Tissue Distribution and Subcellular Localization of Mammalian Myosin I

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Abstract. Myosin I, a nonfilamentous single-headed actin-activated ATPase, has recently been purified from mammalian tissue (Barylko, B., M. C. Wagner, O. Reizes, and J. P. Albanesi. 1992. Proc. Natl. Acad. Sci. USA. 89:490–494). To investigate the distribution of this enzyme in cells and tissues mAbs were generated against myosin I purified from bovine adrenal gland. Eight antibodies were characterized, five of them (M4–M8) recognize epitope(s) on the catalytic “head” portion of myosin I while the other three (M1–M3) react with the “tail” domain. Immunoblot analysis using antiadrenal myosin I antibody M2 demonstrates the widespread distribution of the enzyme in mammalian tissues. Myosin I was immunolocalized in several cell types including bovine kidney (MDBK), rat kidney (NRK), rat brain, rat pheochromocytoma (PC12), fibroblast (Swiss 3T3), and CHO cells. In all cases, myosin I was concentrated at the cell periphery. The most intense labeling was observed in regions of the cell usually associated with motile activity (i.e., filopodia, lamellipodia and growth cones). These results are consistent with earlier observations on protozoan myosin I that suggest a motile role for the enzyme at the plasma membrane.

Many examples of cell motility including endocytosis, exocytosis, protein and organelle translocation, and overall cell shape changes, occur at or near the plasma membrane (Trinkaus, 1984). Since the actin cytoskeleton is concentrated beneath the plasma membrane, it is likely that actin-dependent motors may generate the force required for movement at the cell cortex. Myosin I, a single-headed actin-activated ATPase, is a good candidate for such a motor (Korn and Hammer, 1990; Pollard et al., 1991). Myosin I has been immunolocalized to the plasma membrane of Acanthamoeba (Baines and Korn, 1989), Dictyostelium (Fukui et al., 1989), and intestinal brush border (Matsudaira and Burgess, 1979; Coudrier et al., 1981; Glenney et al., 1982) and the purified myosin I enzymes from those sources have been shown to bind directly to biomembranes and phospholipid vesicles (Adams and Pollard, 1989; Miyata et al., 1989; Hayden et al., 1990). If myosin I is really involved in essential functions at the cell surface, then it should be expressed in all eukaryotic cells. However, until recently, its distribution appeared to be limited to the two protozoans mentioned above and, in higher eukaryotes, to those sources have been shown to bind directly to biomembranes and phospholipid vesicles. The availability of pure adrenal myosin I has allowed us to generate mAbs against this enzyme and to re-examine its distribution in various cells and tissues. In this report we show by immunofluorescence microscopy that myosin I is concentrated at the active, motile edges of cells, including growth cones, filopodia, and lamellipodia. In addition, immunoblot analysis indicates that myosin I is present in many rat tissues, suggesting that the enzyme may perform essential functions in all cells.

Materials and Methods

Purification of Myosin I and Myosin I Fragments

Myosin I was purified from whole bovine adrenal glands using our published protocol (Barylko et al., 1992). The procedure involved the following sequential steps: extraction of tissue with ATP at low ionic strength and coprecipitation with actin, followed by gel filtration on Sepharose CL-4B, anion exchange chromatography on Q-Sepharose, and affinity chromatography on ATP-agarose.

Myosin I was digested with chymotrypsin at 1:50 (wt/wt) enzyme to myo-
Figure 1. Binding of antibodies generated against adrenal myosin I to a total protein extract from bovine adrenal medulla. Lanes are immunoblots with M1, M2, M3, M4, and M5 antibodies, respectively. The chemiluminescence detection method of Gillespie and Hudspeth (1991) was used.

sin ratio in the presence of 0.1 mM CaCl2 for 30 min at room temperature. The reaction was terminated by adding PMSF to final concentration of 2 mM. The proteolytic products were analyzed by SDS/PAGE and used for testing different mAbs on immunoblots.

Production and Purification of Antibodies
mAbs to myosin I were produced by a modification of the method of Brodsky (1985). Purified myosin I (~0.5 mg/ml) was injected into the footpads of 5 Balb/c mice on eight occasions separated by 2-3 d each. Ribi adjuvant (monophosphoryl lipid A + trehalase dimycolate emulsion) (Ribi ImmunoChem Research, Inc., Hamilton, MT) was used for the first two injections. A tail bleed was performed on day 21 and all five sera contained antibodies to myosin I as determined by both Western and dot blotting. The next day popliteal lymph node lymphocytes were fused with NS-I myeloma cells using a solution of 37% polyethylene glycol and 5% DMSO, and a ratio of two lymphocytes per myeloma cell. Wells with colonies growing in HAT (hypoxanthine-aminopterin-thymidine) medium were screened by immunoblotting. Hybridoma cultures that secreted antibodies to myosin I were cloned two times by limiting dilution. All antibodies are IgG1 isotype as determined by double immunodiffusion in agar. Antibodies were isolated from ascites fluids as pure IgG using Bio-Rad’s (Richmond, CA) Affi-Gel Protein A MAPS II kit as described by the manufacturer. Purified antibodies were dialyzed into PBS.

Tissue Culture and Immunofluorescence Microscopy
Stable lines of hybridoma cells and normal rat kidney (NRK)1 were grown in RPMI 1640 medium, Madin-Darby bovine kidney (MDBK), PC12, Swiss 3T3 cells, and CHO cells were cultured in DME. Gentamycin and 10% calf serum (Hyclone Laboratories, Logan, UT) were included in all media. Rat primary brain cultures were prepared and maintained according to the method of Bloom and Vallce (1983). PC12 cells were plated on poly-l-lysine (50 μg/ml, 70-150 kD) coated coverslips in the presence of 50 ng/ml nerve growth factor (NGF) to induce differentiation. For some experiments, PC12 cells were fused using 50% polyethylene glycol followed by trypsin and separation on a 10-30% serum gradient (Hageleus, 1987). All tissue culture reagents, except calf serum, were obtained from Sigma Chemical Co. (St. Louis, MO).

Indirect immunofluorescence microscopy was performed using standard procedures. Fixation and permeabilization of cells was accomplished by immersing coverslips in methanol at −20°C for 5 min, or placing them in a 3.7% solution of formaldehyde in PBS followed by a 5-min incubation in 0.5 or 1% Triton X-100 in PBS. No differences were observed between fixation methods. Purified preparations of antimyosin I (10 μg/ml) and irrelevant monoclonal (H2, kinesin, Pfister et al., 1989) at 10 μg/ml served as primary antibodies. TRITC-labeled donkey anti-mouse IgG (Jackson Immunoresearch Laboratories, Inc., WestGrove, PA) was used as a secondary antibody at 10 μg/ml. For colocalization of actin and myosin I, rhodamine phalloidin (final concentration of 0.04 μM) (Molecular Probes, Eugene, OR) was used in combination with the myosin I antibody. The secondary antibody was Cy5-labeled (Cy5.18-OSU) donkey anti-mouse IgG (Jackson Immunoresearch Laboratories, Inc., WestGrove, PA) used at a concentration of 10 μg/ml. In all experiments coverslips were washed with PBS between antibody incubations.

Images were obtained using a Photometrics cooled CCD camera (Photometrics Inc., Woburn, MA) coupled to a Perceptics 9200 microscope (Carl Zeiss, Inc., Thornwood, NY). Fluorescent images were observed with either a 63×/N.A. 1.4 or a 100×/N.A. 1.3 using epi-illumination. A custom made TRITC/Cy5 filter set was used to view fluorescence (Omega Optical, Brattleboro, VT). No overlap of fluorophore signals was observed. Background images at each wavelength were digitally subtracted using BioVision software (Perceptics). Any additional image enhancement was done using National Institutes of Health Image Software.

1. Abbreviations used in this paper: MDBK, Madin-Darby bovine kidney; NGF, nerve growth factor; NRK, normal rat kidney.
Figure 3. Detection of myosin I in rat tissues by immunoblotting. Rat tissues were extracted with 20 mM Tris, pH 7.5, 2 mM EGTA, 1% SDS and protease inhibitors, and total extracts were run on SDS-PAGE and blotted with antibody M2. ~100 µg of protein was loaded in each lane. The first lane contains a sample of purified adrenal myosin I.

Other Methods

Protein concentration was determined either as described by Bradford (1976) or according to Meloche et al. (1986), using BSA as a standard. SDS-PAGE was carried out on 10% polyacrylamide slab gels according to the method of Matsudaira and Burgess (1978). Molecular mass standards (Bio-Rad, Richmond, CA) included myosin, 200 kD; galactosidase, 116 kD; phosphorylase b, 97.4 kD; BSA, 66 kD; ovalbumin, 45 kD; carbonic anhydrase, 31 kD; or prestained standards (Bio-Rad) myosin, 205 kD; galactosidase, 116 kD; BSA, 80 kD; and ovalbumin, 49.5 kD. For immunoblotting, samples were resolved by SDS-PAGE, and transferred by electroblotting to nitrocellulose. The nitrocellulose was incubated with 10 µg/ml of primary antibodies followed by alkaline phosphatase-conjugated goat anti-mouse IgG (Calbiochem Corp., San Diego, CA).

Results

Preparation and Characterization of mAbs against Adrenal Myosin I

Eight mAbs were raised against native myosin I purified from bovine adrenal gland (medulla and cortex). The specificity of these antibodies was tested on whole extracts from bovine adrenal medulla. Five of them, labeled M1–M5, strongly recognized one polypeptide of 115–120 kD, corresponding to the electrophoretic migration of myosin I (Fig. 1). The other three antibodies (M6–M8) were less sensitive and recognized myosin I weakly in whole adrenal extracts, though they recognized myosin I in fractions enriched for this protein more strongly, e.g., the actomyosin pellet (data not shown). To confirm that the protein recognized by our mAbs was indeed myosin I, immunoblots of pure myosin I mixed with proteolytic fragments of myosin I were carried out. Chymotryptic digestion of adrenal myosin I in the presence of CaCl₂ yields a fragmentation pattern including a 74-kD catalytic domain and several polypeptides of 30–40 kD containing calmodulin-binding sites believed to be derived from the COOH-terminal, “tail” domain (Barylko et al., 1992). As shown in Fig. 2, all antibodies recognize intact myosin I but five antibodies (M4–M8) also recognize the 74-kD polypeptide, while the other antibodies (M1–M3) recognize the lower molecular weight tail fragments.

Tissue Distribution of Myosin I

The presence of myosin I was examined in 15 rat tissues by immunoblot analysis (Fig. 3). Approximately equal amounts of whole tissue extracts prepared in the presence of 1% SDS were immunoblotted with antibody M2 that binds to an epitope on the tail domain of adrenal myosin. This antibody was expected to be most specific for the myosin I isoform against which it was raised since the tail domains of myosin I express only slight sequence similarity while the catalytic domains of all myosins are highly homologous (Pollard et al., 1991). In all tissues, a protein of 115–120 kD that corresponds to the molecular weight of myosin I was detected by the antibody. Myosin I appears to be enriched in spleen, esophagus, heart, lung, adrenal, and stomach. Interestingly, the tissues which show the least reactivity were brain, kidney, and small intestine. Despite tissue differences in the abundance of myo-
Figure 5. Localization of myosin I in MDBK cells with antibody M3 (B) and M2 (C); in NRK cells with antibody M3 (A) and in a CHO cell with antibody M3 (E). Actin (D) and myosin I (C) were colocalized in MDBK cells using rhodamine-phallloidin for actin and Cy5 secondary antibody for myosin I. Note that in all cases the myosin I antibodies stain the peripheral membrane region. F shows the result with the primary antibody omitted. Bar, 10 μm.
Figure 6. Localization of myosin I in 3T3 cells. Cells are stained with either M2 (D and E), M3 (A-C) or rhodamine phalloidin (F). Distinct staining is observed in the lamellipodium (A), filopodia (B and D) and ruffles (C and D). The cell in E was double labeled for myosin I (E) and actin (F). Bar, 10 μm.
Localization of Myosin I in Cultured Cells

The antibodies M2 and M3 were used to immunolocalize myosin I in six cell types: MDBK, rat primary brain, PC12, CHO, NRK, and Swiss 3T3 fibroblasts. Fig. 4 shows the specificity of these antibodies against whole cell homogenates prepared by direct addition of SDS sample buffer to the tissue culture dishes. In all cells one band of \( \sim 116 \) kD reacted strongly with the antibodies. In addition, in PC12, MDBK, and CHO cells there is another lighter band of \( \sim 110 \) kD. This lower molecular weight band is either a proteolytic fragment of myosin I or an isoform of the enzyme. Immunofluorescence microscopy revealed that myosin I was localized at the periphery of all cells examined. However, some cytoplasmic staining was consistently observed. Fig. 5, A (2 focal planes shown) and B clearly demonstrate the presence of myosin I at the leading edge of NRK and MDBK cells. Some MDBK and 3T3 cells were double-labeled for myosin I and actin (Fig. 5, C and D, and 6, E and F). Actin, as detected by rhodamine-phalloidin, is enriched along with myosin I at the periphery of the cell. However, in the cytoplasm, myosin I gives a fine punctate pattern while actin has a typical fibrillar appearance. Fig. 5 E shows a CHO cell with prominently stained ruffles. In 3T3 cells, the presence of myosin I is also evident in filopodia (Fig. 6, B and D) and ruffles (Fig. 6, C and D), consistent with the hypothesis that the enzyme participates in cell locomotion events.

This hypothesis gains further support from examination of PC12 and brain cells. Fig. 7 shows three examples of primary rat brain cells stained with antibody M3. Portions of at least three cells are shown in Fig. 7 A, each having a cellular process or axon which terminates in a highly stained region, i.e., the growth cone. In Fig. 7 B, a neuronal cell with multiple processes has intense staining at most tips while in Fig. 7 C there is staining in fine processes similar to retraction fibers frequently found in fibroblast cells. Fig. 8 confirms that myosin I is found in the growth cone and details the location of the enzyme in NGF-stimulated PC12 cells at different stages of neurite outgrowth (Fig. 8, B–F). In early stages (Fig. 8, B and C), myosin I is found concentrated at the perimeter of cells at several discrete spots. A few of these primitive growth cones begin extension (Fig. 8, D and E) while some remain closely associated with the cell body. Further extension of the process(es) continues and myosin I is seen to be unambiguously enriched in the growth cone (Fig. 8 F).

The precise nature and role of the cortical and cytoplasmic myosin I will require further investigation. Preliminary experiments in which cells were extracted with Triton X-100 before fixation and staining show that some myosin I is removed, but most is retained, suggesting an interaction with the cytoskeleton.

Discussion

Two important observations are presented in this paper: (a) Despite previous results to the contrary, (Hoshimaru and Nakaniishi, 1987; Garcia et al., 1989; Bikle et al., 1991; Kawakami et al., 1992) myosin I is distributed in many, if not all, tissues; and (b) myosin I is concentrated at the highly motile peripheral regions of cells from higher eukaryotes. The technical advance which allowed us to make these observations was the recent purification of bovine adrenal myosin I (Barylko et al., 1992), which was used as the antigen for the production of mAbs. Bovine adrenal and brush border myosins I have similar domain structure, multiple calmodulin binding sites, and express Ca\(^{2+}\)-sensitive actin-activated ATPase activities (Mooseker et al., 1991). However, antibodies and nucleotide probes obtained from brush border myosin I failed to detect the protein or its mRNA in tissues other than the intestine and placenta, suggesting that brush border and nonbrush border myosins I are structurally distinct. In fact, our cDNA cloning and sequence analysis confirms that the two isoforms of myosin I have considerably
Figure 8. Localization of myosin I in PC12 cells. Cells were stained with either M2 (B, D, and F), M3 (C and E) or rhodamine-phalloidin (A). Note the distinct staining in the tips of processes and in F the arrowheads point to either the end of a long process or the cell body from which it originated. Bar, 10 μm.
different amino acid sequences, particularly in the carboxyl-terminal third of the protein, particularly in the tail domains (Reizes, O., J. P. Albanesi, and T. C. Sudhof, manuscript in preparation).

The distribution of myosin I was examined in numerous rat tissues by immunoblot analysis using a mAb specific for the tail (COOH-terminal) domain of myosin I. Within the myosin I molecules which have been studied, the tail domains exhibit the greatest variation in amino acid sequence while the sequences of the catalytic head domains are highly conserved (Pollard et al., 1991). Our results clearly show that higher eukaryotic cells and tissues contain at least one isoform of myosin I. It is possible that the well-characterized brush border isoform is more specific for intestinal epithelium. A 105-kD myosin I was recently identified from the intestine, have a clearly defined brush border. Until now, myosin I does not appear to associate extensively with the plasma membrane, prompting speculation that the enzyme is involved in motile events at the cell surface (Pollard et al., 1991). We have examined the distribution of myosin I in several different cell types including kidney (MDBK and NRK), rat brain, rat pheochromocytoma (PC12), rat fibroblast (Swiss 3T3) and CHO. In each of these, mAbs to adrenal myosin I gave the most intense staining at the cell periphery and, in particular, in regions commonly associated with motile activity such as filopodia, lamellipodia, ruffles, and growth cones. In addition, at the cell periphery there is often a colocalization of myosin I and actin which is not observed in other cell regions. For example, in Swiss 3T3 cells myosin I does not appear to associate extensively with the pronounced actin stress fibers found in the interior of these cells. This is in marked contrast with the codistribution of myosin II with fibroblast stress fibers (Tomasek et al., 1982; Honer and Jockusch, 1988). Perhaps the most striking examples of specific myosin I localization were seen in rat brain and PC12 cells, in which the enzyme is especially concentrated at the growth cones, the tips of extending neurites. Our observations support several recent models of nerve growth cone motility which implicate myosin I in the process of neurite extension (Mitchison and Kirschner, 1988; Smith, 1988; Sheetz et al., 1990). We are currently attempting to further clarify the location of myosin I in these growth cones using EM techniques.

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References

Adams, R. J., and T. D. Pollard. 1989. Binding of myosin-I to membrane lipids. Nature (Lond.). 340:565-568.

Baines, I. C., and E. D. Korn. 1989. Localization of myosin IC and myosin II in Acanthamoeba castellani by indirect immunofluorescence and immunogold electron microscopy. J. Cell Biol. 111:1885-1904.

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.

Bikle, D. D., S. Munson, and M.-L. Mancianti. 1991. Limited tissue distribution of the intestinal brush border myosin I protein. Gastroenterology. 100:395-402.

Bloom, G. S., and R. B. Vallee. 1983. Association of microtubule-associated protein 2 (MAP2) with microtubules and intermediate filaments in cultured brain cells. J. Cell Biol. 108:1453-1463.

Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72:248-254.

Brodaty, F. M. 1985. Clathrin structure characterized with monoclonal antibodies. I. Analysis of multiple antigenic sites. J. Cell Biol. 101:2047-2054.

Coluccio, L. M. 1991. Identification of the microvillus 110-kDa cytoskeleton protein myosin-I in kidney. J. Cell Biol. 113:255-264.

Coudrier, E., H. Reggio, and D. Louvard. 1981. Immunolocalization of the 110,000 molecular weight cytoskeletal protein of intestinal microvilli. J. Mol. Biol. 152:49-66.

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.

Garcia, A., E. Coudrier, J. Carboni, J. Anderson, J. Vanderpoute, M. Merk, M. D'Onofrio, D. Louvard, and M. Arpin. 1989. Partial deduced sequence of the 110-kDa cytoskeletal protein of rat intestinal microvilli shows that this microfilament protein is a member of the myosin I family. J. Cell Biol. 109:2895-2903.

Gillespie, R. G., and A. J. Hudspeth. 1991. Cholinaminocuron detection of proteins from single cells. Proc. Natl. Acad. Sci. USA. 88:2562-2567.

Glenney, J. R., Jr., M. Osborn, and K. Weber. 1982. The intracellular localization of the microvillus 110K protein, a component considered to be involved in the fine structure of the filament attachment of F-actin. Exp. Cell Res. 138:199-205.

Halegoua, S. 1987. Changes in the phosphorylation and distribution of vinculin during nerve growth factor induced neurite outgrowth. Dev. Biol. 121: 570-574.

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.

Honer, B., and B. M. Jockusch. 1988. Stress fiber dynamics as probed by antibodies against myosin. Eur. J. Cell Biol. 47:14–21.

Hosokawa, M., and S. Nakanishi. 1969. Identification of a new type of mammalian myosin heavy chain by molecular cloning. J. Biol. Chem. 262:14625-14632.

Hoshimaru, M., Y. Fujio, K. Sekou, T. Sugimoto, and S. Nakanishi. 1989. Immunohistochemical evidence that myosin I heavy chain-like protein is identical to the 110-kilodalton brush-border protein. J. Biochem. 106:455-459.

Kawakami, H., K. Moriysogi, T. Utsumi, and S. Nakanishi. 1992. Structural organization and expression of the gene for bovine myosin I heavy chain. J. Biochem. 111:302-309.

Korn, E. D., and J. A. Hammer. 1990. Small Myosins. Curt. Opin. Cell Biol. 2:57-61.

Matsuda, P. T., and D. R. Burgess. 1978. SDS microsomal linear gradient gradient polyacrylamide gel electrophoresis. Anal. Biochem. 87:386-396.

Matsuda, P. T., and D. R. Burgess. 1979. Identification and organization of the components in the isolated microvillus cytoskeleton. J. Cell Biol. 83:651-673.

Melote, S., H. Ong, M. Cantin, and A. De Lean. 1986. Molecular characterization of the solubilized atrial natriuretic factor receptor from bovine adrenal zona glomerulosa. Mol. Pharmacol. 30:537-543.

Miyata, H., B. Bowers, and E. D. Korn. 1989. Plasma membrane association of Acanthamoeba myosin-I. J. Cell Biol. 109:1519-1528.

Mitchison, T., and M. Kirschner. 1988. Cytoskeletal dynamics and nerve growth. Neuron. 1:761-772.

Mooseker, M. S., J. S. Wolenski, T. R. Coleman, S. M. Hayden, R. E. Cheney, E. Espedale, M. B. Heintzelman, and M. D. Peterson. 1991. Structural and functional dissection of a membrane-bound microfilament: brush border myosin I in Current Topics in Membranes, vol. 38. Academic Press, Inc., Dallas, MN. 315-355.

Pfister, K. K., M. C. Wagner, D. L. Stenoien, S. T. Brady, and G. S. Bloom. 1989. Monoclonal antibodies to kinesin heavy and light chains of rat brain microtubule complex (myosin-I). J. Neurosci. 9:1453-1463.

Pollard, T. D., S. M. K. Doerner, and H. G. Zot. 1991. Myosin-I. Annu. Rev. Physiol. 53:653-681.

Sheetz, M. P., N. I. Baumrad, D. B. Wayne, and A. L. Pearman. 1990. Concomitance of membrane association by fast transport and trapping in neuronal growth cones. Cell. 61:231-241.

Smith, S. J. 1988. Neuronal cytoarchitecture: the actin-based motility of growth cones. Science (Wash. DC). 242:708-715.

Swanljung-Collins, H., and J. H. Collins. 1991. Rapid, high-yield purification of intestinal brush border myosin I. Methods Enzymol. 196:3-11.

Tomasek, J. J., J. E. Hay, and K. Fujiwara. 1982. Collagen modulates cell shape and cytoskeleton of embryonic cornea and fibroblast fibroblasts: distribution of actin, actinin, and myosin. Dev. Biol. 92:107-122.

Trinkaus, J. P. 1984. Cells into Organs. Prentice-Hall, New York.