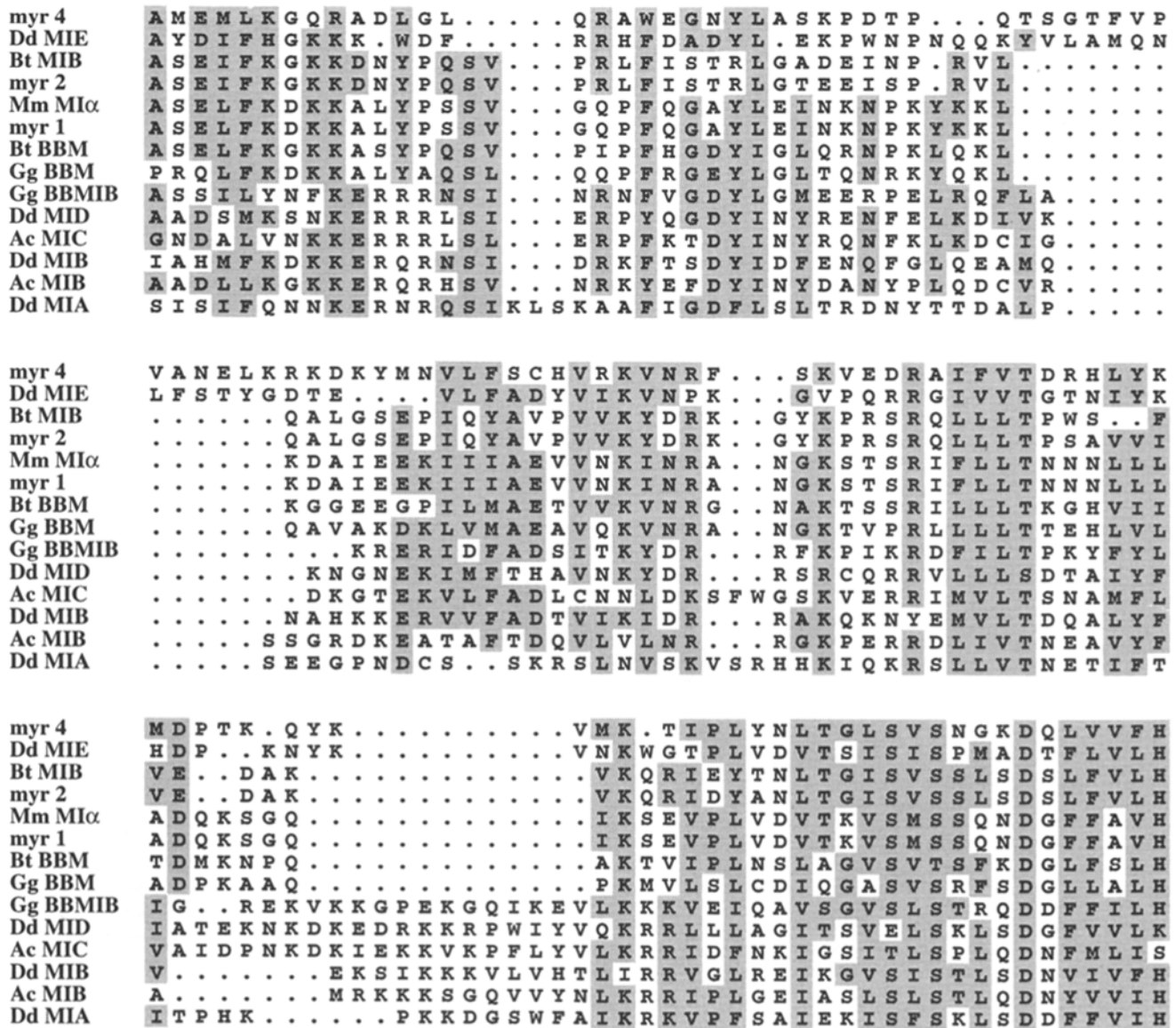


Figure 5. Identification of a myosin I tail homology motif present in all currently available myosin I tail sequences. An alignment of the amino acid sequences is shown. Myr 4, amino acids 806–920; myr 1c, amino acids 886–1050 (53); myr 2, amino acids 841–945 (Ruppert, C., J. Godel, J. Reinhard, and M. Bähler, accession number X74800); *A. castellanii* myosin-IB (Ac MIB) amino acids 701–821 (37); *A. castellanii* myosin-IC (Ac MIC), amino acids 696–818 (35); *D. discoideum* myosin-IA (Dd MIA), amino acids 771–890 (59); *D. discoideum* myosin-IB (Dd MIB), amino acids 721–860 (36); *D. discoideum* myosin-ID (Dd MID), amino acids 720–840 (34); *D. discoideum* myosin-IE (Dd MIE), amino acids 801–912 (60); bovine brush border myosin I (Bt BBM), amino acids 850–956 (32); bovine myosin-IB (Bt MIB), amino acids 842–945 (Reizes, O., C. Li, C. Sudhof, and J. P. Albanesi, accession number Z22852); chicken brush border myosin I (Gg BBM), amino acids 808–914 (24); chicken brush border myosin-IB (Gg BBMIB), amino acids 720–835 (Knight, A., and J. Kendrick-Jones, accession number X70400). Shaded residues denote identical or similar amino acids.

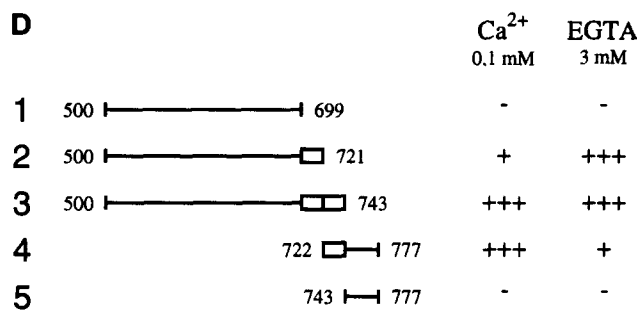
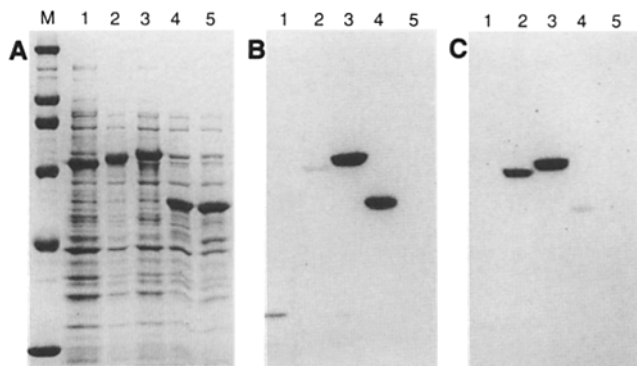
7). The CaM-binding activity of this construct was dependent on calcium (data not shown). To define the tail binding region, we expressed a series of myr 4 tail fragments fused to maltose-binding protein (Fig. 7). CaM binding could be achieved with fragments containing amino acids 776–874. Fragments including only part of this region either did not bind CaM at all or showed only marginal CaM binding activity. Even stronger CaM binding was observed with fragments starting at amino acid 776 and ending between amino acids 896 and 1006, but not with a fragment encoding amino acids 743–898. These results suggested that the myr 4 tail

CaM-binding activity was not solely dependent on a short stretch of amino acid sequence, as demonstrated for the IQ motifs.

#### Distribution of myr 4

Antisera were raised against a bacterially expressed myr 4 fragment (amino acids 513–1,006) tagged with 6xHis residues (Tü 14) and a synthetic myr 4 peptide (Tü 12, 13), and affinity purified over a bacterially expressed myr 4 tail fragment (amino acids 776–1,006) fused to maltose-binding





**E** IQ motifs

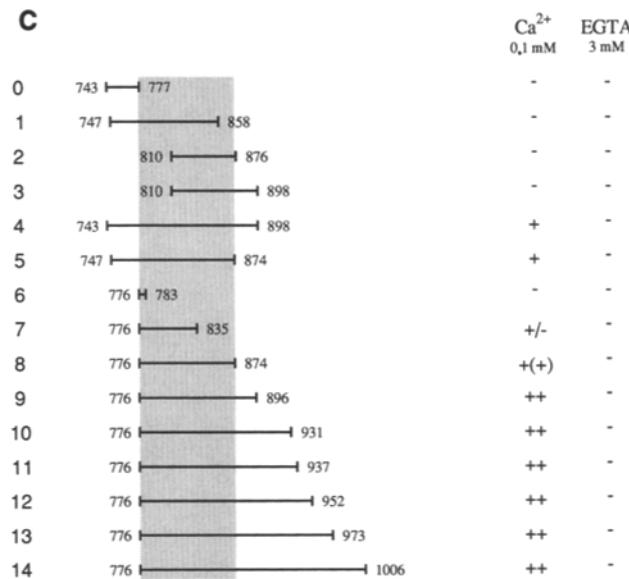
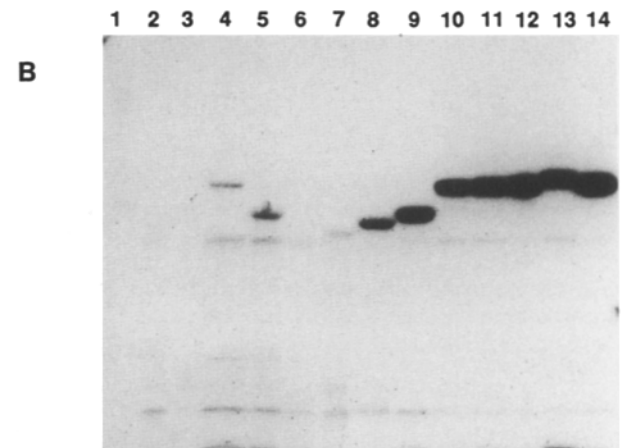
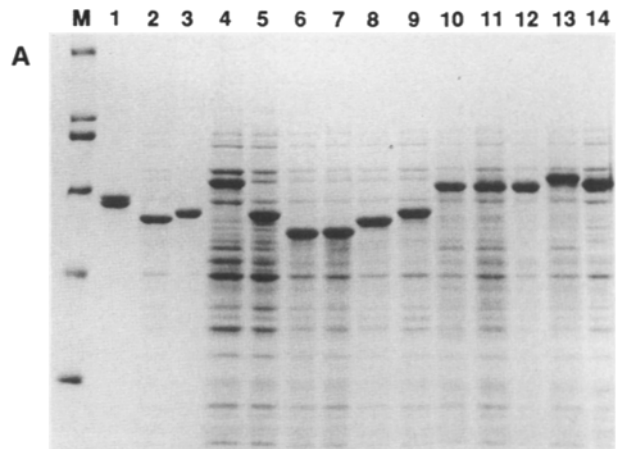
neuromodulin 33 **KAATKIQ**ASFRGHITRKKLKG<sup>55</sup>

myr 4 I 700 RVVLF**LQK**VWRGTLARMRYKRT<sup>721</sup>

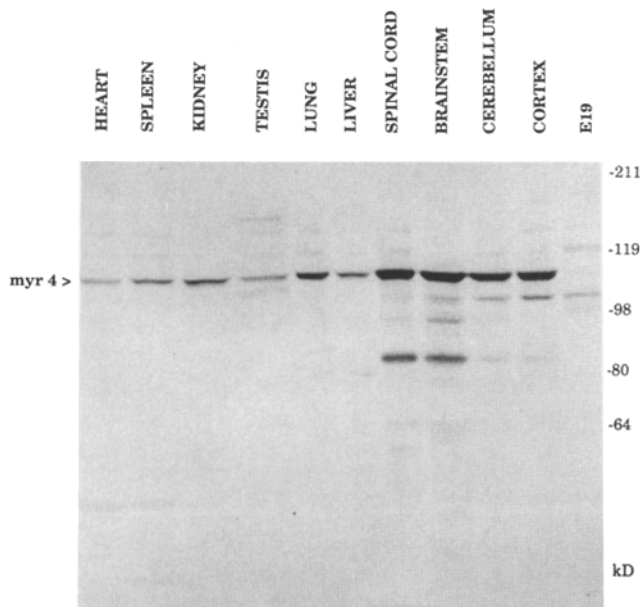
myr 4 II 722 **KAALTIIRY**RYKVKSYIHEV<sup>743</sup>

**Figure 6.** The two IQ motifs present in myr 4 bind calmodulin in a differentially Ca<sup>2+</sup>-sensitive manner. Coomassie brilliant blue-stained SDS-gel (A) and autoradiographs of identical gels incubated with <sup>125</sup>I-calmodulin in the presence (0.1 mM Ca<sup>2+</sup>) (B) or absence of free calcium ions (3 mM EGTA) (C) are shown. Fusion proteins of maltose-binding protein with myr 4 fragments spanning variable regions of the regulatory domain were expressed in *E. coli*. Whole-cell lysates were separated by SDS-PAGE. Expressed myr 4 fragments separated on lanes 1-5 are schematically depicted in (D) and numbered accordingly. IQ motifs (boxes) are indicated. Lane M includes the following markers: myosin heavy chain, 205 kD;  $\beta$ -galactosidase, 116 kD; phosphorylase b, 97.4 kD; bovine serum albumin, 66 kD; ovalbumin, 45 kD; and carbonic anhydrase, 29 kD. Calmodulin-binding results obtained with the various myr 4 fragments (B and C) are schematically summarized in (D). No binding is indicated by (-), and increasing strength of binding is indicated by numbers of (+). (E) The amino acid sequences of the two myr 4 IQ motifs are aligned with each other and the IQ motif of the calmodulin binding protein neuromodulin (5). Residues conserved between neuromodulin and myr 4 are printed in bold type.

protein. These antibodies recognized specifically the identical 110-kD protein that we had identified as myr 4 with our myosin I consensus antibody. Tissue distribution studies with these myr 4-specific antibodies demonstrated that myr 4 was most prominently expressed in the adult brainstem and spinal cord, and at slightly lower levels in cortex and cerebellum (Fig. 8). Reduced levels of myr 4 as compared to the four brain regions were detected in lung, kidney, spleen, liver, testis, and heart muscle. Barely detectable levels were observed in embryonic brain tissue.



**Figure 7.** Ca<sup>2+</sup>-dependent calmodulin binding of myr 4 tail constructs. Various myr 4 tail constructs (lanes 1-14), schematically depicted in (C), were expressed in *E. coli* and separated on SDS-PAGE. Coomassie blue-stained SDS-gel (A) and autoradiograph of an identical gel incubated with <sup>125</sup>I-calmodulin in the presence (0.1 mM) of free calcium ions (B) are shown. Size standard markers (M) in A are identical to those in Fig. 5. Calmodulin binding of the various myr 4 tail constructs is summarized in (C). The shaded area denotes the region involved in calmodulin binding. No calmodulin binding is indicated by (-), and the increasing strength of binding is indicated by numbers of (+).



**Figure 8.** Tissue distribution of myr 4. Equal amounts of protein from different tissues and brain regions were separated on SDS-PAGE and immunoblotted with the affinity-purified myr 4 antibody Tü 12, followed by a secondary antibody coupled to alkaline phosphatase. The respective position of myr 4 is indicated. Molecular mass standards in kilodaltons are shown on the right. Tissues are indicated on top of each lane, with E19 denoting embryonic day 19 rat brain.

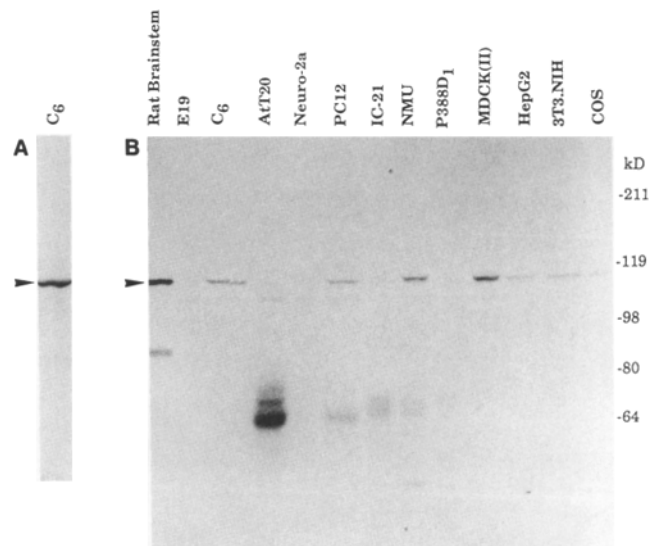
Several cell lines were screened for myr 4 expression by immunoblotting. As shown in Fig. 9, myr 4 was detected in C6, PC12, NMU, MDCK, HepG2, NIH3T3, and COS cells. Myr 4 was barely detectable in AtT20, IC-21, and P388D<sub>1</sub> cells, and not detectable in Neuro-2A cells. These data demonstrated that myr 4 is expressed in many tissues and cell lines, albeit at varying levels. Similar results were obtained by Northern blot analysis, which demonstrated that myr 4 mRNA is also relatively abundant in intestine (data not shown).

#### Expression of myr 4 during Rat Forebrain Development

We investigated the expression of myr 4 during rat forebrain development. As shown in Fig. 10, expression of myr 4 protein was barely detectable at embryonic and early postnatal days, markedly increased during postnatal weeks 2–3, and remained at high levels until adulthood. This result demonstrated that myr 4 expression in brain tissue is developmentally regulated.

#### Immunofluorescence Localization of myr 4 in Brain Sections and in Cultured Cells

We determined the localization of myr 4 in two regions of the adult rat brain (Fig. 11). The cell bodies and apical dendrites of a subpopulation of neurons were prominently labeled in the cortex and thalamus. In the cortex, a subpopulation of pyramidal cells in layers 5 and 6 exhibited punctate staining (Fig. 11A) reminiscent of an organellar association. A subpopulation of neuronal cell bodies and apical dendrites was also stained in the thalamus (Fig. 11B). Based on their morphology, these cells are likely to represent projection



**Figure 9.** Expression of myr 4 protein in various cell lines. Equal amounts of protein from various cell lines, rat brainstem, and embryonic day 19 rat brain tissue were separated on SDS-PAGE and immunoblotted with Tü 14 (A) or Tü 12 (B) antibody. Cell lines or brain tissues are indicated on top of each lane. Molecular weight markers are given in kilodaltons. Note the specificity of Tü 12 and Tü 14 antibodies in the C6-cell line.

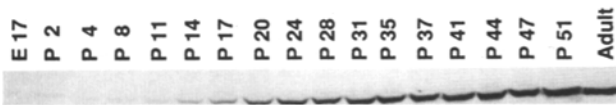
neurons. In both brain regions, a weaker general punctate labeling that was not easily attributable to particular structures was also evident. A similar punctate myr 4 immunoreactivity was observed in subpopulations of neurons in the brainstem (data not shown).

In the rat C6 glioma cell homogenate, the antibodies Tü 12 and Tü 14 reacted monospecifically with myr 4 (Fig. 9). This cell line was therefore used to localize myr 4 by indirect immunofluorescence (Fig. 12). As in the neurons stained *in situ*, a punctate staining pattern throughout the C6 cells was observed (Fig. 12). The punctae sometimes appeared to line up along invisible tracks. However, double immunofluorescence staining with phalloidin failed to demonstrate an obvious colocalization of myr 4-positive punctae with actin filaments (Fig. 12, B and C). The observed punctate staining pattern was reminiscent of an organellar localization. However, double immunofluorescence studies with organellar markers failed so far to identify a candidate organelle. In particular, no apparent colocalization with  $\alpha$ -adaptin or cavolin was observed (data not shown).

## Discussion

### Myr 4 Defines a New Subclass of Myosin I

Using our previously described myosin-I consensus antibody (53), we have identified myr 4, a novel mammalian myosin I. The characterization of myr 4 as a class I myosin molecule was based on biochemical and sequence data. We demonstrated that it is extracted from tissue homogenates by MgATP, that it binds in a nucleotide-dependent manner to F-actin, and that it binds calmodulin. Sequence data revealed that myr 4 consisted of a typical myosin head domain, a regulatory light chain-binding domain, and a tail domain.



**Figure 10.** Developmental expression of myr 4 in rat forebrain. Equal amounts of protein from embryonic day 17 (E17), postnatal days 2–51 (P2–P51) and adult rat forebrain tissue homogenates were separated on SDS-PAGE and transferred to nitrocellulose. The nitrocellulose was incubated with antibody Tü 12 followed by a secondary antibody coupled to alkaline phosphatase.

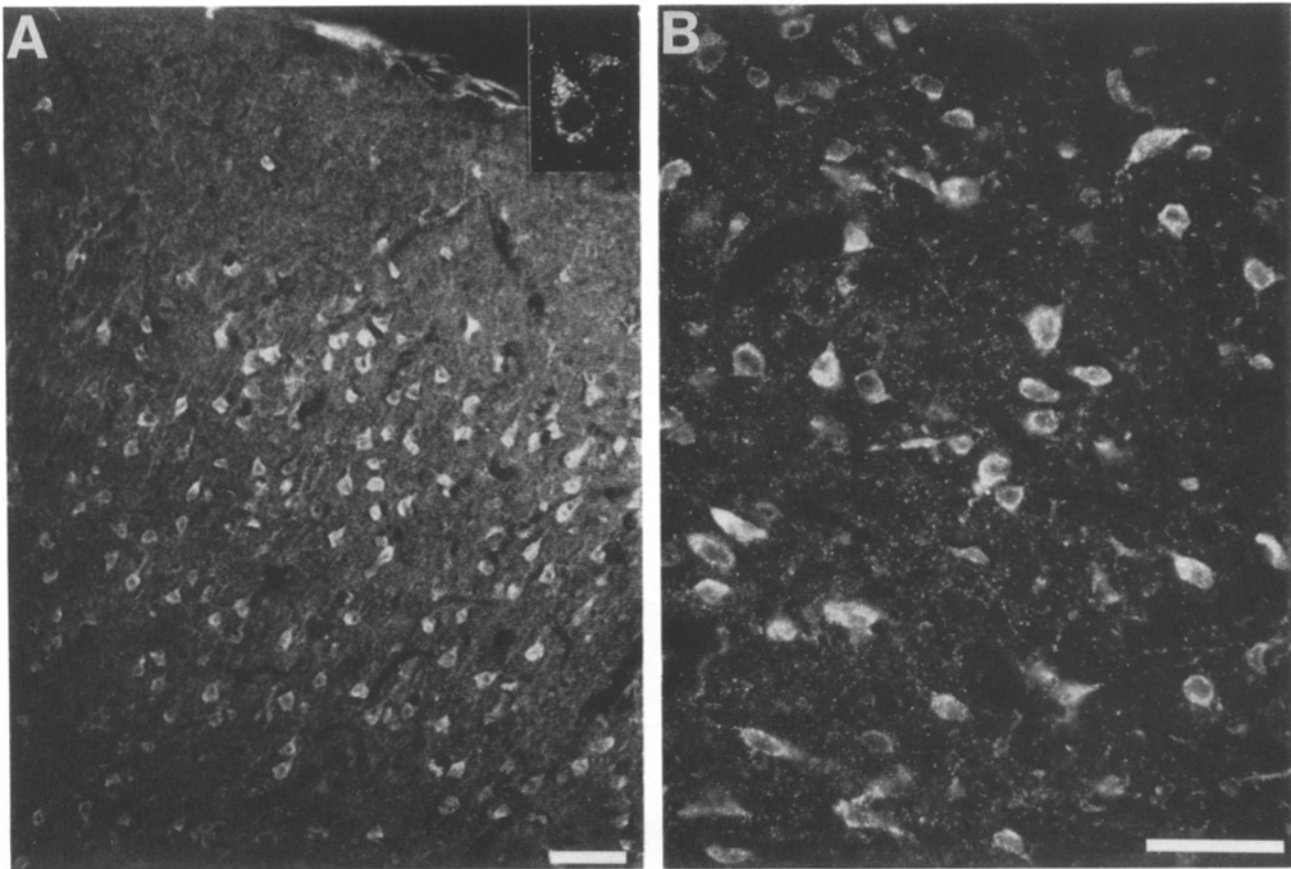
Comparison of the myr 4 head domain amino acid sequence with the amino acid sequences of other myosin head domains demonstrated that myr 4 is a class I myosin (for nomenclature see reference 7). However, myr 4 could not be grouped unequivocally with any of the known myosin I subclasses. It was not significantly more homologous to the vertebrate brush border type myosin I subclass, which includes myr 1, the only other identified myosin I from rat, than to myosin IA and IE from *D. discoideum*. Myr 4 may therefore represent a member of a novel myosin I subclass. This notion is supported by the analysis of several unpublished myosin I amino acid sequences (Fig. 3) and the lack of extensive sequence homology between myr 4 and other myosin I tail domain sequences, except for the myosin I tail homology motif.

### Identification of a Myosin I Tail Homology Motif

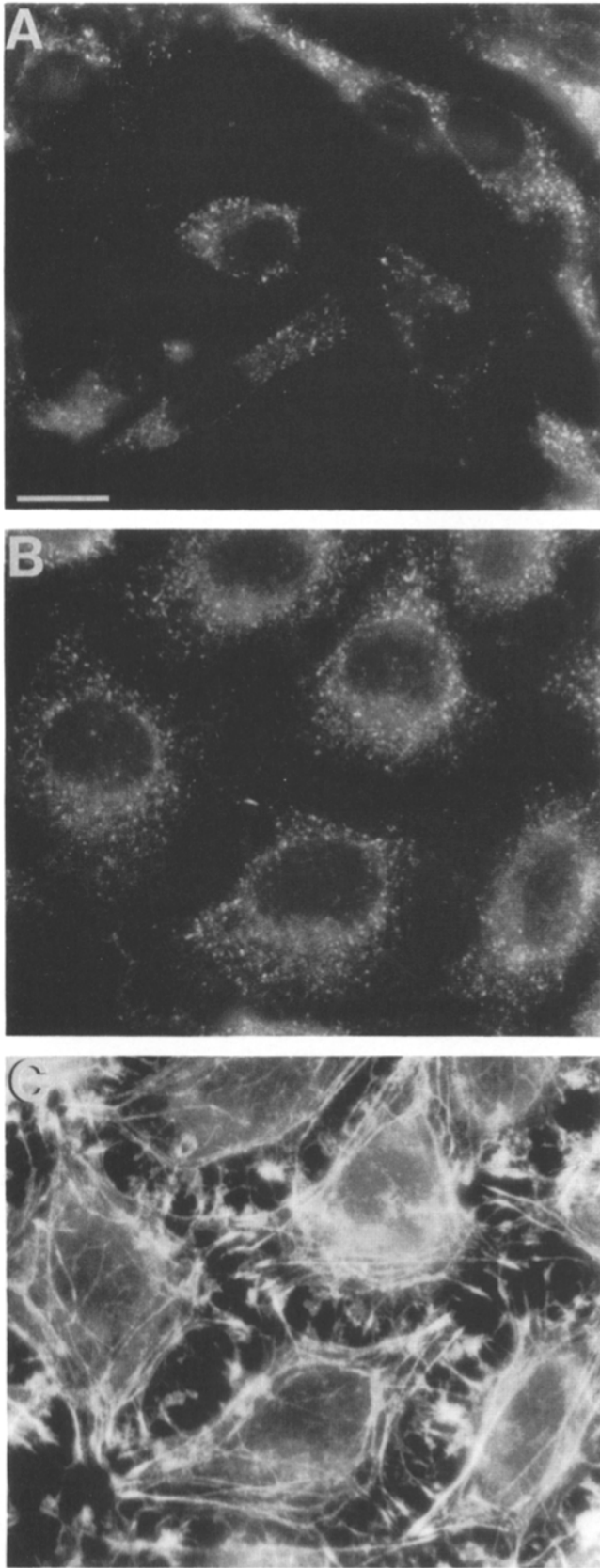
Analysis of divergent myosin I tail domain sequences revealed a common amino acid motif characteristic of myosin I tails. This motif was identified in all presently available myosin I tail sequences, but not in tail sequences of other myosins, and it is therefore diagnostic of myosin I tail domains. The tail homology motif forms part of the tail homology 1 region previously defined by comparison of protozoan myosins I (34). It consists of three regions with particular conservation that may be separated by short myosin I subclass (defined by head sequence comparisons) specific inserts. The high degree of conservation implies that this motif is of functional significance. The only common function ascribed to myosin I tail domains is their capability to interact with membranes. A phospholipid-binding site has been localized to a tail fragment (amino acids 701–888) of *Acanthamoeba* myosin IC that contains most of the tail homology motif (amino acids 696–818) (17). Further experiments will have to address whether the tail homology motif plays a role in membrane binding or any other as yet unidentified function common to myosin I's.

### Differences between myr 4 and myr 1

Unlike myr 1, which is closely related to brush border myosin I, myr 4 defines a new subclass of myosins I. It represents



**Figure 11.** Immunolocalization of myr 4 in two brain regions. Coronal cryosections of rat cortex (A) and thalamus (B) were labeled by indirect immunofluorescence with antibodies to myr 4 (A, Tü 12, and B, Tü 14) using a secondary antibody coupled to Cy3. Prominent punctate immunoreactivity is seen in pyramidal cell bodies and their apical dendrites of cortical layers 5 and 6, as well as in relay neurons in the thalamus. Inset in (A) shows a confocal image to demonstrate punctate staining. Bar, 50  $\mu$ m.



**Figure 12.** Immunolocalization of myr 4 in rat glioma C6 cells. C6 cells were labeled by indirect immunofluorescence with antibody Tü 14 (A) using a secondary antibody coupled to Cy3, with antibody Tü 12 (B) using a secondary antibody coupled to rhodamine,

the first mammalian myosin I identified that is not closely related to brush border myosin I. As opposed to myr 1 that exists in three alternatively spliced forms, we have so far detected no evidence for alternatively spliced forms of myr 4.

Myr 4 and myr 1 differed in their extraction behavior from tissues. Myr 4 was more difficult to solubilize than myr 1. Upon homogenization of brain tissue, virtually all myr 4 protein, but only ~50% of myr 1, was recovered in the particulate fraction. The nucleotide ATP readily released most of the particulate myr 1, but myr 4 was released only in conjunction with high ionic strength. Detergent and elevated pH values aided in the release of myr 4 (Kieferle, S., and M. Bähler, unpublished observations). The tail domain of myr 4 did not contain sequences present in the tail domains of amoeboid myosin I molecules, which are involved in nucleotide-independent actin binding (17, 43). The harsh treatments needed for myr 4 solubilization are therefore unlikely to result from nucleotide-independent actin binding. Furthermore, partially purified myr 4 was readily released from F-actin by MgATP. It will therefore be of interest to determine with which molecule(s) myr 4 is associated.

Expression levels of myr 4 and myr 1a exhibited a strikingly converse regulation during brain development. Myr 4 protein levels increased during postnatal rat brain development and were highest in the adult brain, whereas myr 1a levels were high in embryonic brain tissue and decreased during postnatal development. Currently, we do not know whether myr 4 and myr 1a are expressed in the same or different cells. Myr 1 was reported to be expressed in neurons (56). We demonstrated in this report that myr 4 is expressed in subpopulations of neurons in the adult rat brain. Myr 4 expression might be induced by synaptogenesis or gliogenesis because both processes in rats mostly take place during postnatal development. So far, we have not been able to relate any functional differences between neurons to myr 4 expression.

Both myr 4 and myr 1 were found to be expressed in all tested tissues. However, the relative expression levels in the various tissues differed for the two proteins. In tissues from adult rats, myr 1 was most prominently expressed in lung, whereas myr 4 was most prominently expressed in brain. These results together with the different developmental regulation suggest that myr 4 and myr 1 play general but nonidentical roles.

#### ***Differential Ca<sup>2+</sup> Sensitivity of Calmodulin Binding by IQ Motifs***

Myosin molecules contain various numbers of light chains. It was recently suggested by Cheney and Mooseker (6) that a motif, called the IQ motif, found in the regulatory domains of myosins, provides a binding site for proteins of the calmodulin/EF-hand superfamily, and that these sites generally retain their binding activity, even in the absence of calcium. Two consecutive imperfect repeats of 22 amino acids

or with fluoresceine-phalloidin (C), respectively. Double immunofluorescence labeling for myr 4 and F-actin is shown in B and C. Bar, 10  $\mu$ m.

conforming to the IQ motif were detected in the regulatory domain of myr 4. Preparations of myr 4 purified from rat brain contained calmodulin. This finding indicated that calmodulin is complexed with myr 4. Because of the small amounts of purified myr 4 available, no stoichiometry of calmodulin binding could be determined. Direct binding of calmodulin to myr 4 was demonstrated in a  $^{125}\text{I}$ -calmodulin overlay assay. By producing several constructs that encoded either a single IQ motif, both IQ motifs, or no IQ motif, we were able to demonstrate that each of the two IQ motifs represented a calmodulin binding site. The two IQ motifs differed in their dependency on calcium for calmodulin binding. The  $\text{NH}_2$  terminal IQ motif bound calmodulin well only in the absence of free calcium, whereas the COOH-terminal IQ motif-bound calmodulin well only in the presence of free calcium. No apparent cooperativity between these two calmodulin binding sites was detected in the construct encoding both IQ motifs. These results demonstrate, for the first time, that IQ motifs can exhibit drastically different dependencies on calcium for calmodulin binding, and that an IQ motif can bind calmodulin in a  $\text{Ca}^{2+}$ -dependent manner. It has been noted for chicken brush border myosin I that micromolar concentrations of calcium cause a partial release of bound calmodulin (9, 10, 58). The affinities for calmodulin binding depending on free  $\text{Ca}^{2+}$  concentrations remain to be determined for the two myr 4 IQ motifs. This information will be needed to speculate on the numbers of calmodulin molecules associated with myr 4 under physiological conditions. In addition, structural data will be needed to determine which residues in IQ motifs confer differential  $\text{Ca}^{2+}$  sensitivity for calmodulin binding. In the motif IQX-XXRGXXXR, the glutamine and glycine residues are exchanged for an isoleucine and an arginine, respectively, in the carboxy-terminal myr 4 IQ motif. The amino-terminal myr 4 IQ motif and the neuromodulin IQ motif, which both bind calmodulin stronger in the absence of  $\text{Ca}^{2+}$ , conform to the consensus sequence (reference 5 and this report). From the presented calmodulin-binding results, it seems likely that myr 4 activity will be regulated by  $\text{Ca}^{2+}$ /calmodulin.

Upon analysis of calmodulin binding by the myr 4 regulatory domain, we noticed that a construct encoding the tail domain exhibited calcium-dependent calmodulin-binding activity in  $^{125}\text{I}$ -calmodulin gel overlays. A variety of constructs designed to identify the tail calmodulin binding sequence demonstrated that amino acids 776–874 were necessary for calmodulin binding. This region is considerably larger than the calmodulin-binding regions of known calmodulin-binding proteins (e.g., the IQ motifs). Furthermore, the relative amounts of calmodulin bound to constructs with different  $\text{NH}_2$  termini containing the critical region were variable. Recently, the phospholipid/membrane binding domain of the plasma membrane  $\text{Ca}^{2+}$  pump was shown to bind calmodulin (21). Several members of the EF-hand superfamily, including calmodulin, are known to bind membranes or hydrophobic supports in the presence of calcium (19, 63). Therefore, it seems possible that in vivo, this region represents a phospholipid/membrane binding site rather than a calmodulin-binding site. This region (amino acids 776–874) partially overlaps with the myosin I tail homology motif (amino acids 805–920), which represents a candidate phospholipid binding site. Future experiments will have to address this possibility.

#### Localization of myr 4

To understand the possible roles of myosin I molecules in cellular trafficking, one needs to determine their subcellular distributions. Examination of the subcellular localization of myr 4 revealed a punctate distribution. This punctate distribution was reminiscent of a membranous, organellar localization. Double immunofluorescence staining with F-actin demonstrated no apparent colocalization, indicating that under steady-state conditions myr 4 localization within cells is mainly determined via its tail domain rather than the actin-binding head domain. So far, we have been unable to determine the organelle that myr 4 is associated with. Determination of the organellar membrane that myr 4 is associated with will require immunoelectron microscopy and identification of an organellar marker that colocalizes with myr 4. Although we cannot conclusively rule out that myr 4 is not associated with an organellar/vesicular membrane, myr 4 consistently fractionated on sucrose gradients with a density of 1.14–1.16  $\text{g}/\text{cm}^3$ , the same density as, e.g., plasma membrane (Kroschewski, R., R. Müller, and M. Bähler, unpublished observations).

The localization of myr 4 contrasts with the localizations reported for other vertebrate myosin I molecules. Brush border myosin I, a bovine myosin I purified from adrenal gland, and a 120-kD protein of Swiss 3T3 fibroblasts cross-reactive with an antibody raised against chicken brush border myosin I were all found to be concentrated in actin-rich cortical regions (13, 18, 46, 61). These observations prompted speculations that the mentioned myosin I's are involved in motile events at the cell surface. However, all three proteins are possibly also located on membranous organelles. Some brush border myosin I was detected on vesicles (18), a punctate perinuclear staining was observed in some cells for the bovine adrenal myosin I (61), and discrete, punctate spots were observed in the lamellae, as well as in the perinuclear region of Swiss 3T3 cells, with the antibody against chicken brush border myosin I (13). The respective myosin I motor domains, responsible for actin binding, might therefore play different roles in determining the steady-state localizations. Alternatively, the observed differences in apparent colocalization with actin filaments might simply reflect associations with different membrane compartments. It remains to be determined whether vertebrate myosin I molecules are involved in cell shape changes, membrane dynamics, or both.

In conclusion, myr 4 represents a widely distributed myosin I that defines a new subclass of myosin I. All currently sequenced myosin I molecules contain a conserved motif in their tail domains. Myr 4 exhibits three in vitro mapped calmodulin-binding sites, whereby one IQ motif, contrary to previously characterized calmodulin binding by myosins, required free  $\text{Ca}^{2+}$  for optimal calmodulin binding activity. Myr 4 is likely to play a role in membrane traffic that is regulated by  $\text{Ca}^{2+}$ /calmodulin.

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