Evidence for the Presence of Myosin I in the Nucleus*

(Received for publication, February 26, 1997, and in revised form, April 30, 1997)

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We produced and affinity-purified polyclonal antibodies to adrenal myosin I. These antibodies recognize adrenal myosin I by Western blot analysis (116 kDa) and inhibit the actin-activated ATPase activity of purified adrenal myosin I. They also recognize a 120-kDa protein in extracts prepared from many different cell lines. Fluorescence microscopy demonstrated the presence of immunoreactive material in the perinuclear region, the leading edges, and the nuclei of 3T3 cells. Fluorescence microscopy also demonstrated nuclear staining in mouse oocytes at the germinal vesicle stage and in the pronuclei during fertilization. Confocal and immunoelectron microscopy confirmed the intranuclear localization. Electron microscopy also demonstrated staining of structures in nucleoli that are thought to be associated with rDNA transcription. Western blot analyses revealed the presence of the 120-kDa protein in extracts prepared from nuclei that are apparently free of cytosolic contamination. The same nuclear protein binds 125I-calmodulin and is photoaffinity labeled with [a-32P]ATP. The 120-kDa protein was partially purified from twice washed nuclei using ammonium sulfate fractionation and gel filtration chromatography. Column fractions containing 120-kDa protein as revealed by Western blot analysis also contain K+\-EDTA ATPase activity. The 120-kDa protein was also shown to bind actin in the absence, but not the presence, of ATP. Since K+\-EDTA ATPase activity, actin, and ATP binding are defining features of the members of the myosin superfamily of proteins, we propose that the 120-kDa protein is a previously undescribed myosin I isoform that is an intranuclear actin-based molecular motor.

Myosin I is a single-headed, monomeric, actin-activated ATPase (1). First described in Acanthamoeba castellanii (1), myosin I is now known to be widely distributed in metazoan cells (1–12). As additional myosin I proteins have been identified, it has become clear that there are at least four different subclasses of myosin I (1). All myosin I proteins consist of a 110–150-kDa heavy chain and 1–6 light chains located in the neck region between the head and tail (1). This light chain has been shown to be calmodulin in vertebrate myosin I proteins (1). Immunofluorescence studies of mammalian cells have shown that myosin I is diffusely distributed throughout the entire cytoplasm and that it concentrates near cortical surfaces and in the perinuclear region (12, 13). Although evidence for specific roles of myosin I proteins in metazoan cells is lacking, there is speculation, based on localization studies, that myosin I proteins are molecular motors involved in plasma membrane extension (12, 13), vesicle and organelle transport (14), and mecha-nochemical regulation of calcium channels in hair cells (15). To investigate the role of myosin I in mammalian cells, we produced and affinity-purified polyclonal antibodies to adrenal myosin I. These antibodies recognize a 120-kDa protein that is found in the cytoplasm and the nucleus. Moreover, we present immunological and biochemical data that support the notion that this 120-kDa protein is an intranuclear, actin-based ATPase of the myosin I subfamily of molecular motors.

EXPERIMENTAL PROCEDURES

Production and Purification of Antibodies—Adrenal myosin I was purified as described previously (3) except that we used the entire bovine adrenal gland. Polyclonal antibodies to bovine adrenal myosin I were raised in rabbits as described previously (16). Specific antibodies were purified from the immune serum on an adrenal myosin I-Sepha-rose 4B column and all the data reported used these purified antibodies. Control antibodies were purified from normal rabbit serum by affinity chromatography on a protein A-Sepharose column. Antibodies to non-muscle myosin II were produced, affinity-purified, and characterized as described previously (17). Monoclonal antibodies to bovine adrenal medullary myosin I (M2 clone) directed against the tail region (12) were a gift from Dr. Joseph P. Albanesi (University of Texas Southwestern Medical Center, Dallas, Texas). Polyclonal (rabbit) anti-78-kDa glucose-regulated protein/BiP (anti-GRP78) antibodies were purchased from Affinity Bio-Reagents Inc. (Neshanic Station, NJ).

Cell Culture—NIH 3T3 fibroblasts and J774 and MDCK cells were cultured in Dulbecco’s modified Eagle’s medium plus 10% calf serum and antibiotics at 37 °C in an atmosphere containing 5% CO2.

Light Microscopy—Cells grown on coverslips were fixed by incubating in freshly made 3% formaldehyde in phosphate-buffered saline for 7 min at room temperature. Cells were then washed in phosphate-buffered saline and stained with 2 μg/ml affinity-purified myosin I or myosin II antibodies followed by Texas red-conjugated anti-bodies to rabbit IgG (Jackson Immunoresearch Laboratories, Inc., West Grove, PA). The coverslips were mounted in Vectorstain and photographed using a Zeiss IM 35 inverted photomicroscope using a Planapochromat ×63, 1.4 numerical aperture objective. Confocal images were obtained using a Bio-Rad MRC 600 laser-scanning confocal microscope equipped with a krypton-argon mixed gas laser. The scanhead was mounted on a Nikon Optiphot, and the COSMOS operating software was installed on a Dell 468 computer. A ×60, 1.4 numerical aperture objective was used with the spatial filter set at 0 to obtain approximately 0.5-μm sections. Oocytes were collected and

* This work was supported in part by the Grant Agency of the Academy of Sciences of the Czech Republic Grant A5039701 (to P. H.), National Institutes of Health (NIH) Grants HD 12912, HL 32887 (to G. S.), and HL 02411 (to P. de L.), and National Science Foundation (NSF) Grant MCB 9631833 (to P. de L.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ Supported by NIH Grant HL 076922.

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FIG. 1. Western blot analysis of myosin I distribution. Purified adrenal myosin I (M-I) and total cell extracts prepared from NIH 3T3, J774, and Madin-Darby canine kidney cells (MDCK, left panel) and cytoplasmic (Cyto) and cytoplasmic (Cyto) and nuclear (Nuc) fractions prepared from NIH 3T3 cells (right panel) were separated by SDS-PAGE and transferred to nitrocellulose. The left panel was probed with affinity-purified polyclonal antibodies to adrenal myosin I (M-IpAb). The right panel was probed (top to bottom) with polyclonal antibodies to nonmuscle myosin II (M-IIAb), polyclonal antibodies to myosin I (M-IpAb), monoclonal antibodies to tail domain of adrenal myosin I (M-ImAb), and polyclonal antibodies to GRP78 (GRP78Ab). Note that the polyclonal and monoclonal antibodies to adrenal myosin I react with different proteins. Moreover, the antibody to myosin II, the monoclonal antibody to myosin I, and the GRP78 antibody identify only cytoplasmic proteins.

In contrast, the polyclonal antibody to adrenal myosin I recognizes protein bands in both the cytoplasmic and nuclear fractions.

Table

| Antibody combination | Specific activity (nmol/min/mg myosin) |
|----------------------|---------------------------------------|
| Myosin I + actin + control antibody | 50.7 |
| Myosin I + actin + myosin I antibody | 0.0 |

Adrenal myosin I (0.12 μm) was assayed at 25 °C in the presence or absence of 10 μM F-actin. The myosin I preparation was reconstituted with control antibody (5 μM, final concentration) or with affinity-purified antibodies to myosin I (3.4 μM, final concentration) for 15 min on ice. ATPase assays performed as previously described (2) were linear with respect to time.

Western blot analysis—Whole cell extracts and cytoplasmic and nuclear fractions (100 μg each) were separated by SDS-PAGE on 5–20% gradient slab gels and transferred to 0.2-μm nitrocellulose paper (17). The nitrocellulose sheets were incubated with affinity-purified antibodies to myosin I (0.4 μg/ml), anti-myosin II antibodies (0.4 μg/ml), anti-GRP78 antibodies (0.5 μg/ml), or mouse monoclonal anti-myosin I antibodies (10 μg/ml) followed by peroxidase-conjugated secondary antibodies.

Partial purification of the 120-kDa protein—Cells grown to confluence in 150 15-cm dishes were harvested and fractionated, and the isolated nuclei were isolated using a tworesuspension/centrifugation washes through a sucrose cushion as described above. They were suspended in 1 M NaCl, 1 mM DTT, 0.5% Nonidet P-40, 0.4 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 10 μg/ml pepstatin, 10 mM MgCl2, 40 μg/ml DNase, 20 mM Tris-HCl, pH 7.5, and extracted by passing them through hyperdermic needles of decreasing gauge down to a 27-gauge needle. The supernatant was collected by centrifugation, ATP added to a final concentration of 10 mM, and subjected to ammonium sulfate fractionation. The protein precipitating between 20 and 65% ammonium sulfate was dissolved in 0.5 M NaCl, 5 mM EDTA, 1 mM EGTA, 1 mM DTT, 50 mM Tris, pH 7.5, and MgCl2 and ATP were added to final concentrations of 12 mM and 10 mM, respectively. This solution was clarified by centrifugation (50,000 × g, 10 min) and applied to a 1.25 × 70-cm Sepharose 4B column (85 ml).

Actin binding assays—Fractions from the gel filtration column that contained the 120-kDa protein were pooled, dialyzed in 50 mM KCl, 1 mM DTT, 1 mM EDTA, 20 mM Tris, pH 7.5, concentrated, spin at 100,000 × g for 20 min, and incubated with 0.2 mg/ml F-actin, 50 mM KCl, 1 mM DTT, 1 mM EDTA, 2 mM MgCl2, 1 mM CaCl2, 20 mM Tris, pH 7.5 (100 μl total volume) for 60 min at 4 °C. ATP (10 mM, final concentration) was added to some samples and incubated for another 10 min. All samples were then spun at 100,000 × g for 20 min. The entire pellet and supernatant fractions were separated by SDS-PAGE and transferred to nitrocellulose, and probed with the polyclonal antibody to myosin I.

Other methods—Myosin II was purified from adrenal glands. Calmodulin overlay (20), ATP photolabeling (21), K+-EDTA ATPase (2) and protein (22) assays were performed as described.

RESULTS

Characterization of Antibodies—We produced and affinity-purified polyclonal antibodies to bovine adrenal myosin I. Western blot analyses demonstrated that the purified antibodies recognized purified adrenal myosin heavy chain (Fig. 1). The antibodies predominantly recognized a slightly larger, 120-kDa protein in extracts prepared from a number of mammalian cell lines (Fig. 1). In some cells (e.g., Madin-Darby canine kidney cells (MDCK) in Fig. 1), the antibodies reacted with a 116-kDa protein that co-migrated with adrenal myosin I. It was also possible to visualize this 116-kDa protein upon longer development of the color reaction in all cell types. Antibodies to myosin I also inhibited the actin-activated ATPase activity of purified

| Protein | M-I | 120 | 116 |
|---------|-----|-----|-----|
| Myosin I | 1.2 | 1.0 | 0.8 |
| Myosin II | 1.1 | 0.9 | 0.7 |

1 The abbreviations used are: WGA, wheat germ agglutinin; ConA, concanavalin A; DTT, dithiothreitol; PAGE, polyacrylamide gel electrophoresis; GRP78, 78-kDa glucose-regulated protein/BiP.
adrenal myosin I (Table I). Antibodies to myosin II, which were used as a control antibody, only recognized myosin II by Western blot analysis (Fig. 1).

Analysis of Cellular Fractions—Nuclei isolated from interphase cells were used to investigate the presence of the 120-kDa protein. These nuclei and all those used in subsequent experiments were washed twice by resuspension/centrifugation through a sucrose cushion to eliminate cytoplasmic contamination. The purified nuclei, when fixed, permeabilized, and immunostained, showed myosin I staining but no myosin II staining (data not shown). Western blot analysis of nuclei and cytoplasmic fractions (Fig. 1) showed that myosin II and GRP78, a marker for the endoplasmic reticulum (48), are present only in the cytoplasmic fraction. The same is true for the 116-kDa myosin I isoform recognized by the well characterized M2 monoclonal antibody to adrenal myosin I (12). In contrast, the polyclonal antibodies to adrenal myosin I described here recognize proteins with a slightly higher molecular weight (Mr 120,000) in both the cytoplasmic and nuclear fractions (Fig. 1). The difference in the molecular weights of the myosin I isoforms identified by the monoclonal and polyclonal antibodies was confirmed by reprobing the nitrocellulose sheets with the complementary antibodies, i.e. first polyclonal and then monoclonal and vice versa (not shown). Thus, the 116-kDa protein appears to be present only in the cytoplasm, while the 120-kDa protein is found in both the cytoplasm and the nucleus.

Fluorescence, Confocal, and Electron Microscopy—Immunofluorescence analysis using fluorescence and confocal microscopy showed that myosin I is located in the perinuclear region and the leading edges of migrating cells (Fig. 2) as expected from earlier work (12, 13). The myosin I antibodies also stained the nucleus (Fig. 2). Optically sectioning cells through the nucleus using confocal microscopy demonstrated intranuclear staining (Fig. 2). The myosin I antibodies also stained

FIG. 2. Localization of myosin I in interphase NIH 3T3 fibroblasts. Panels A and B are phase contrast and epifluorescence images of the same cells. The nuclei in all of these cells are stained by the myosin I antibodies. Panels C and D are confocal images taken at the same magnification. Panel C shows an optical slice through the nucleus that clearly demonstrates intranuclear localization of myosin I. Panel D shows an optical slice that emphasizes the perinuclear and membrane localization of myosin I. Panels E and F are phase contrast (E) and epifluorescence (F) images of the same cells that were stained with antibodies to myosin II and the same secondary antibody used in panels B–D. Note the absence of nuclear staining in panel F. Bars, 10 μm.
nucleoplasm of the germinal vesicle of mouse oocytes before fertilization (Fig. 3A) and the pronuclei during fertilization (Fig. 3E). In contrast, the antibodies stained vesicles distributed throughout the cytoplasm following breakdown of the germinal vesicle (Fig. 3C). The relocation of myosin I to the interphase pronucleus after meiosis completion was also investigated by injecting unfertilized oocytes with either ConA or WGA and then parthenogenetically activating the oocytes. WGA, unlike ConA, blocks the completion of pro-nuclear formation by binding to a nucleopore glycoprotein and preventing the uptake of nonchromosomal proteins (23). WGA also blocks the movement of myosin I into the haploid female pronucleus (Fig. 3, I-L).

The staining pattern seen in Fig. 2 was retained when cells were fixed in methanol (12) or fixed, permeabilized, and stained in cytoskeletal stabilizing buffer (13), procedures previously used to visualize myosin I in the cytoplasm. Preabsorbing the myosin I antibodies with purified adrenal myosin I removed the staining pattern in both 3T3 cells (not shown) and oocytes (Fig. 3G). Antibodies to myosin II, in contrast, stained stress fibers in 3T3 fibroblasts and never stained the nucleus (Fig. 2). It is noteworthy that the same secondary antibody was used to visualize the myosin I and myosin II antibodies in Fig. 2.

Electron microscopy was also used to locate the protein recognized by the antibodies to adrenal myosin I (Fig. 4). These experiments clearly demonstrated the presence of immunoreactive material in the nucleoplasm of 3T3 cells (Fig. 4, top panel). Moreover, the protein recognized by the myosin I antibody co-localizes with actin in the nucleus (Fig. 4, inset). High power examination of nucleoli demonstrated that the antibody stained mainly the dense fibrillar component where transcription of rDNA takes place (Fig. 4, bottom panel). This component surrounds fibrillar centers that are rich in actin (24, 25).

Biochemical Characterization—A calmodulin overlay assay performed on purified nuclei showed the presence of a 120-kDa protein that binds 125I-calmodulin (Fig. 5). Extracts were also prepared under nondenaturing conditions, photolabeled with [α-32P]ATP, separated by SDS-PAGE, and transferred to nitrocellulose (Fig. 5). An autoradiogram of the nitrocellulose sheet demonstrated photolabeling of a 120-kDa protein in nuclei that had been washed once or twice. Following autoradiography, the nitrocellulose sheets from the calmodulin binding and ATP photolabeling assays were probed with polyclonal antibodies to myosin I. These experiments showed that the 120-kDa protein is not recognized by the antibodies to myosin I.

To characterize this protein further, the 120-kDa protein was partially purified from twice washed, 3T3 nuclei using ammonium sulfate fractionation and gel filtration chromatography. Column fractions were assayed using Western blotting and K'-EDTA ATPase assays. K'-EDTA ATPase activity is an unique feature of myosin molecules that was used in the original investigation that identified myosin I in *Acanthamoeba* (2). Fig. 6 shows that fractions containing the 120-kDa protein also contain K'-EDTA ATPase activity. Column fractions containing the 120-kDa protein were pooled, concentrated, and tested for actin binding because members of the myosin superfamily bind actin in the absence, but not the presence, of ATP (1). Fig. 7 shows the 120-kDa protein binds actin in the absence, but not in the presence of ATP.

**DISCUSSION**

The data presented above demonstrate the following: (a) the presence of a 120-kDa protein in nuclei apparently free of cytosolic contamination that is recognized by polyclonal antibodies to adrenal myosin I; (b) immunostaining of the nucleus with affinity-purified polyclonal antibodies to adrenal myosin I that inhibit the actin-activated ATPase activity of purified adrenal myosin I; (c) differential staining of the germinal vesicle, cytoplasm, and pronuclei in the female mouse oocyte development; (d) co-localization of the 120-kDa protein and actin in the nucleus; (e) elimination of the nuclear staining when pronuclear formation is blocked or when the antibodies are preincubated with purified adrenal myosin I; (f) the immunoreactive 120-kDa protein binds 125I-calmodulin and is photoaffinity-labeled with ATP; (g) the presence of K'-EDTA
M-I protein following gel filtration chromatography of nuclear extracts for protein (Fractions (0.5 ml each) were analyzed for protein (○) and K^-EDTA ATPase activity (△) and by Western blotting to detect the presence of the 120-kDa protein (inset). The same fractions contain the 120-kDa protein and K^-EDTA ATPase activity. M-I, purified adrenal myosin; N, nuclear extract.

ATPase activity in column fractions containing the 120-kDa protein following gel filtration chromatography of nuclear extracts apparently free of cytoplasmic contaminants; (h) actin binding by the 120-kDa protein in the absence, but not the presence, of ATP. K^-EDTA ATPase activity, actin, and ATP binding are defining features of the myosin superfamily of proteins, and calmodulin binding is a signature feature of all unconventional myosins (1). Therefore, we conclude that the 120-kDa protein is a previously undescribed myosin I isoform that localizes to the nucleus.

Previous experiments have demonstrated the presence of myosin I in the leading edges of Dictyostelium amoeba (28) and fibroblasts (12, 13), growth cones in neural cells (12), the tips of stereocilia in hair cells (15), Golgi-derived vesicles from epithelial cells (14), and the midbody during cytokinesis (27). These studies did not report nuclear staining, perhaps due to restricted reactivity of the antibodies used in these experiments with individual myosin I isoforms. For instance, antibodies to Acanthamoeba myosin IB do not stain structures containing myosin IC (28), and antibodies to brush border myosin I do not react with adrenal myosin I (12). Furthermore, the polyclonal antibodies to adrenal myosin I described here have a broader range than monoclonal antibodies to adrenal myosin I (12). The monoclonal antibodies only recognize a cytosolic protein with a molecular weight of approximately 116,000, whereas the polyclonal antibodies recognize the 116-kDa protein and a protein with a slightly higher molecular weight (M, 120,000) (Fig. 1). This difference in antibody reactivity was confirmed by preparing a whole cell extract from 3T3 cells and subjecting it to ammonium sulfate fractionation and gel filtration chromatography. Column fractions were then separated by SDS-PAGE, transferred to nitrocellulose, and probed with either the polyclonal or the monoclonal antibodies to adrenal myosin I. This experiment (not shown) demonstrated that the monoclonal antibody recognized a protein (M, 116,000) that eluted from the column later (peak at fraction 96) than the 120-kDa protein recognized by the polyclonal antibodies (peak at fraction 81 as shown in Fig. 6, inset). Thus, the polyclonal antibodies to adrenal myosin I described in this paper apparently react with a novel myosin I isoform.

Fluorescence microscopy performed on mouse oocytes double stained with the antibodies to myosin I and a DNA dye demonstrated that the myosin I antibodies stained predominantly the germinal vesicle (Fig. 3). In contrast, the 120-kDa protein associates with vesicles distributed throughout the cytoplasm following breakdown of the germinal vesicle (Fig. 3C). Following fertilization, the myosin I staining decreases in the cytoplasm and is seen mainly in the pronuclei (Fig. 3E). This movement of myosin I into the nucleus could be blocked by microinjecting oocytes with the lectin WGA and then pantherogenically activating the oocytes (Fig. 3F). WGA, but not other lectins such as ConA, inhibits the uptake of nonchromosomal proteins into reforming nuclei and blocks full nuclear decondensation after telophase by binding to a 62-kDa pore glycoprotein (23). The demonstration that WGA blocks both myosin I import (Fig. 3F) and the completion of female pronuclear formation (Fig. 3L) strongly support the idea that myosin I, like other nuclear proteins (29, 30), is a cytosolic protein in meiosis that is retargeted to the nucleus postactivation.

The presence of molecular motors in the nucleus is likely for a number of reasons. Nascent DNA tightly associates with a nuclear “matrix” (31), and the presence of a filamentous network in the nucleus has been demonstrated (31, 32). Although there is no consensus as to the composition of this network, it has been suggested that replication occurs as templates move through replication “factories” that are attached to the nuclear “matrix” (31), and the presence of a filamentous network in the nucleus has been demonstrated (33, 34). Transcription of ribosomal genes has been shown to take place in the “dense fibrillar component” on the surface of the fibrillar centers, and a dynamic model in which templates move through an array of RNA polymerases on the surface of the fibrillar centers has been suggested (24, 25). Here we show that these fibrillar centers are rich in actin and that the dense fibrillar component contains myosin I.

The presence of actin and myosin I in the same nuclear structure is of potential importance. Actin has previously been described in the nucleus (35–39), frequently in association with the nuclear matrix (36–38) but not in association with a myosin. The presence of nucleus-specific actin-binding proteins has also been demonstrated (40, 41). It has also been suggested that actin interacts with small ribonucleoproteins in the processing and transport of RNA (37). In addition, injection of anti-actin antibodies blocks chromosome condensation in Xenopus oocytes (42) and transcription of lampbrush chromosomes.

FIG. 6. Fractionation of twice washed nuclear extracts by Sepharose 4B chromatography. Fractions (0.5 ml each) were analyzed for protein (○) and K^-EDTA ATPase activity (△) and by Western blotting to detect the presence of the 120-kDa protein (inset). The same fractions contain the 120-kDa protein and K^-EDTA ATPase activity. M-I, purified adrenal myosin; N, nuclear extract.

FIG. 7. Actin binding to the 120-kDa protein. Fractions 65–93 in Fig. 6 were pooled, concentrated, and incubated in the absence of F-actin, the presence of F-actin (+Actin) or the presence of F-actin and 10 mM ATP (+Actin, +ATP) and incubated for another 10 min. All samples were then spun at 100,000 × g for 20 min. The pellet (P) and supernatant (S) fractions were subjected to a Western blot analysis using polyclonal antibodies to myosin I. MW, molecular weight markers; M-I, purified adrenal myosin I (M, 116,000). Note the presence of the 120-kDa protein in the pellet only when incubated with F-actin in the absence of ATP.
remain to be seen. and whether myosin I functions as a nuclear molecular motor involved in transcription. Whether such interactions take place transcription (Fig. 4) add credence to the idea that myosin I is structures in the nucleolus thought to be involved in rDNA and actin in the nucleoplasm and the localization of myosin I in movement by simultaneously binding to actin through the ac-

positively charged C-terminal tail that is known to interact with phospholipids and to power the movement of membrane fragments on actin filaments (47). Similarly, myosin I could bind to negatively charged nuclear components and power their movement by simultaneously binding to actin through the actin binding site on the head (1). The co-localization of myosin I and actin in the nucleoplasm and the localization of myosin I in structures in the nucleolus thought to be involved in rDNA transcription (Fig. 4) add credence to the idea that myosin I is involved in transcription. Whether such interactions take place and whether myosin I functions as a nuclear molecular motor remain to be seen.

Acknowledgments—We thank Joe Albanesi and Barbara Barylko for providing the M2 monoclonal antibody to myosin I, Jim Sellers for providing actin, and Sergei Popov for comments on the manuscript.

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