

# Biochemical and Immunological Characterization of p190-Calmodulin Complex from Vertebrate Brain: A Novel Calmodulin-binding Myosin

Foued S. Espindola, Enilza M. Espreafico, Milton V. Coelho, Antonio R. Martins,\* Flavia R. C. Costa, Mark S. Mooseker,† and Roy E. Larson

Departments of Biochemistry and \*Pharmacology, Faculdade de Medicina de Ribeirão Preto, Universidade de São Paulo, Ribeirão Preto, 14049, São Paulo, Brazil; and †Department of Biology, Yale University, New Haven, Connecticut 06511

**Abstract.** We have recently identified a novel 190-kD calmodulin-binding protein (p190) associated with the actin-based cytoskeleton from mammalian brain (Larson, R. E., D. E. Pitta, and J. A. Ferro. 1988. *Braz. J. Med. Biol. Res.* 21:213–217; Larson, R. E., F. S. Espindola, and E. M. Espreafico. 1990. *J. Neurochem.* 54:1288–1294). These studies indicated that p190 is a phosphoprotein substrate for calmodulin-dependent kinase II and has calcium- and calmodulin-stimulated MgATPase activity. We now have biochemical and immunological evidence that this protein is a novel calmodulin-binding myosin whose properties include (a) Ca<sup>2+</sup> dependent actin activation of its Mg-ATPase activity, which seems to be mediated by Ca<sup>2+</sup> binding directly to calmodulin(s) associated with p190 (maximal activation by actin requires the presence of Ca<sup>2+</sup> and is further augmented by addition of exogenous calmodulin); (b) ATP-sensitive cross-linking of skeletal muscle F-actin, as demonstrated by the low-speed actin sedimentation assay; and (c) cross-reactiv-

ity with mAbs specific for epitopes in the head of brush border myosin I. We also show that p190 has properties distinct from conventional brain myosin II and brush border myosin I, including (a) separation of p190 from brain myosin II by gel filtration on a Sephacryl S-500 column; (b) lack by p190 of K<sup>+</sup>-stimulated EDTA ATPase activity characteristic of most myosins; (c) lack of immunological cross-reactivity of polyclonal antibodies which recognize p190 and brain myosin II, respectively; (d) lack of immunological recognition of p190 by mAbs against an epitope in the tail region of brush border myosin I; and (e) distinctive proteolytic susceptibility to calpain. A survey of rat tissues by immunoblotting indicated that p190 is expressed predominantly in the adult forebrain and cerebellum, and could be detected in embryos 11 d post coitus. Immunocytochemical studies showed p190 to be present in the perikarya and dendritic extensions of Purkinje cells of the cerebellum.

**C**ALCIUM ions have direct and modulatory roles in several neuronal functions: neurotransmitter release (Augustine et al., 1987; Smith and Augustine, 1988), synaptic plasticity (Kandel, 1981; Kennedy, 1989), and specialized cellular motility, such as axoplasmic transport (Ochs, 1982), neurite extension (Lankford and Letourneau, 1989), and growth cone motility (Kater et al., 1988). Calmodulin is the major intracellular receptor of Ca<sup>2+</sup>, and mediates Ca<sup>2+</sup> action in at least some aspects of all three of these neuronal functions. Cytoskeletal organization and contractile elements are exquisitely involved in these functions and several cytoskeletal and associated proteins in brain have been identified as calmodulin-binding proteins (Sobue et al., 1987).

The actin cytoskeleton is concentrated at the leading edge of active growth cones (Letourneau, 1981; Smith, 1988) and is essential for their motility (Forscher and Smith, 1988). An actin network of filaments has been visualized in postsynaptic densities (Ratner and Mahler, 1983) and an hypothesis of synaptic plasticity based on contraction-generated dendritic-spike movements has been proposed (Crick, 1982). Also, the dynamic assembly of the actin cytoskeleton has been demonstrated in synaptosomes and during transmitter release (Bernstein and Bamberg, 1989). These studies lead to the question of the identity of the force generators in the actin cytoskeleton.

Myosin II has been purified and characterized biochemically from brain of several vertebrate species (Barylko and Sobieszek, 1983; Hobbs and Frederiksen, 1983; Malik et al., 1983; Matsumura et al., 1989) and identified by immunolocalization in synaptic junctions (Beach et al., 1981), neurites (Kuczmarski and Rosenbaum, 1979), and growth

Foued S. Espindola's present address is Department of Physiological Sciences, Universidade Federal de Uberlândia, Uberlândia 38400, Minas Gerais, Brazil.

cones (Bridgman and Daily, 1989). Although brain myosin II has been postulated to be involved with actin in contractile events mentioned above (see Letourneau, 1981), as of yet no direct demonstration of this has been forthcoming and the exact role of brain myosin II remains unknown. Considerable biochemical work in amoeba (for review see Pollard et al., 1991) and recent advances in molecular genetics (see Kiehart, 1990) have shown "myosins" to be a family of distinct molecules which include two major types, myosin isoforms I and II, as well as examples of unconventional myosins whose classification is still not clear—such as the ninaC proteins of *Drosophila retina* (Montell and Rubin, 1988), the product of the MYO2 gene in *Saccharomyces cerevisiae* (Johnston et al., 1991), and the gene product of the mouse dilute locus (Mercer et al., 1991). Recently, a myosin I has been localized to the leading edge of locomoting amoebae (Fukui et al., 1989), whereas myosin II localized to the posterior region, suggesting distinct roles in the same cell for these myosins in mechanisms of cellular motility. Extending these findings to higher organisms, one might expect novel myosins to be involved in specific aspects of actin-based cytoskeletal dynamics, including those related to neuronal function.

We have recently identified a novel calmodulin-binding protein (p190) in mammalian brain, associated with the actin-based cytoskeleton (Larson et al., 1988; Larson et al., 1990). In this paper we present biochemical and immunological evidence that p190 has myosin-like properties which make it a potential molecular motor, yet is distinct from conventional brain myosin II. Immunolocalization studies show this molecule to be enriched in brain and demonstrable in Purkinje cells of the cerebellum. Preliminary reports of these findings have been published in abstract form (Larson et al. 1988. *Society for Neurosci. Abstr.* 14:436; Espreafico et al., 1990. *J. Cell Biol.* 111:167a.).

## Materials and Methods

### Materials

Alkaline phosphatase conjugated to goat anti-rabbit IgG(Fc) and goat anti-mouse IgG(H+L) were purchased from Promega Corp. (Madison, WI); rabbit antiplatelet myosin (human) was from Biomed. Technol. Inc. (Baltimore, MD). PMSF, trifluoperazine (TFP),<sup>1</sup> bromochloroindolyl phosphate, nitroterazolium blue, ATP, DTT, and EGTA were purchased from Sigma Chem. Co. (St. Louis, MO). Octyl-Sepharose, Q-Sepharose Fast Flow, and Sephacryl S-500 were purchased from Pharmacia LKB Biotechnology Inc. (Piscataway, NJ). Nitrocellulose sheets were Hybond-C extra from Amersham Corp. (Arlington, IL). Other chemicals were of analytical grade.

### Preparation of Proteins

Brain actomyosin (BAM) was prepared from rabbit, rat, and chick (1–5-d-old) brains as described previously (Larson et al., 1990); a p190-enriched preparation (PEP) was obtained from these animals by a modification of Larson et al. (1990) as summarized below. Immunoblot analysis of each fraction along the purification scheme was performed to provide a rough estimate of protein yield. All procedures were performed on ice. Brains were rapidly removed from decapitated animals and dropped into ice-cold homogenization buffer A (~12 ml/g of brain tissue) consisting of 25 mM Tris-HCl, pH 8.5, 10 mM EDTA, 5 mM ATP, 2 mM 2-mercaptoethanol, and 0.3 mM PMSF. Brains were homogenized with a high-speed shearing

homogenizer in batches so that the maximum time from decapitation to homogenization was <5 min. The total homogenate was centrifuged at 17,000 g for 40 min. Variable amounts of brain myosin II relative to p190 can be obtained at this step—longer extraction time before centrifugation, i.e., >20 min, increases the amount of myosin II in the final preparation. The resulting supernatant, which by immunoblot analysis generally contained about half the total p190 present in the initial homogenate, was filtered through glass wool and dialyzed for 20 h against two changes (4 liters each) of 20 mM Tris-HCl, pH 7.5, 1 mM EDTA, 50 mM KCl, 2 mM 2-mercaptoethanol, and 0.2 mM PMSF. The fine precipitate that formed (about half of the total p190 present in the dialysate) was collected by centrifugation at 17,000 g for 40 min and resuspended in 25 mM Tris-HCl, pH 8.0, 50 mM KCl, 10 mM EGTA, and 10 mM EDTA (~3 ml/5 g of brain). Triton X-100 was added to 1%; the solution was warmed to 25°C, and then homogenized and centrifuged at 17,000 g for 30 min at 4°C. The pellet was washed in 25 mM Tris-HCl, pH 8.0, and recentrifuged. Finally, the pellet was resuspended in 25 mM Tris-HCl, pH 8.0, and kept on ice until used.

Calpain was purified from rat brain based on the method of Fox et al. (1985), with modifications. Briefly, brains from 30 rats were homogenized in 120 ml of buffer B (25 mM Tris-HCl, pH 8.0, 1 mM DTT, 0.3 mM PMSF, and 1 mM Na<sub>3</sub>N) containing 10 mM EDTA and 10 mM EGTA, and centrifuged at 15,000 g for 30 min. The supernatant was recentrifuged at 100,000 g for 60 min, and this supernatant was loaded onto a Q-Sepharose column (1.5 × 20 cm) equilibrated in buffer B containing 1 mM EDTA and eluted with 125 ml of 300 mM KCl in the same buffer. The eluate was applied to an octyl-Sepharose column (1.5 × 20 cm) equilibrated at 25°C in buffer B containing 300 mM KCl and 1 mM EDTA. The column was washed with 10 vol of the same buffer and then eluted with buffer B adjusted to pH 7.2. Fractions containing calpain were identified as possessing Ca<sup>2+</sup>-dependent casein proteolytic activity. These fractions are probably composed of a mixture of calpain I and II. They were stored at 2–4°C until used.

Calmodulin was purified from bovine brain as described by Gopalakrishna and Anderson (1982). Skeletal muscle actin was prepared as described by Spudich and Watt (1971). Protein concentration was determined by the method of Hartree (1972).

### Assay for ATPase Activity

ATPase activity was determined by measuring the inorganic phosphate released from ATP by method of Heinonen and Lahti (1981). The reaction was carried out at 37°C in a final volume of 200  $\mu$ l. MgATPase activity was assayed in 25 mM imidazole-HCl, pH 7.5, 1 mM EGTA, 0.2 mM DTT, 1 mM ATP, 5 mM MgCl<sub>2</sub>, 30–100  $\mu$ g/ml of protein, and where appropriate, 2 mM CaCl<sub>2</sub>, 1.5  $\mu$ M purified bovine brain calmodulin, and 50  $\mu$ M TFP. K-EDTA ATPase activity was assayed in 25 mM imidazole-HCl, pH 8.0, 1 mM EDTA, 0.2 mM DTT, 1 mM ATP, 30–100  $\mu$ g/ml of protein, and either 0.06 M or 0.6 M KCl. Reactions were initiated by the addition of ATP and stopped after 30–60 min by the addition of the acid-molybdate-acetone solution of the Pi assay.

### Low-Speed Actin Sedimentation Assay

Actin cross-linking activity of p190 was assessed by the induction of actin sedimentation at low centrifuge speed as described by Pollard and Cooper (1982). The reaction media contained 10 mM Tris-HCl, pH 8.0, 170 mM KCl, 1 mM MgCl<sub>2</sub>, 1.0 mM DTT, and 0.02% Na<sub>3</sub>N in a final volume of 100  $\mu$ l. To the appropriate samples, 300  $\mu$ g/ml of F-actin and/or 58  $\mu$ g of p190 (pooled fraction from a Sephacryl S-500 column) were added and assayed in the presence or absence of 1 mM ATP. Reactions were incubated at room temperature for 15 min and then centrifuged at 8,800 g for 15 min. The supernatants were carefully removed and the pellets resuspended in an equal volume of buffer. Both were analyzed by SDS-PAGE.

### Gel Electrophoresis and Immunoblotting

SDS-PAGE was carried out in 7–15% gradient minislab gels, unless otherwise stated, with the discontinuous system of Laemmli and Favre (1973). Electrophoretic transfer (Western blotting) to nitrocellulose membranes was performed as described by Towbin et al. (1979). Polyclonal antibodies were generated in rabbits by inoculation with brain myosin heavy chain and p190, respectively, purified from rats by electroelution of the polypeptides from BAM samples separated by SDS-PAGE on standard size 5% gels. The IgG fraction of the immune sera was isolated by DEAE chromatography (Harlow and Lane, 1988). The specificity of the antibody was determined by immunoblot analysis. Immunodetection of specific antigens on Western

1. Abbreviations used in this paper: BAM, brain actomyosin; BB, brush border; NC, nitrocellulose; PEP, p190-enriched protein; TFP, trifluoperazine.

blots was done using alkaline phosphatase conjugates of goat anti-rabbit (for polyclonals) or anti-mouse (for monoclonals) antibodies and developed with bromochloroindolyl phosphate and nitrotetrazolium blue (Harlow and Lane, 1988).

### Immunolocalization in Histochemical Slices

Affinity-purified anti-p190 was prepared from anti-p190 IgG by the method of Olmsted (1986). p190 from rat brain was purified by electroelution from 5% SDS-PAGE gels (Hunkapiller et al., 1983) and bound to a 1 × 2 cm strip of nitrocellulose (NC) which was subsequently blocked by incubation with 5% dry milk in TBS-Tween. Anti-p190 IgG solution diluted 1:10, ~0.5 mg in 1 ml of TBS was incubated with the NC strip for 2 h. This solution was removed and the NC strip washed in TBS containing 0.05% Tween 20. Monospecific antibodies against p190 were eluted off the NC strip by incubation for 5 min in 0.5 ml of 100 mM triethylamine, pH 11.5. The supernatant was immediately dialyzed overnight at 4°C in TBS containing 0.05% Tween 20 and 0.02% NaN<sub>3</sub>. The NC strip was reusable after a 5-min wash in 2 M guanidine-HCl and several washes in TBS-Tween 20. A control solution, "preabsorbed anti-p190," was prepared with the same system except that the anti-p190 IgG solution diluted 1:10 was incubated twice with the NC strip; thus all the specific anti-p190 antibodies were removed based on Western blot test.

Adult rats were anesthetized with sodium pentobarbital, heparinized, and perfused through the left ventricle with PLP fixative (10 mM periodate, 75 mM lysine, and 0.5% paraformaldehyde in 37 mM sodium phosphate buffer, pH 7.4) as described by McLean and Nakane (1974). Brains were removed and postfixed in the same fixative for 6–8 h at 4°C, and then placed in 20% sucrose in phosphate buffer until sinking. The cerebellum was then either embedded in paraffin or quick frozen. 10-μm sections were cut and mounted on gelatin-subbed slides. Hydrated slices were incubated for 2 h in TBS containing 1% Triton X-100, 10% bovine or sheep serum, and 0.02% NaN<sub>3</sub>, then 24 h at room temperature with affinity-purified anti-p190 (or preabsorbed anti-p190, as a control) in TBS containing 0.1% Triton X-100, 10% bovine or sheep serum, and 0.02% NaN<sub>3</sub>. Incubation with anti-rabbit IgG conjugated with alkaline phosphatase was for 2 h and the reaction was developed in the dark with bromochloroindolyl phosphate and nitrotetrazolium blue in the presence of 2 mM levamisol (Harlow and Lane, 1988).

## Results

### p190 Is a Novel Calmodulin-binding Protein Associated with Brain Actomyosin from Several Vertebrates

We have previously identified a 190-kD calmodulin-binding protein (p190) associated with the actin-based cytoskeleton in mammalian brain (Larson et al., 1988; Larson et al., 1990). Applying similar extraction procedures to several vertebrate species we show in Fig. 1 that p190 and calmodulin are present in actomyosin preparations (BAM) from avian and mammalian brains, and can be enriched in fractions obtained from each species by a simple modification of this extraction procedure (see PEP in Materials and Methods). p190 is easily detected in BAM by SDS-PAGE in 7–15% gradient gels, migrating slightly ahead of the heavy chain of brain myosin II, a well-characterized, conventional nonmuscle myosin. In the PEP fraction p190 is the predominant Coomassie blue-stained peptide, although small amounts of myosin II can be detected which vary slightly from preparation to preparation. Brain actin and endogenous calmodulin are also present with p190 in the PEP fractions.

Polyclonal antibodies raised in rabbits against p190 from rats specifically recognize p190 in rat and chick preparations on Western blots, as shown in Fig. 2. This figure also shows that polyclonal antibodies against platelet myosin, which, according to the manufacturer, recognize several nonmuscle myosins but not skeletal, smooth, or cardiac

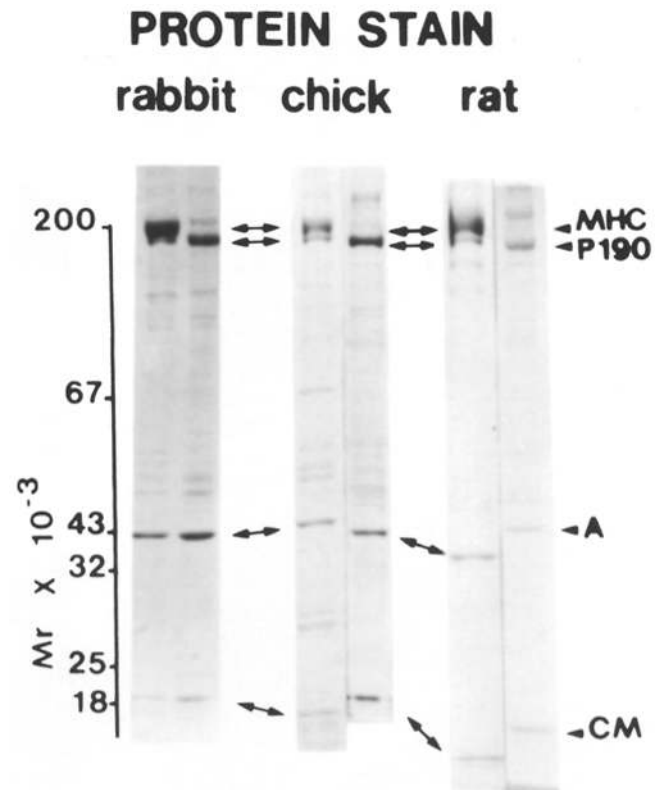


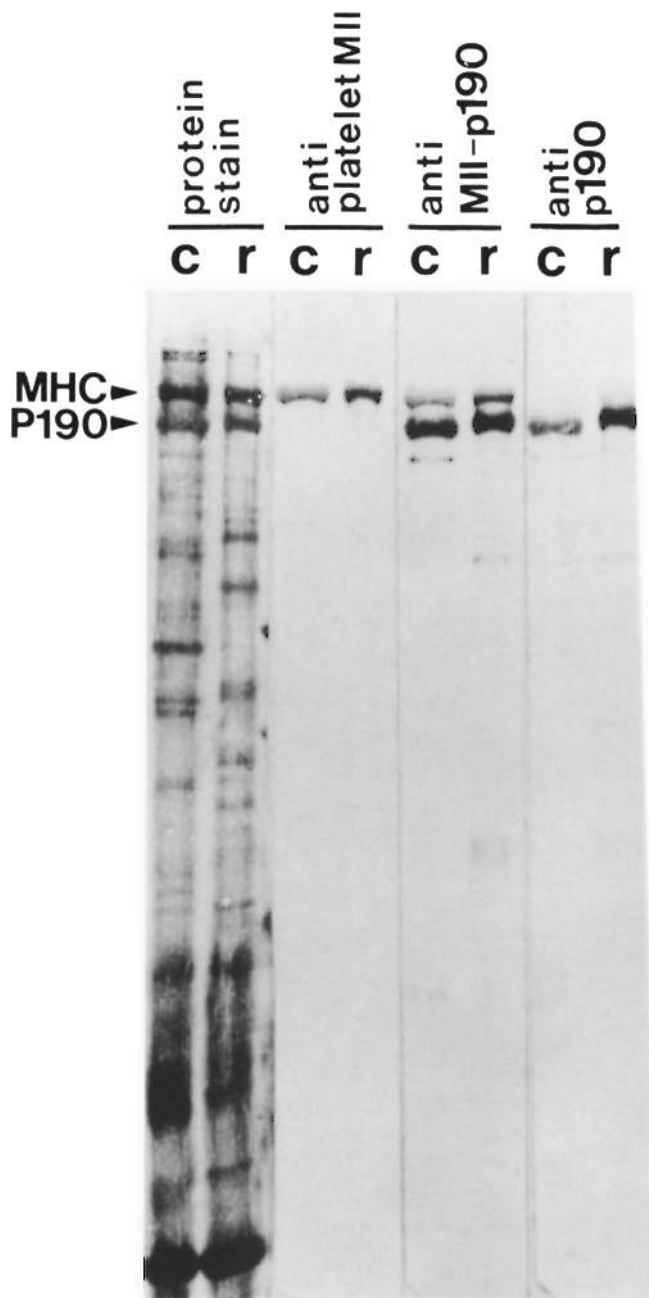
Figure 1. Actin cytoskeletal preparations from brain of several vertebrate species contain p190. For each species a brain actomyosin preparation (left) is compared with a p190-enriched preparation (right). Samples were analyzed by SDS-PAGE on 7–15% gradient gels stained with Coomassie blue R-250. These fractions had very similar polypeptide profiles for each of the species, including enrichment of brain myosin heavy chain (MHC) in BAM and of p190 in the PEP fraction. A, brain actin; CM, endogenous calmodulin.

myosins, clearly recognized brain myosin II heavy chain, but not p190.

Together, these experiments demonstrate that p190 and calmodulin are associated with the actin cytoskeleton in brain from several vertebrate species and that p190 is a distinct molecule from brain and other nonmuscle myosins.

### Purification of p190–Calmodulin Complex

To separate p190 from brain myosin II and actin, BAM preparations from rabbits were solubilized in an ATP-pyrophosphate buffer, and clarified by centrifugation; and the supernatants were applied to a Sephacryl S-500 column. This purification procedure resulted in yields of purified p190 in the range of 1–10% based on qualitative assessment of immunoblots of the various fractions throughout purification (100–200 μg from 12 rabbits). A typical fractionation analyzed by SDS-PAGE is shown in Fig. 3 A. p190 was eluted in the fractions following the peak of myosin II identified by its 200-kD heavy chain and three light chains (16–18 kD). Note that the endogenous calmodulin coeluted with the p190, well ahead of its exclusion volume, implicating its association with p190 during chromatography. Lastly, fractions enriched in brain actin were eluted. Purified brain myosin II and p190 were obtained from pools 1 and 2, respectively, by precipitation in Tris buffer (Fig. 3 B).



**Figure 2.** p190 is immunologically distinct from conventional non-muscle myosin. Western blots of brain actomyosin preparations with approximately equivalent amounts of p190 and brain myosin II are shown for chicks (c) and rats (r). A blot stained for protein with india ink is shown on left. Equivalent blots were probed with polyclonal antibodies against platelet myosin II (*anti-platelet MII*), p190 (*anti-p190*), and a mixture of both antibodies (*anti-MII-p190*), as indicated. *MHC*, myosin heavy chain.

#### **p190-Calmodulin Complex Has Actin-activated ATPase Activity in the Presence of $Ca^{2+}$**

Pooled and concentrated fractions of purified p190 and brain myosin II (Fig. 3 B) obtained from Sephacryl S-500 chromatography were assayed for ATPase activity (Table I). The p190 fraction had low MgATPase activity which was not affected by the addition of  $Ca^{2+}$ , purified bovine calmodu-

lin, or TFP. The addition of the brain actin fraction or purified muscle actin increased the MgATPase activity by 1.9-fold and 3.5-fold, respectively, although these data are not statistically significant. However, the addition of brain actin fraction to p190 in the presence of  $Ca^{2+}$  and  $Ca^{2+}$ /calmodulin stimulated the ATPase activity by 20- and 15-fold, respectively. Purified muscle actin mimicked these effects but to a lesser degree. Brain myosin II also has MgATPase activity which, in the presence, but not in the absence, of  $Ca^{2+}$ /calmodulin was stimulated almost twofold by either brain or muscle actin (see also Tanaka et al., 1986). The maximum specific activity attained by brain myosin II was only 40% of that of p190. These data indicate that p190, in the presence of  $Ca^{2+}$ , is an actin-activated ATPase and that the brain actin fraction may contain factors which potentiate this activity for p190 but not for brain myosin II.

Although the p190 fraction has actin-activated MgATPase activity similar to myosin, its K-EDTA ATPase activity was not typical of myosins, since increasing KCl from 0.06 to 0.6 M did not stimulate ATPase activity in the presence of EDTA (Table I). Brain myosin II, on the other hand, showed typical K-EDTA ATPase activity.

#### **p190 Induces and Accompanies Actin Sedimentation in an ATP-sensitive Manner under Low-Speed Centrifugation**

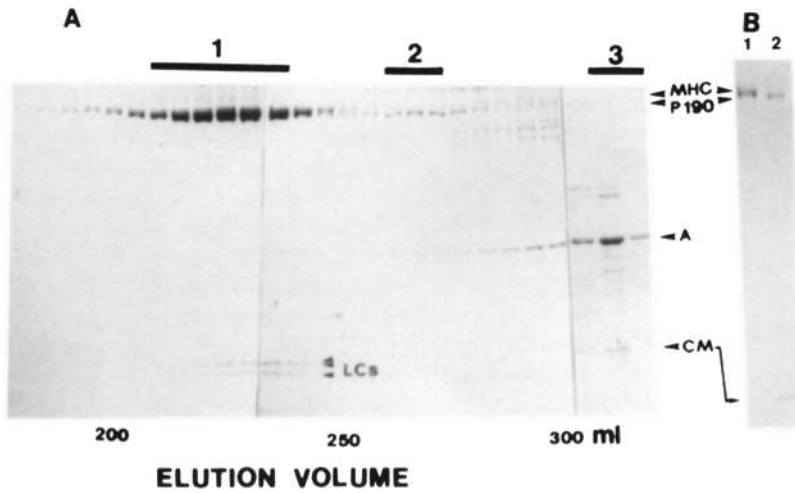
Another way to assess the interaction of actin with myosin or other actin-binding proteins is by determining their effect on actin sedimentation under low-speed centrifugation (Pollard and Cooper, 1982). Under the conditions of this assay F-actin alone did not sediment during low-speed centrifugation (Fig. 4). However, the addition of a low concentration of p190 resulted in the cosedimentation of actin and p190 when ATP was absent but not when ATP was present. The results show that p190 is effective in inducing the sedimentation of actin and cosediments with actin in an ATP-sensitive manner.

#### **p190 Is Recognized by mAbs Against the Head Domain of Brush Border Myosin I**

Western blots of PEP samples from rabbit, rat, and chick brains were probed with mAbs generated against brush border myosin I (BB myosin I) (Fig. 5). The mAbs, CX-1 and CX-2, which recognize epitopes localized to the 25 kD  $NH_2$ -terminal, head domains of both BB myosin I and skeletal muscle myosin II (Carboni et al., 1988) also recognized p190 from all three species. Brain myosin II from chick was also labeled by these mAbs. Thus, there is a conserved epitope shared between three distinct myosins (i.e., chick BB myosin I, rabbit skeletal muscle myosin II, and chick brain myosin II) and p190 from all three species examined. Included as a control, a mAb specific for the tail region of BB myosin I, CX-7 (Hayden et al., 1990), did not react with p190 from any of the species.

#### **p190 Is Selectively Cleaved by Calpain**

Calpain is a  $Ca^{2+}$ -dependent protease whose substrates include many calmodulin-binding proteins (Wang et al., 1989). Thus, it was of interest to verify if p190 was also a substrate for this protease. Indeed, p190 and a high molecular weight



**Figure 3.** p190 is separated from brain myosin II and actin on a Sephacryl S-500 column. (A) SDS-PAGE analysis of fractions from rabbit BAM applied to a Sephacryl S-500 column shows the isolation of brain myosin II, p190, and brain actin. BAM from 12 rabbits was solubilized in buffer C (25 mM Tris-HCl, pH 7.8, containing 600 mM KCl, 5 mM MgCl<sub>2</sub>, 45 mM Na<sub>2</sub>P<sub>2</sub>O<sub>7</sub>, 5 mM ATP, 0.2 mM PMSF, and 3 mM 2-mercaptoethanol). The clarified supernatant (20,000 g for 30 min) containing 10–13 mg of protein was applied to a column of Sephacryl S-500 (2.1 × 117 cm) equilibrated and eluted with buffer C containing 2 mM ATP. 5-ml fractions were collected and pooled as indicated in the figure: pool 1, brain myosin II; pool 2, p190; pool 3, brain actin. (B) The pooled fractions were dialyzed against 25 mM Tris-HCl, pH 7.8, containing 0.2 mM PMSF and 3 mM 2-mercaptoethanol and concentrated by evaporation

tion from a dialysis sack hung in front of an airstream in the coldroom. Brain myosin II (lane 1) and p190 (lane 2) were collected by centrifugation (10,000 g for 15 min) and resuspended in 25 mM Tris-HCl, pH 7.8, containing 400 mM KCl. The final yield of p190 was 100–200 μg from 12 rabbits. LCa, brain myosin II light chains.

protein which we suspect to be fodrin, a well-characterized calpain substrate (Harris and Morrow, 1990), were readily cleaved by calpain in the presence of Ca<sup>2+</sup>, whereas myosin II heavy chain, actin, and endogenous calmodulin in this same sample remained intact under these conditions (Fig. 6). Several relatively stable peptides were generated from p190 by calpain, as indicated by arrowheads to the right of the figure, which were identified by probing immunoblots with p190-reactive antibodies (data not shown).

#### **p190 Is Enriched in Adult Rat Brain and Detected in 11-d-old Embryos**

To evaluate the tissue distribution and relative abundance of p190 in the rat, Western blots of homogenates from several brain regions as well as other tissues dissected from adult rats were probed with anti-p190 polyclonals. As can be seen in Fig. 7 A, p190 is easily visualized in most brain regions but not in the other tissues examined, although a faint signal,

not revealed on the photograph, could be seen on the nitrocellulose for several of these tissues. p190 expression during embryonic development was also examined by immunoblots (Fig. 7 B) and was detected in rat embryos as early as 11 d post coitus, increasing rapidly after day 15. Considering immunological identity, these results indicate that p190 is relatively abundant in brain tissue in comparison to the other tissues examined and is expressed early in development. However, p190 does not represent a major protein in any of the tissues so far analyzed, including brain.

#### **p190 Is Demonstrable by Immunocytochemistry in Purkinje Cells of the Cerebellum**

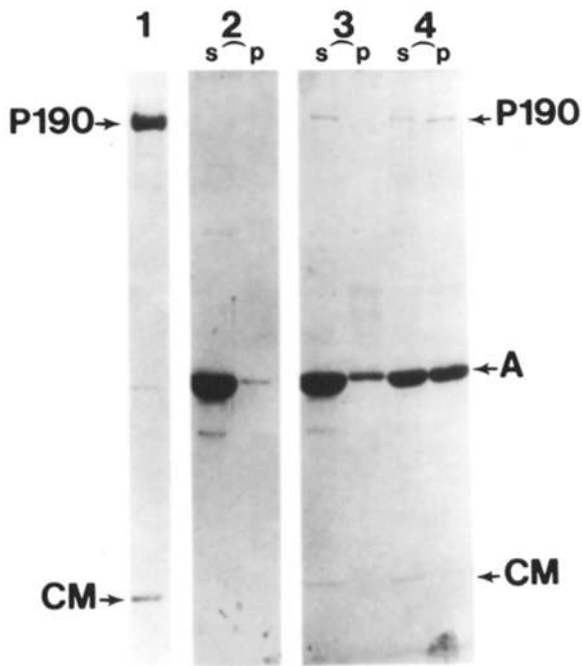
Polyclonal antibodies generated against p190 electroeluted from SDS-PAGE gels were quite specific for p190 on Western blots of whole brain homogenates, as shown in Fig. 8. For immunocytochemistry studies, we further purified these antibodies by affinity purification (see Materials and Meth-

**Table I.** ATPase Activities of p190 and Brain Myosin II Fractions from the Sephacryl S-500 Column

Assay conditions	ATPase activity (nmol Pi/mg per min)					
	p190	p190 + Brain actin	p190 + Skeletal muscle actin	Brain myosin II	Brain myosin II + Brain actin fraction	Brain myosin II + Skeletal muscle actin
<b>Mg-ATPase</b>						
No additions	10 ± 7 (2)	19 ± 11 (2)	35 ± 23 (3)	33 ± 14 (3)	23 ± 9 (2)	30 ± 8 (3)
+Ca	6 ± 4 (3)	122 ± 22 (2)	64 ± 17 (2)			
+Ca, CaM	13 ± 6 (3)	191 ± 3 (2)	96 ± 36 (3)	42 ± 15 (3)	77 ± 19 (2)	72 ± 16 (3)
+Ca, CaM, TFP	13*	43*	36*			
<b>K-EDTA ATPase</b>						
0.06 M KCl	53 ± 21 (4)			62 ± 19 (4)		
0.6 M KCl	47 ± 12 (4)			450 ± 63 (4)		

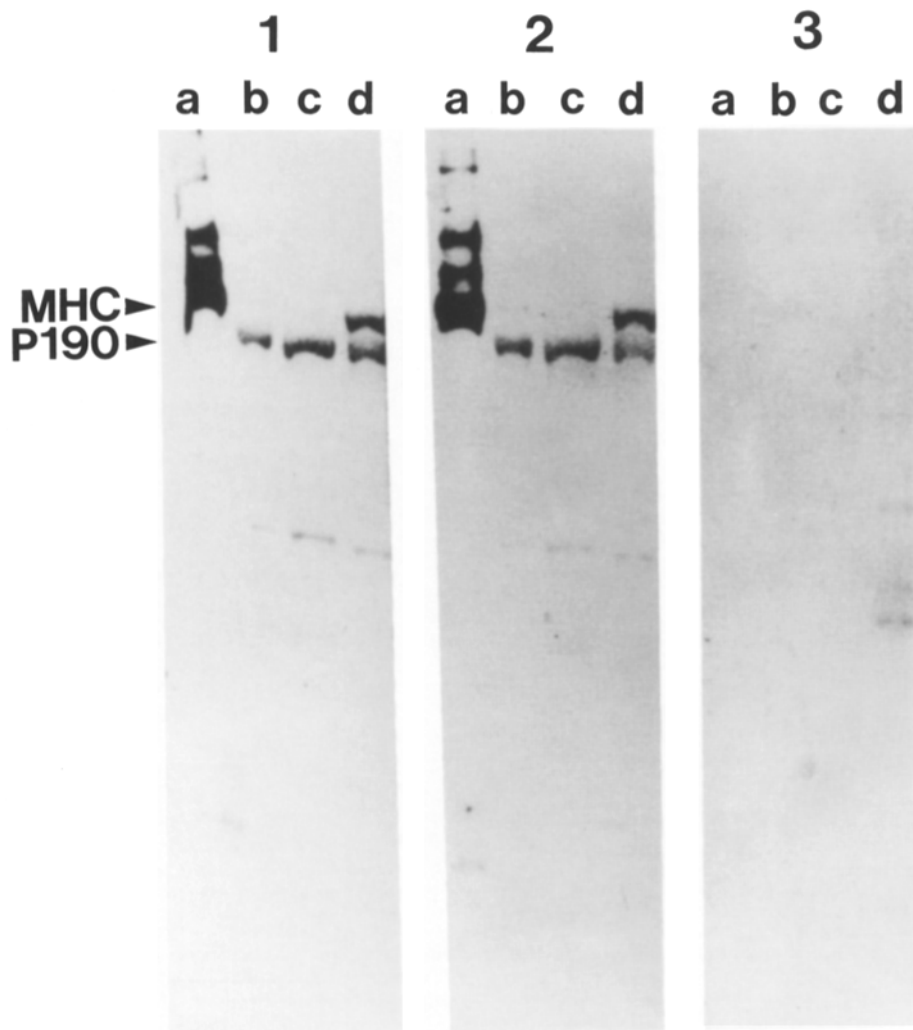
Average values ± SD of reactions done in duplicate for the number of preparations given in parentheses. ATPase assays were performed as described in Materials and Methods for reactions containing 32 μg/ml of p190 pooled fraction from the Sephacryl S-500 column, 50 μg/ml of brain myosin, 60 μg/ml of brain actin fraction, and 160 μg/ml of purified skeletal muscle actin as indicated. The brain actin fraction was adjusted to 50 mM KCl and 5 mM MgCl<sub>2</sub> before addition to the reaction media to induce polymerization to F-actin. ATP hydrolysis attributable to brain or muscle actin alone was determined in separate reactions to be <4% of the ATP hydrolysis of p190 plus brain actin.

\* Average value of duplicate assays of a single preparation.



**Figure 4.** p190 induces actin sedimentation and cosediments with actin in an ATP-sensitive manner. The low-speed actin sedimenta-

tion assay was performed as described in Materials and Methods. Lane 1 shows a sample of the p190 fraction used in this assay, purified from a Sephacryl S-500 column, concentrated five times by precipitation in 5% TCA and 0.15% deoxycholate (Peterson, 1983) and solubilized in SDS-PAGE sample buffer. Supernatants (*s*) and pellets (*p*) of the following experiments were analyzed by SDS-PAGE: (lane 2) purified muscle F-actin alone; (lane 3) F-actin plus p190 in the presence of ATP; (lane 4) F-actin plus p190 in the absence of ATP. Traces of tropomyosin (36 kD) can be detected by SDS-PAGE in the purified actin preparation.



**Figure 5.** p190 is recognized by mAbs specific for the head domain of myosin. Immunoblot analysis of PEP samples after SDS-PAGE on 7% gels probed with mAbs generated against (BB myosin I). Sets of rabbit skeletal muscle myosin (*a*) and PEP samples from rabbit (*b*), rat (*c*), and chick (*d*) brains on Western blots were probed with mAbs specific for the head domain (mAb CX-1, set 1, and mAb CX-2, set 2) or tail region (mAb CX-7, set 3) of BB myosin I and developed with anti-mouse, alkaline phosphatase conjugate.

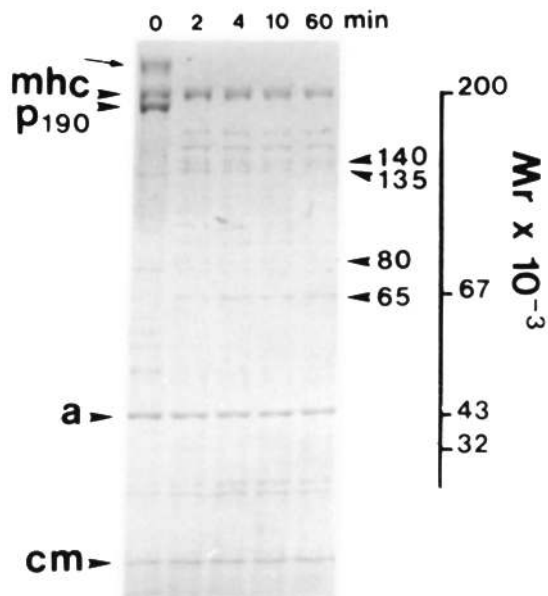


Figure 6. p190 is selectively cleaved by calpain. SDS-PAGE analysis of the proteolysis of p190 by calpain was made on samples from

## Discussion

In this paper we describe the biochemical and immunological properties of a novel calmodulin-binding myosin enriched in neuronal cells of the vertebrate central nervous system. Similar to other known nonmuscle myosins, p190 has MgATPase activity which is activated by actin in the presence of  $\text{Ca}^{2+}$  and calmodulin. Also, ATP-sensitive cross-linking of muscle F-actin by p190 has been demonstrated by

rat brain containing equivalent amounts of p190 and brain myosin II, 1.5 mg protein/ml, and incubated with calpain (0.1 mg/ml) at 27°C in 25 mM Tris-HCl, pH 7.2, containing 1 mM DTT. The reaction was initiated by the addition of  $\text{Ca}^{2+}$  to 5 mM; aliquots were removed at 2, 4, 10, and 60 min as indicated and stopped by mixing with SDS-PAGE sample buffer containing 10 mM EGTA. An aliquot removed before  $\text{Ca}^{2+}$  addition is indicated as time 0. Arrow to the left of the figure indicates a high molecular weight polypeptide which like p190 was also rapidly proteolysed by calpain. Polypeptides generated from p190 were identified by immunoblots probed with antibodies which recognized p190 (data not shown) and are indicated by arrowheads to the right of the figure.

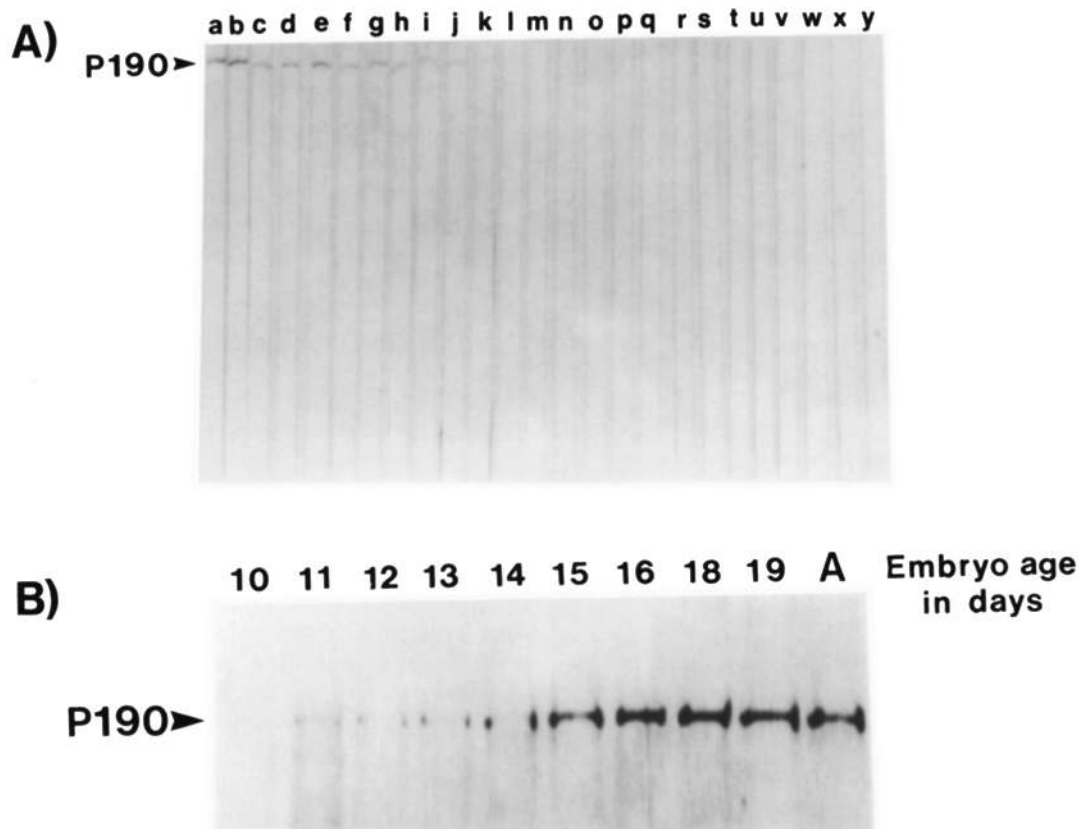
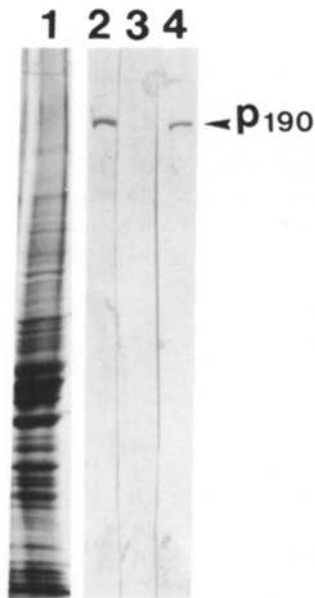


Figure 7. p190 is enriched in homogenates from various brain regions and can be detected in embryo brain. (A) Immunoblot analysis of total homogenates of adult rat tissues and brain regions probed with anti-p190 polyclonal antibodies. SDS-PAGE samples were prepared from homogenates of freshly dissected tissues in buffer A (see Materials and Methods), boiled for 2 min, ultrasonicated to break up DNA, and stored frozen until used. Samples were loaded in nearly equal amounts (35  $\mu\text{g}$ ) of total protein. Forebrain (a), cerebral cortex (b), olfactory bulb (c), corpus striatum (d), hippocampus (e), hypothalamus (f), hypophysis (g), colliculus (h), pons (i), medulla (j), cerebellum (k), spinal cord (l), thymus (m), adrenal gland (n), ovary (o), testis (p), uterus (q), pancreas (r), lung (s), spleen (t), liver (u), kidney (v), heart (w), stomach (x), and ileum (y). (B) Immunoblot analysis of total homogenates of embryos and fetal rat brains probed with anti-p190 polyclonal antibodies. Whole embryo, 10 d post coitum, embryo head, 11 d post coitum, and embryo brain, 12-16, 18, and 19 d post coitum, as consecutively indicated on the photograph, and adult brain (A).





**Figure 8.** Affinity-purified anti-p190 polyclonal antibodies are monospecific. Characterization of polyclonal antibodies generated against p190 was done on Western blots of whole brain homogenates. Lane 1 shows a Coomassie blue-stained gel of whole brain homogenate. Lanes 2-4 are immunoblots of whole brain homogenates probed with polyclonal antibodies against p190, diluted 1:1,000 (lane 2), with the preabsorbed antibody fraction, diluted 1:1,000 (lane 3), and with affinity-purified anti-p190 fraction, diluted 1:20 (lane 4).

the low-speed sedimentation assay. Structural homology between p190, BB myosin I, skeletal muscle myosin II, and brain myosin II has been demonstrated by cross-reactivity of a series of mAbs, generated against chicken BB myosin I, with specificity for epitopes in the head domain (Carboni et al., 1988). Thus, p190 has the critical enzymatic, structural, and actin-binding properties characteristic of myosins.

On the other hand, our data also show that p190 is a novel protein with properties clearly distinct from brain myosin II, BB myosin I, and known myosins from vertebrate tissues. p190 has a distinct migration on SDS-PAGE and can be separated from myosin II by gel filtration chromatography (Fig. 3; Larson et al., 1988). Also, p190 does not have typical myosin-like K-EDTA ATPase activity as does brain myosin II (Table I) and other vertebrate myosins. Calpain selectively cleaves p190 but not brain myosin II (Fig. 6). Polyclonal antibodies against p190 do not recognize brain myosin II (Fig. 2), myosins II from skeletal, cardiac, and smooth muscle, or BB myosin I (Fig. 7 A and results not shown). Nor is p190 recognized by polyclonal antibodies against brain myosin II (Larson et al., 1990), platelet myosin (Fig. 2), or monoclonals against the tail region of BB myosin I (Fig. 5).

### **p190 Binds Calmodulin**

Our biochemical studies have shown that p190 copurifies with calmodulin as a stable complex. Gel filtration chromatography shows the coelution of p190 and calmodulin in fractions well above the exclusion limits of free calmodulin, implicating the association of p190 and calmodulin during the chromatography (Fig. 3; Larson et al., 1988). In preparations where p190 was enriched and brain myosin II was absent, polypeptides in the 16-20-kD range other than calmodulin were not detected in SDS-PAGE gels, suggesting that calmodulin is the analogous light chain of this myosin-like protein. In comparison with other known calmodulin-binding proteins, p190 is most similar to the heavy chain of BB myosin I, in that calmodulin remains bound in the absence of  $Ca^{2+}$ . Indeed, all buffers used for purification contain EGTA. However, some loss of bound calmodulin during

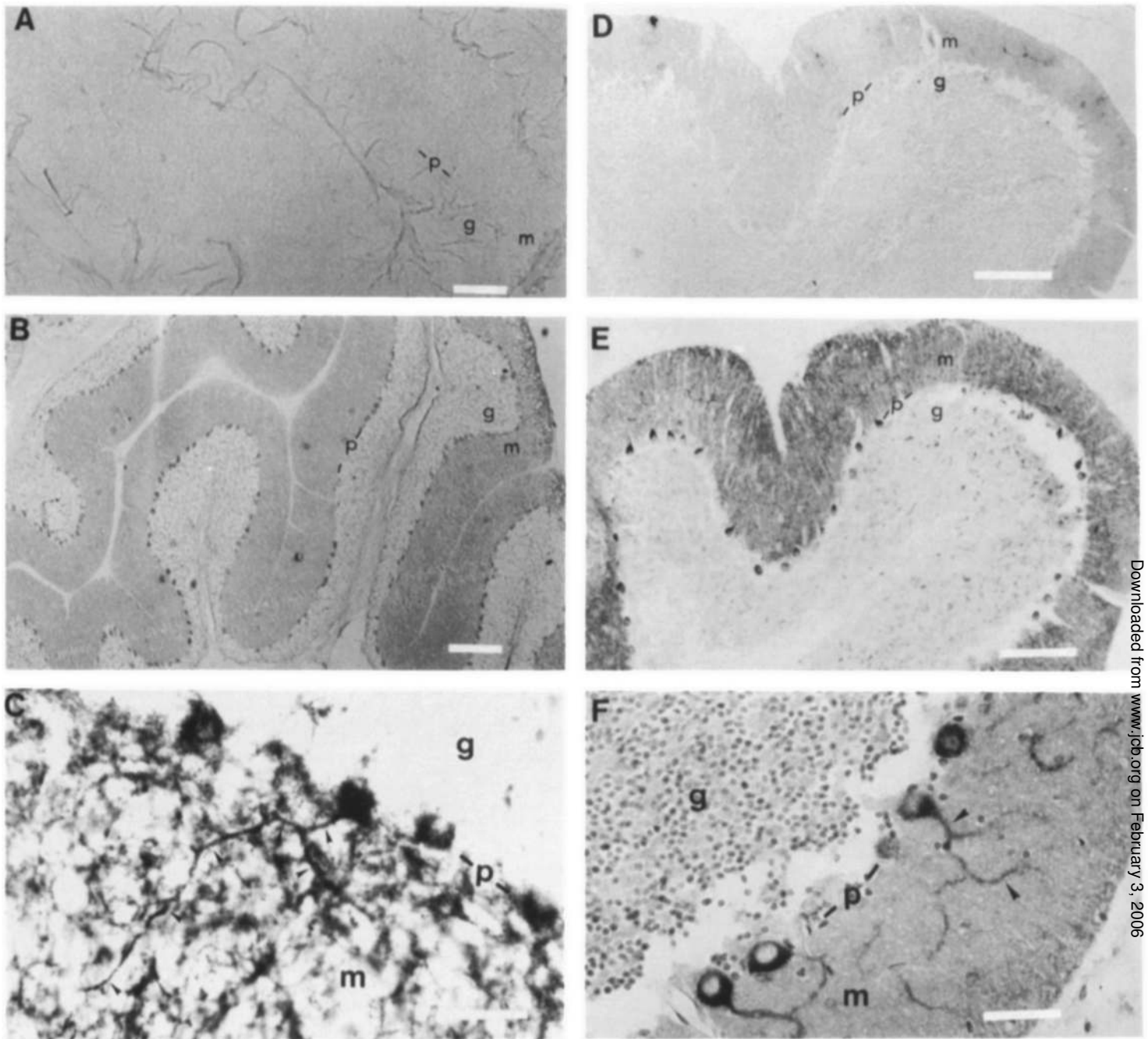
the purification of p190 appears to be occurring (see Fig. 3); similar variability in calmodulin content has been observed during purification of BB myosin I (for review and references see Mooseker et al., 1991). Future studies are required to define the precise stoichiometry of p190-calmodulin interaction, and to determine how that association might be regulated by such factors as  $Ca^{2+}$  and p190 phosphorylation. For example, it will be important to determine if, like BB myosin I, partial dissociation of its calmodulin light chains occurs at elevated  $Ca^{2+}$  (Collins et al., 1990; Swanlung-Collins and Collins, 1991).

### **Actin-activated ATPase Activity of p190 Requires $Ca^{2+}$ -sensitive Factors**

In the presence of  $Ca^{2+}$ , actin activates the MgATPase activity of p190 purified from rabbits on a Sephacryl S-500 column. Since calmodulin copurifies with p190 in the column fractions and since TFP at low concentrations (50  $\mu$ M) inhibits the  $Ca^{2+}$  effect, it seems reasonable to conclude that the stimulatory effect of  $Ca^{2+}$  occurs via the bound calmodulin. Addition of purified bovine calmodulin further stimulated the ATPase activity indicating that either the functional sites were not fully occupied by endogenous calmodulin or other factors utilizing soluble calmodulin were coming into play. Phosphorylation of myosins by calmodulin-dependent kinases is a well-known regulatory process for smooth and nonmuscle myosins. In fact, we previously reported that p190 is a phosphoprotein substrate for calmodulin-dependent kinase II under conditions where the ATPase activity is highly stimulated by  $Ca^{2+}$  and calmodulin (Larson et al., 1990). Recent experiments in our laboratory have shown that under conditions where p190 was highly phosphorylated, EGTA or TFP still inhibited the MgATPase activity to basal levels (Espindola, F. S., and R. E. Larson, unpublished observations), indicating that phosphorylation was not in itself sufficient to activate the ATPase activity. Although these experiments do not directly address what role phosphorylation may have in p190 activity, they do suggest that factors other than phosphorylation are essential. In summary, our data suggest two ways in which  $Ca^{2+}$ /calmodulin may regulate p190 activity: (1) via calmodulin directly bound to sites on the p190 molecule, and (2) via phosphorylation by  $Ca^{2+}$ /calmodulin-dependent protein kinase II.

In conclusion, the studies presented here establish that the p190-calmodulin complex possesses biochemical properties characteristic of a myosin but with properties unique from that for conventional brain myosin II. The calmodulin-binding properties of p190 suggest that it may be a member of an emerging class of unconventional myosins that contain calmodulin light chains. In addition to BB myosin I, newly characterized members include myosins I purified from adrenal medulla (Barylko et al., 1992) and renal BBs (Coluccio, 1991). Two other possible calmodulin-binding myosins, based on the presence of BB myosin I-like calmodulin-binding domains, include the predicted proteins encoded by the MYO2 gene of *S. cerevisiae* (Johnston et al., 1991) and the dilute gene of mouse (Mercer et al., 1991). In the next paper in this series (Espreafico, E., R. Cheney, M. Matteoli, P. De Camilli, R. Larson, and M. Mooseker, manuscript in preparation) we will present results of the molecular cloning of chick brain p190 (European Molecular Bi-





**Figure 9.** Photomicrographs of coronal sections through rat cerebellum incubated with anti-p190 IgG and developed by the reaction of alkaline phosphatase conjugated to anti-rabbit IgG. A, B, and C are frozen sections and D, E, and F are paraffin-embedded sections. A/B and D/E are paired sequential sections incubated with preabsorbed anti-p190 IgG (A and D) as controls and affinity-purified anti-p190 IgG (B and E). C and F are enlargements showing the intense decoration of Purkinje cell perikarya and dendritic extensions by anti-p190 antibodies. m, molecular layer; g, granular layer; and p, Purkinje layer in cerebellum. Bars, (A, B, D, and E) 200  $\mu$ m; (C and F) 20  $\mu$ m.

ology Laboratory sequence accession No. Z11718), demonstrating that p190 is, in fact, a chick homolog of the mouse dilute protein. Thus the present study represents the first biochemical characterization of a member of this novel class of calmodulin binding, unconventional myosins.

We thank Domingos E. Pitta for his expert technical assistance and Silvia Regina Andrade for auxiliary support throughout this project. We are grateful to Marcelo da Costa Gonçalves for the photomicrographs and Maria da Graça Cabeira for expert advice and materials for the preparation and manipulation of rat embryos.

Financial support was received from FAPESP 88/3914-0, CNPq

500247/89-7, and 403877/88-1 (R. E. Larson), National Institutes of Health grant AM 25387 (M. S. Mooseker), and a basic research grant from the Muscular Dystrophy Association (M. S. Mooseker). F. S. Espindola, E. M. Espreafico, and M. V. Coelho received predoctoral fellowships from PIDC-CAPES, CAPES, and CNPq, respectively. F. R. C. Costa received an undergraduate training stipend from CNPq.

Received for publication 12 August 1991 and in revised form 15 February 1992.

#### References

Augustine, G. J., M. P. Charlton, and S. J. Smith. 1987. Calcium action in synaptic transmitter release. *Annu. Rev. Neurosci.* 10:633-696.

- Barylko, B., and A. Sobieszek. 1983. Phosphorylation and actin activation of brain myosin. *EMBO (Eur. Mol. Biol. Organ.) J.* 2:369-374.
- Barylko, B., M. C. Wagner, O. Reizes, and J. P. Albanesi. 1992. Purification and characterization of a mammalian myosin I. *Proc. Natl. Acad. Sci. USA.* 89:490-494.
- Beach, R. L., P. T. Kelly, J. A. Babitch, and C. W. Cotman. 1981. Identification of myosin in isolated synaptic junctions. *Brain Res.* 225:75-93.
- Bernstein, B. W., and J. R. Bamberg. 1989. Cycling of actin assembly in synaptosomes and neurotransmitter release. *Neuron.* 3:257-265.
- Bridgman, P. C., and M. E. Dailey. 1989. The organization of myosin and actin in rapid frozen nerve growth cones. *J. Cell Biol.* 108:95-109.
- Carboni, J. M., K. A. Conzelman, R. A. Adams, D. A. Kaiser, T. D. Pollard, and M. S. Mooseker. 1988. Structural and immunological characterization of the myosin-like 110-KD subunit of the intestinal microvillar 110K-calmodulin and complex: evidence for discrete myosin head and calmodulin-binding domains. *J. Cell Biol.* 107:1749-1757.
- Collins, K., J. R. Sellers, and P. T. Matsudaira. 1990. Calmodulin dissociation regulates brush border myosin-I (110K-calmodulin) activity in vitro. *J. Cell Biol.* 110:1137-1147.
- Coluccio, L. M. 1991. Identification of the 110kD protein-calmodulin complex (myosin I) in kidney. *Eur. J. Cell Biol.* 56:286-294.
- Crick, F. 1982. Do dendritic spines twitch? *TINS (Trends Neurosci.)* 5:44-46.
- Forscher, P., and S. J. Smith. 1988. Actions of cytochalasins on the organization of actin filaments and microtubules in a neuronal growth cone. *J. Cell Biol.* 107:1505-1516.
- Fox, J. E. B., D. E. Goll, C. C. Reynolds, and D. R. Phillips. 1985. Identification of two proteins (actin-binding protein and P235) that are hydrolyzed by endogenous  $Ca^{2+}$ -dependent protease during platelet aggregation. *J. Biol. Chem.* 260:1060-1066.
- Fukui, Y., T. J. Lynch, H. Brzeska, and E. D. Korn. 1989. Myosin I is located at the leading edges of locomoting Dictyostelium amoebae. *Nature (Lond.)* 341:328-331.
- Gopalakrishana, R., and W. Anderson. 1982. Ca-induced hydrophobic site on calmodulin: application for purification of calmodulin by phenyl-sepharose affinity chromatography. *Biochem. Biophys. Res. Commun.* 104:P830-836.
- Harlow, E. and D. Lane. 1988. *Antibodies: A Laboratory Manual.* Cold Spring Harbor Laboratory, Cold Spring Harbor, NY. pp. 298-305, 379, 505.
- Harris, A. S., and J. S. Morrow. 1990. Calmodulin and calcium-dependent protease I coordinately regulate the interaction of fodrin with actin. *Proc. Natl. Acad. Sci. USA.* 87:3009-3013.
- Hartree, E. F. 1972. Determination of protein: a modification of the Lowry method that gives a linear photometric response. *Anal. Biochem.* 48:422-427.
- Hayden, S. M., J. S. Wolenski, and M. S. Mooseker. 1990. Binding of brush border myosin I to phospholipid vesicles. *J. Cell Biol.* 111:443-451.
- Heinonen, J. K., and R. J. Lahti. 1981. A new and convenient colorimetric determination of inorganic orthophosphate and its application to the assay of inorganic pyrophosphatase. *Anal. Biochem.* 113:313-317.
- Hobbs, D. S., and D. W. Frederiksen. 1983. Physical and enzymatic properties of myosin from porcine brain. *Biophys. J.* 32:705-718.
- Hunkapiller, M. W., E. Lujan, F. Ostrander, and L. E. Hood. 1983. Isolation of microgram quantities of proteins from polyacrylamide gels for amino acid sequence analysis. *Meth. Enzymol.* 91:227-236.
- Johnston, G. C., J. A. Prendergast, and R. A. Singer. 1991. The Saccharomyces cerevisiae MYO2 gene encodes an essential myosin for vectorial transport of vesicles. *J. Cell Biol.* 113:539-551.
- Kandel, E. R. 1981. Calcium and the control of synaptic strength by learning. *Nature (Lond.)* 293:697-700.
- Kater, S. B., M. P. Mattson, C. Cohan, and J. Connor. 1988. Calcium regulation of the neuronal growth cone. *TINS (Trends Neurosci.)* 11:315-321.
- Kennedy, M. B. 1989. Regulation of synaptic transmission in the central nervous system: long-term potentiation. *Cell.* 59:777-787.
- Kiehart, D. P. 1990. Molecular genetic dissection of myosin heavy chain function. *Cell.* 60:347-350.
- Kuczmarzski, E. R., and J. L. Rosenbaum. 1979. Studies on the organization and localization of actin and myosin in neurons. *J. Cell Biol.* 80:356-371.
- Laemmli, U. K., and M. Favre. 1973. Maturation of the head of bacteriophage T4. *J. Mol. Biol.* 80:575-599.
- Lankford, K. L., and P. C. Letourneau. 1989. Evidence that calcium may control neurite outgrowth by regulating the stability of actin filaments. *J. Cell Biol.* 109:1229-1243.
- Larson, R. E., D. E. Pitta, and J. A. Ferro. 1988. A novel 190 KDa calmodulin-binding protein associated with brain actomyosin. *Braz. J. Med. Biol. Res.* 21:213-217.
- Larson, R. E., F. S. Espindola, and E. M. Espreafico. 1990. Calmodulin-binding proteins and calcium/calmodulin-regulated enzyme activities associated with brain actomyosin. *J. Neurochem.* 54:1288-1294.
- Letourneau, P. C. 1981. Immunocytochemical evidence for colocalization in neurite growth cones of actin and myosin and their relationship to cell-substratum adhesions. *Dev. Biol.* 85:113-122.
- Malik, M. N., M. D. Fenko, L. Scotto, P. Merz, J. Rothman, H. Tuzio, and H. M. Wisniewski. 1983. Purification and characterization of myosin from calf brain. *J. Neurochem.* 40:1620-1629.
- Matsumura, S., K. Ohmori, T. Chiba, and A. Kumon. 1989. Physical, enzymatic, and contractile properties of brain myosin with anti-brain myosin Fab fragment bound on its tail. *J. Biochem.* 105:803-812.
- McLean, I. W., and P. K. Nakane. 1974. Periodate-lysine-paraformaldehyde fixative. A new fixative for immunoelectron microscopy. *J. Histochem Cytochem.* 22:1077-1083.
- Mercer, J. A., P. K. Seperack, M. C. Strobel, N. G. Copeland, and N. A. Jenkins. 1991. Novel myosin heavy chain encoded by murine dilute coat colour locus. *Nature (Lond.)* 349:709-713.
- Montell, C., and G. Rubin. 1988. The Drosophila ninaC locus encodes two photoreceptor cell specific proteins with domains homologous to protein kinases and the myosin heavy chain head. *Cell.* 52:757-762.
- Mooseker, M. S., J. S. Wolenski, T. R. Coleman, S. M. Hayden, R. E. Cheney, E. Espreafico, M. B. Heintzelman, and M. D. Peterson. 1991. Structural and functional dissection of a membrane-bound mechanoenzyme: brush border myosin I. *Current Topics in Membr. Trans.* 38:31-55.
- Ochs, S. 1982. Calcium and the mechanism of axoplasmic transport. *Federation Proc.* 41:2301-2306.
- Olmsted, J. B. 1986. Analysis of cytoskeletal structures using blot-purified monospecific antibodies. *Methods Enzymol.* 134:467-472.
- Peterson, G. L. 1983. Determination of total protein. *Methods Enzymol.* 91:95-119.
- Pollard, T. D., and J. A. Cooper. 1982. Methods to characterize actin filament networks. *Methods Enzymol.* 85:211-233.
- Pollard, T. D., S. K. Doberstein, and H. G. Zot. 1991. Myosin-I. *Annu. Rev. Physiol.* 53:653-681.
- Ratner, N., and H. R. Mahler. 1983. Structural organization of filamentous proteins in post synaptic density. *Biochemistry.* 22:2446-2453.
- Smith, S. J. 1988. Neuronal cytomechanics: the actin-based motility of growth cones. *Science (Wash. DC).* 242:708-715.
- Smith, S. J., and G. J. Augustine. 1988. Calcium ions, active zones and synaptic transmitter release. *TINS (Trends Neurosci.)* 10:458-464.
- Sobue, K., T. Tanaka, K. Kanda, and N. Ashino. 1987. Control of cytoskeleton mediated by cytochalasins (cytoskeleton-related calmodulin-binding proteins). In *The Role of Calcium in Biological Systems.* L. J. Anghileri, editor. CRC Press, Inc. IV:21-35.
- Spudich, J. and S. Watt. 1971. The regulation of rabbit skeletal muscle contraction I. Biochemical studies of the interaction of the tropomyosin-troponin complex with actin and the proteolytic fragments of myosin. *J. Biol. Chem.* 246:4866-4871.
- Swanlung-Collins, and J. H. Collins. 1991.  $Ca^{2+}$  stimulates the  $Mg^{2+}$  ATPase activity of brush border myosin I with three or four calmodulin light chains but inhibits with less than two bound. *J. Biol. Chem.* 266:1312-1319.
- Tanaka, E., K. Fukunaga, H. Yamamoto, T. Iwasa, and E. Miyamoto. 1986. Regulation of the actin-activated Mg-ATPase of brain myosin via phosphorylation by the brain  $Ca^{2+}$ , calmodulin-dependent protein kinases. *J. Neurochem.* 47:254-262.
- Towbin, H., T. Staehelin, and J. Gordon. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. USA.* 76:4350-4354.
- Wang, K. K. W., A. Villalobo, and B. D. Roufogalis. 1989. Calmodulin-binding proteins as calpain substrates. *Biochem. J.* 262:693-706.