Human myosin-IXb is a mechanochemically active motor and a GAP for rho

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INTRODUCTION

A myosin superfamily of actin-based molecular motors is now well established (reviewed by Mermall et al., 1998; Mooseker and Cheney, 1995; Cope et al., 1996). To date, this myosin family has been empirically divided into 14 different classes (I-XIV), based on degree of sequence similarity within the head (motor) domain of myosins in a given class. All known myosins consist of a head (motor) domain that contains the ATP and actin binding sites, a neck domain with one or more light chain binding sites (termed IQ motifs) and a tail domain of varied size and structure. It is likely that the tail domain plays a critical role in determining a myosin’s functional properties by specifying where and with what a given myosin interacts within the cell. Cell locomotion, organelle transport and signal transduction are just a few of the diverse functions proposed for the myosin superfamily.

Perhaps the most striking examples of the diverse nature of myosin tail structures are the class IX myosins. Class IX myosins were initially discovered in a PCR screen for novel myosins (Bement et al., 1994). The two fully sequenced heavy chains of this class, human myosin-IXb (M9b) and rat myr5, both contain within their tails a GTPase activator protein (GAP) domain that is structurally homologous to members of the rhoGAP family. This is quite remarkable since several members of the rho family (rac, rho and cdc42) are involved in remodeling the actin cytoskeleton, specifically membrane ruffling, stress fiber formation and filopodial extension (Ridley, 1996; Tapon and Hall, 1997). The head domain of these myosins is also unusual; relative to muscle myosin II, both proteins have a novel amino-terminal domain of about 140 amino acids with no sequence identity to any other protein and a large 126-amino-acid insertion that lies within the presumed actin binding site (based on comparisons with primary and atomic structures of muscle myosin II head domain; Rayment et al., 1993). The neck domain of this myosin class consists of four IQ motifs, although the light chains associated with class IX myosins have not yet been identified.

Little functional information is currently known about class IX myosins. We have previously shown that human M9b transcript is most highly expressed in peripheral blood leukocytes (Wirth et al., 1996). The expression of leukocyte M9b protein increases 4.5-fold upon macrophage-like differentiation of a human promyelocytic cell line (HL60), while M9b distribution changes from a cortical localization to a more cytoplasmic localization (Wirth et al., 1996). These changes suggest a role for M9b in leukocyte differentiation. Most recently, overexpression of the rat ortholog of human M9b, myr5, or its rho GAP domain in NRK and HeLa cells caused a rounded cellular morphology with loss of actin filaments and cell contacts, suggesting that the rho GAP domain is functional in vivo (Müller et al., 1997).

In terms of biochemical information, the Bähler laboratory has reported that myr5 binds F-actin in an ATP-sensitive manner and that bacterially expressed fusion proteins of the GAP domain are a GAP for rho (Müller et al., 1997; Reinhard et al., 1995). This is an unexpected result since the class IX myosin GAP domain is structurally most similar to that of

SUMMARY

The heavy chains of the class IX myosins, rat myr5 and human myosin-IXb, contain within their tail domains a region with sequence homology to GTPase activating proteins for the rho family of G proteins. Because low levels of myosin-IXb expression preclude purification by conventional means, we have employed an immunoadsorption strategy to purify myosin-IXb, enabling us to characterize the mechanochemical and rho-GTPase activation properties of the native protein. In this report we have examined the light chain content, actin binding properties, in vitro motility and rho-GTPase activity of human myosin-IXb purified from leukocytes.

The results presented here indicate that myosin-IXb contains calmodulin as a light chain and that it binds to actin with high affinity in both the absence and presence of ATP. Myosin-IXb is an active motor which, like other calmodulin-containing myosins, exhibits maximal velocity of actin filaments (15 nm/second) in the absence of Ca²⁺. Native myosin-IXb exhibits GAP activity on rho. Class IX myosins may be an important link between rho and rho-dependent remodeling of the actin cytoskeleton.

Key words: Myosin-I, Myosin-IX, Myosin (unconventional), GAP, Leukocyte, Motility
chimerins, which are GAPs for rac and not rho (Reinhard et al., 1995; Wirth et al., 1996).

In this report, we have sought to characterize further the biochemical properties of M9b. Here, we demonstrate that immunopurified, native M9b contains calmodulin light chains and is a Ca^{2+}-regulated, mechanochemically active motor that exhibits rho GAP activity.

**MATERIALS AND METHODS**

**Gel electrophoresis and immunoblotting**

Proteins were separated on 5%-20% SDS-PAGE minigels and transferred to PVDF membrane (BIO-RAD, Hercules, CA). Membranes were probed with M9b tail antibody. Calmodulin blots were performed as described by Sacks et al. (1991) and probed with calmodulin monoclonal antibody (Upstate Biotechnology Inc., Lake Placid, NY, USA).

**Production of myosin-lbx antibodies**

Antibodies were raised against three domains of the M9b heavy chain: the N-terminal portion (‘nose’ antibody), the majority of the tail domain (‘tail’ antibody) and the C-terminal domain of a splice variant, M9b2 (‘tip’ antibody). Tail and nose antibodies were produced as described in Wirth et al. (1996). The tip antibody was raised against the C-terminal domain of a newly identified splice form of M9b (see Fig. 1), which differs from our originally reported molecule at the C-terminal tip. The C-terminal tip domain (amino acids 1954-2126) was bacterially expressed using two different expression vectors: pGEX vector (glutathione-S-transferase-containing fusion protein; Amrad Corp. Melbourne, Australia) and Qia-Express vector (histidine-tagged fusion protein; Qiagen Inc., Chatworth, CA, USA). Both fusion proteins were purified by affinity chromatography (on glutathione and Ni columns, respectively) following manufacturers’ recommended protocols. The glutathione-S-transferase-containing fusion protein was used as immunogen and the histidine-tagged protein coupled to CNBr-activated Sepharose (Pharmacia-LKB Biotechnology Inc., Piscataway, NJ, USA) was used for affinity purification. The tip immunogen does not include the GAP domain.

**Protein purification**

Medically discarded human leukocytes were homogenized for 3x30 second pulses with an Omni Mixer (DuPont Instruments, Newtown, CT) in buffer A (10 mM imidazole, 50 mM KCl, 2.5 mM MgCl2, 1 mM EGTA, 2.5 mM ATP, 1 mM DTT, 1 mM Pefabloc, 5 µg/ml leupeptin, 5 µg/ml pepstatin, 5 µg/ml aprotinin). The extent of homogenization was monitored by light microscopy; cells and nuclei appeared to be completely disrupted. The homogenate was centrifuged at 100,000 g for 1 hour. The resulting supernatant was chromatographed on a Q-Sepharose column (Sigma Chemical Company, St Louis, MO). The column was washed with buffer A and M9b was eluted with a 50-650 mM KCl gradient in buffer A. M9b-containing fractions were identified by dot blot analysis (Settleman and Foster, 1995), pooled, dialyzed against buffer A and clarified. M9b began to elute at 190 mM KCl. M9b-containing fractions were chromatographed on an S-500 column as described by Cheney et al. (1993) for myosin-V. M9b-containing fractions were pooled and dialyzed against buffer B (10 mM imidazole, 75 mM KCl, 2.5 mM MgCl2, 1 mM EGTA, 1 mM DTT, 0.2 mM PMSF). (M9b elutes in the same fractions as myosin-V from the S-500 column.)

Recombinant rho was purified from *E. coli* as previously described (Ren et al., 1996). The rho GAP p190 was expressed in baculovirus (Settleman and Foster, 1995).

**Actin cosedimentation assays**

Samples of M9b (purified as described above for actin cosedimentation assays) were incubated with 6 µM F-actin ± 5 µM MgATP at room temperature in buffer B for 15 minutes. Actin cosedimentations with rho (rho-GTP or rho-GTPS) were done in buffer B or in the GTPase assay buffer (see below). Samples were spun at 100,000 g for 1 hour. Supernatants were removed; pellets were resuspended to the same volume as the supernatant. Gel samples were prepared by adding SDS sample buffer.

**In vitro motility assays**

These assays were a modification of the sliding filament assay (Kron and Spudich, 1986). To prepare motility chambers, nitrocellulose-coated coverslips were incubated with 0.5 mg/ml Protein-A in Tris buffered saline (TBS) for 30 minutes and washed with TBS. Chambers were incubated with 250 µg/ml anti-M9b (nose, tail or tip) or rabbit nonimmune control IgG for 30 minutes, washed with TBS and then blocked with 5% casein, 5% BSA for 30 minutes. Chambers were washed again with TBS and incubated with M9b-containing Q-Sepharose column fractions (described above) overnight. Finally, chambers were washed with high salt wash (buffer B plus 500 mM KCl), then with buffer B. Motility assays were done in motility buffer, consisting of 40 mM imidazole, pH 7.3, 75 mM KCl, 2.5 mM MgCl2, 2 mM MgATP, 100 µg/ml calmodulin, 1 mM DTT, 200 µg/ml glucose oxidase, 100 µg/ml catalase, 2.5 mg/ml glucose, 5 nM BODIPY-TRX-phallacidin (Molecular Probes, Eugene, OR) stabilized actin filaments and 1 mM EGTA or a 4 mM EGTA-Ca^{2+} buffer calculated to give 10 µM free Ca^{2+} (Portzehl et al., 1964). Movement was observed with a VE-1000 FIT camera (Dage-MTI Inc., Michigan City, IN) attached to a Nikon Diaphot 300 equipped for epifluorescence. Images were recorded every 30 seconds with Metamorph (Universal Imaging Corporation, West Chester, PA). Filaments moving in approximately straight lines for at least three consecutive images (1.5 minutes) were used to measure rates. Rates of individual filaments were measured with Track Points (Metamorph) every 30 seconds and averaged over the entire time the filament moved in its path (ranging from 1.5 to 20 minutes). At the end of the experiments, motility chambers were prised open and protein was solubilized with hot SDS sample buffer. The amount of M9b contained in the chambers was quantitated by western blotting against a M9b fusion protein standard curve.

**GAP assays**

Rho GAP assays were performed as described by Chuang et al. (1993) with tip antibody immunoprecipitated M9b, although results were similar with tail antibody immunoprecipitated M9b.

**RESULTS**

**Identification of a novel splice variant of M9b**

We have identified a splice form of M9b (called M9b2; GenBank...
accession number AF020267) that replaces the last 99 amino acids of our originally reported molecule (M9b1) with a different 228-amino-acid domain at the C terminus (Wirth et al., 1996). The N-terminal portion of this tail tip domain is 84% identical to rat myr5, while the C-terminal two-thirds of this domain extends beyond the end of rat myr5 (see Fig. 1). The molecular masses of M9b1 and M9b2 are 228 kDa and 240 kDa, respectively. The M9b nose and tail antibodies used in this study recognize both M9b1 and M9b2 splice forms, but the M9b tip antibody was raised only against the M9b2 novel splice domain (see Fig. 2).

Note that M9b is not expressed at high enough levels to see on a Coomassie blue-stained gel (Fig. 3A), indicating that M9b is not a major protein species in the leukocyte. M9b sometimes runs on our gels as a closely spaced doublet at 230 kDa (Wirth et al., 1996). Since the M9b2 specific antibody gives the same immunoprobe pattern as the M9b antibodies that recognize both M9b1 and M9b2 splice forms, it is unlikely that it represents the two splice forms we have identified. The doublet may represent protein degradation, post-translational modifications, or another (yet unidentified) splice form. A 190 kDa band is also sometimes seen on western blots (see Fig. 3B). This band’s intensity increases upon storage, indicating that it is probably a proteolytic fragment of M9b.

**Purification of native human M9b**

M9b is most abundant in peripheral blood leukocytes in the human body, yet the protein is expressed at extremely low levels in these cells (approx. 0.01% of total protein). Attempts at conventional, biochemical purification were unsuccessful, therefore an immunopurification scheme was devised. First, leukocyte extracts were enriched for M9b through passage over a Q-Sepharose column (Fig. 3A,B). For GAP assays, native M9b was purified from M9b-containing Q-Sepharose column fractions via immunoprecipitation with tail or tip antibodies tethered to Protein-A Sepharose (described in Materials and methods; Fig. 4A,B). For motility assays, native M9b was immunopurified from M9b-containing Q-Sepharose column fractions with tail antibodies bound to Protein A tethered to the motility chamber surface (described in Materials and methods; Fig. 4D). M9b does not precipitate in control precipitations using nonimmune rabbit control IgG (Fig. 4A,B,D).

**Fig. 1.** Comparison of class IX myosin tail domains. Shown are the amino acids of the tail tips of the two splice forms of human M9b (1 and 2) and rat myr5. Identical residues are in bold.

**Fig. 2.** Schematic of the domain structure of the two human M9b heavy chain splice forms, which differ at the tips of their tails. The regions used to generate the three different polyclonal antibodies (nose, tail and tip) used in this study are shown. (A) Box diagram of our originally reported molecule (Wirth et al., 1996) used to generate nose and tail antibodies. (B) Box diagram denoting the newly identified splice form. Nose and tail antibodies recognize both splice forms, while tip antibodies recognize only the M9b2 splice form.
M9b associates with calmodulin

The neck of M9b contains four distinct light-chain binding IQ motifs (approx. 23 amino acid repeats), suggesting binding of four light chains (Wirth et al., 1996; Mooseker and Cheney, 1995). The Ca$^{2+}$-binding protein calmodulin is known to be a light chain for various unconventional myosins (Wolenski, 1995; Houdusse et al., 1996). To test whether calmodulin serves as a light chain, western blots of M9b immunoprecipitates (−Ca$^{2+}$) were probed with anti-calmodulin antibody. Calmodulin cosediments with M9b and undergoes its characteristic gel mobility shift in Ca$^{2+}$ versus EGTA, while calmodulin does not sediment with rabbit nonimmune IgG (Fig. 4C).

M9b binds F-actin in an ATP-insensitive manner

The interaction of M9b with actin was tested in an actin cosedimentation assay. Human leukocyte M9b was partially purified through ion exchange and gel filtration chromatography, as described in Materials and methods. Samples of this preparation (containing approximately 0.5 pmoles of M9b) were incubated with F-actin in the presence or absence of ATP, then centrifuged at 100,000 g to pellet the actin. M9b sedimented nearly 100% with actin in the absence of ATP and only a trace amount of M9b was released in the presence of ATP (Fig. 5). Addition of Ca$^{2+}$ and calmodulin to the cosedimentation assay made no significant difference to these results (not shown). Given the low concentration of M9b in this preparation, this result suggests that M9b has a high affinity for actin even in the presence of ATP, similar to myosin-V (Nascimento et al., 1996).

These actin cosedimentation results are different from those observed by Reinhard et al. (1995). These authors did their
cosedimentation assay in a high salt buffer (with 300-350 mM NaCl and 20 mM KCl) and observed no ATP-independent binding of rat myr5 to actin. Under these high salt conditions, we observed approximately 60% less binding of human M9b to actin, but human M9b still bound to actin in the presence of ATP (data not shown).

The mechanochemical activity of M9b is regulated by calcium

To assess whether or not M9b is mechanochemically active, we tested its motor activity with a modified sliding filament in vitro motility assay (based on Kron and Spudich, 1986). M9b was immunopurified directly into the motility chambers with protein A-tethered tail-directed antibodies, as described in Materials and methods. Roughly 0.13 pmoles of M9b were immunoadsorbed onto the bottom coverslip of motility chambers, giving approx. 500 molecules/μm² on the viewing surface of the chamber (assuming that M9b is single-headed). Robust movement of actin filaments was observed in chambers containing motility buffer with 1 mM EGTA (Fig. 6). Movement persisted for over 60 minutes, with an average of 65% of the filaments moving per chamber (range was 33-100%; see Fig. 7). The average rate of movement was 15±2.8 nm/second. M9b linked to chambers with tip antibodies moved actin filaments at the same rate as M9b linked to chambers with tail antibodies. This suggests that the observed slow rate of actin filament movement by M9b is not due to decreased flexibility of M9b because it is attached to the motility chamber at a region near its neck (Winkelmann et al., 1995).

Three lines of evidence indicate that M9b is responsible for the observed movement of actin filaments. First, control chambers were prepared with protein A-tethered nonimmune rabbit IgG and exposed to the same leukocyte preparation as the experimental chambers. A few actin filaments stuck to the control antibody-coated surface of these chambers, but they never moved (results not shown). Chamber blots of two other myosins present in these cells (myosins -II and -V) indicated that these myosins were not present in the chambers (results not shown). Finally, we perfused antibodies directed against the head/motor domain of M9b into motility chambers. Complete inhibition of movement was obtained with 50 μg/ml antibody, but movement persisted with 50 μg/ml control, rabbit nonimmune IgG. At M9b head antibody concentrations of 25 μg/ml, inhibition was still observed, although some filaments exhibited jittering. Movement persisted with 15 μg/ml M9b head antibody. These results indicate that the motility observed is M9b-mediated.

Calcium inhibits the in vitro motility of some myosins -I and -V (Collins et al., 1990; Cheney et al., 1993; Williams and Coluccio, 1994; Mooseker and Cheney, 1995), presumably by causing a dissociation of the calmodulin light chains from the neck region or via a Ca²⁺-induced conformational change in the calmodulin light chain (Wolenski, 1995; Houdusse et al., 1996). To test the effects of Ca²⁺ on the mechanochemistry of M9b, motility buffer containing 10 μM free Ca²⁺ was flushed into motility chambers. An immediate reduction in filament

![Fig. 5. M9b cosediments with F-actin in an ATP-independent manner. (A) Western blot of actin cosedimentation assay with partially purified human M9b. Partially purified human M9b was incubated with actin in the presence or absence of ATP. M9b binds to actin, whether or not ATP is present. (B) Coomassie blue-stained gel of samples run in A. F-actin (where added) is visible in the pellets (arrow), but the sedimenting M9b cannot be visualized by Coomassie blue stain.](image)

![Fig. 6. M9b translocates actin filaments in an in vitro motility assay. Four examples of M9b translocating actin filaments are shown in 1 mM EGTA over 8 minutes (A-D). The path of each moving filament is traced in the far right column and the rate (in nm/second) is shown to the right of the traces. Bar, 2 μm.](image)
velocity was observed, from 15±2.8 nm/second (in EGTA) to 10±2.5 nm/second (in Ca\(^{2+}\)) (see Fig. 8). The slowing of the actin filament velocity was observed whether chambers containing EGTA were flushed with Ca\(^{2+}\) or whether they were initially prepared with 10 μM Ca\(^{2+}\), and compared to other chambers initially prepared with EGTA. We noticed in preliminary experiments that robust movement of actin filaments could not be obtained without addition of exogenous calmodulin, and that Ca\(^{2+}\) caused a loss of calmodulin light chains from immunoprecipitated M9b (results not shown). These results are comparable to those observed with some myosins-I and myosin-V, suggesting a similar type of motor regulation.

Native M9b has rho GAP activity

M9b contains a GAP domain in its tail that is most like GAPs for the rho family of GTPases (Wirth et al., 1996; Reinhard et al., 1995). Bacterially expressed fusion proteins of the myr5 GAP domain and Sf9 cell lysates containing baculovirus-expressed myr5 have GAP activity primarily on rhoA and little to no activity on cdc42-Hs and rac1 (Müller et al., 1997; Reinhard et al., 1995). These small GTPases switch from an activated (GTP-bound) state to an inactivated (GDP-bound) state via their low intrinsic GTPase activity. This GTPase activity can be increased by the interaction with GAPs, thus inactivating the G-protein more quickly.

In this study we tested the GAP activity of native M9b in a standard GTPase assay on rho (rhoA), rac (rac1) and cdc42 (cdc42-Hs) (Chuang et al., 1993) using M9b immunoprecipitated from human leucocyte extracts (as described in Materials and methods). As a control, immunoprecipitates with rabbit nonimmune IgG from the same cell extracts were used. Incubation of approximately 0.02 pmoles of immunoprecipitated M9b with rho (1 pmole) caused a 34% decrease in the amount of [\(^{32}\)P]GTP bound to rho after 5 minutes at room temperature (Fig. 9). Experiments using [\(^{32}\)P]GTP-bound rho showed little loss of nucleotide from rho (in the presence or absence of M9b), suggesting that the decrease in amount of [\(^{32}\)P]GTP bound to rho reflects GTP hydrolysis and not merely loss of GTP. Incubation of rho with similar concentrations (0.05 pmoles) of a known rhoGAP, p190, caused nearly the same decrease in the amount of GTP bound to rho after a 5 minute incubation at room temperature (44%), indicating that p190 and M9b have similar specific activities (Fig. 9). Addition of control immunoprecipitates to rho did not affect the amount of GTP bound to rho, indicating that other GAPs did not nonspecifically immunoprecipitate in the preparation (Fig. 9). M9b had no significant effect on the amount of GTP bound to constitutively active rho (rhoV14), rac or cdc42 (results not shown). Surprisingly, M9b immunoprecipitated with the tail antibody (whose immunogen includes the GAP domain) has the same GAP activity as that precipitated with the tip antibody (results not shown).

DISCUSSION

The immunopurification strategy used in this report has allowed for the initial biochemical characterization of native M9b, despite its low abundance. This experimental approach should be generally applicable to the characterization of novel unconventional myosins, many of which are also expressed at low levels. There are several key conclusions from the results presented here. First, M9b is a calmodulin-containing, mechanochemically active motor whose activity is regulated by Ca\(^{2+}\). Second, M9b exhibits unusual actin binding and motile properties compared to most other known myosins. Third, M9b exhibits rho GAP activity. Thus, this myosin may play a role in down-regulating the assembly of actin filament arrays upon which it moves.
M9b is a calcium-regulated molecular motor

Our in vitro motility experiments indicate that M9b is mechanochemically active and regulated by Ca\(^{2+}\). At room temperature, M9b translocates actin filaments at 15±2.8 nm/second. In the presence of Ca\(^{2+}\), the velocity slows (10±2.5 nm/second), a response seen for several calmodulin-containing myosins. In comparison, in the sliding filament assay at room temperature, class II myosins translocate actin at rate of 3000-4000 nm/second (Kron and Spudich, 1986), class V myosins at 200-400 nm/second (Cheney et al., 1993), Acanthamoeba myosin-I at 200 nm/second (Zot et al., 1992), brush border myosin-I at 40-65 nm/second (Collins et al., 1990; Wolenski et al., 1993), bovine adrenal gland myosin-I\(\beta\) (baculovirus expressed) at 300-500 nm/second (Zhu et al., 1996) and rat liver myosin-I\(\alpha\) at 12 nm/second (30-50 nm/second at 37°C; Williams and Coluccio, 1994). Thus, in comparison to other myosins, M9b is among the slowest myosins characterized to date.

While M9b may be slow, it is very robust. Compared to other myosins we have tested under similar conditions, M9b is of equivalent (myosin-V) or greater (brush border myosin-I and muscle myosin-II) robustness in terms of percentage of filaments moving per field and longevity of movement. When myosin preparations lose activity upon storage, we generally note a loss of robustness, but no significant change in velocity. Similarly, overnight storage of M9b in motility chambers does not change actin filament velocity; however, the robustness of movement is decreased approximately 50% (P. Post, unpublished observations). Thus, while one should have serious reservations about the significance of velocities obtained from in vitro assays, the slow velocity of M9b is probably not from ‘damage’ during purification. On the other hand, it is possible that the slow velocity is indicative of a down-regulated state of this myosin as it is purified.

The unusual actin binding properties of M9b may contribute to this motor’s slow velocity. Even at the very low concentrations of M9b assayed, this myosin cosediments with actin in both the absence and presence of ATP. It should be noted that in a similar study using a cruder fraction of myr5 in a high salt buffer, no binding of myr5 to actin with ATP was observed (Reinhard et al., 1995). However, we have observed ATP-independent binding of human M9b to actin in this high salt buffer. The basis for this difference is unknown; it could be due to real differences in these myosins, the presence of different contaminating proteins in the crude fractions used for each study, or perhaps differences in their regulatory state as purified.

There are several explanations for the ATP-independent binding of M9b to actin described here. First, like myosin-V (Nascimento et al., 1996), the motor domain may remain

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**Fig. 8.** The in vitro motility of M9b is slowed by Ca\(^{2+}\).
(A) Histogram of rates of 47 moving actin filaments analyzed in 1 mM EGTA. The average rate of movement is 15±2.8 nm/second. (B) Histogram of rates of 51 moving actin filaments in an EGTA-Ca\(^{2+}\) buffer calculated to generate 10 \(\mu\)M free Ca\(^{2+}\). The average rate of movement slows to 10±2.5 nm/second (\(P<0.005\)).

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**Fig. 9.** Native M9b has rhoA GAP activity. Shown are results of representative \([\gamma^{32}P]GTPase assays of rho in the presence (n=6) and absence (n=3) of immunoprecipitated M9b, as well as in the presence of control immunoprecipitates (n=3). As a comparison, the activity of an equal molar amount of p190 rho GAP as M9b is shown. The y-axis displays percentage radioactivity (from \([\gamma^{32}P]GTP) remaining bound to rhoA, meaning that this GTP was not hydrolyzed. Therefore lower numbers represent higher GTPase activity. M9b increases the intrinsic GTPase activity of rhoA.
bound to actin in the presence of ATP, either because its duty cycle is different from conventional myosin II or because the head domain has a second ATP-insensitive actin binding site. In this regard, it is important to recall the large insert (150 amino acids) this myosin has at its presumed contact site with actin. This highly basic domain (pI 11.6) may well contribute to maintaining high affinity binding to actin as it goes through the ATPase cycle. Second, like amoeboid myosins-I (Doberstein and Pollard, 1992; Jung and Hammer, 1994; Rosenfeld and Rener, 1994), M9b could have a second actin binding site in its tail domain (although one might expect that a second actin binding site on the tail would be occluded by the tethering antibody). Third, M9b could be complexed with a second protein that tethers it to actin indirectly.

The slow velocity of M9b could also be indicative of a very slow ATPase cycle. Given the potential significance of a slow duty cycle when considering a function for M9b, it will be critical to assess the ATPase properties of this motor – this task will probably require in vitro expression of this myosin. If M9b binds to actin with high affinity, but with a very slow duty cycle, small numbers of motors could maintain tension (assuming the myosin is tethered somehow) on a filament for a substantial amount of time. Such tension generation could be important, but this would also result in localization of the GAP activity on the tail of each actin-associated motor. Alternatively, assuming that the only cargo (and thus function) that M9b has is its GAP domain, then the myosin would slowly translocate along a filament, while down-regulating the rho-dependent cascade of events that may have evoked that filament’s initial assembly. In this model, M9b programs the removal of its transducing element as it moves along.

Regulation of M9b

Another critical issue in defining the motor properties of M9b will be to determine how it is regulated. The mechanochemical activity of all known myosins is regulated, either through mechanisms that are myosin-linked (e.g. via Ca^{2+} binding, heavy or light chain phosphorylation), actin linked (e.g. via troponin and tropomyosin), or by both types of mechanisms. Ca^{2+}, mediated through calmodulin light chains, presumably plays some role in M9b mechanochemical regulation. It will be critical to assess effects of Ca^{2+} on M9b MgATPase properties since the common ‘slowing’ observed for calmodulin-containing myosins in motility chambers does not correlate well with enzymatic activities of those myosins (Umemoto and Sellers, 1990; Cheney et al., 1993; Wolenski et al., 1993; Houdusse et al., 1996).

Given the presumed role of M9b in ‘down-regulating’ rho-mediated events, it will be critical to determine whether this myosin is a substrate for kinases implicated in rho cascades. The rho family interacts with several kinases, some of which phosphorylate other myosins. Rho interacts with protein kinase N (Amano et al., 1996b; Watanabe et al., 1996) and rho kinase (Kimura et al., 1996); rho kinase affects the phosphorylation state of myosin II regulatory light chain as well as inducing fibroblast stress fiber formation, focal adhesion formation and smooth muscle contraction (Amano et al., 1996a, 1997, Kureishi et al., 1997). Rac- and cdc42-GTP bind to p65 PAK; this kinase-G-protein complex phosphorylates Acanthamoeba myosin I, activating enzymatic activity (Brzeska et al., 1997). Since M9b has an acidic residue at the conserved, head domain phosphorylation site, it is probably not regulated by phosphorylation here (Bement and Mooseker, 1995), but this does not exclude phosphorylation at another site. The studies presented here provide the means with which to test possible regulators of M9b by these kinases.

Rho GAP activity of M9b

The results presented here have demonstrated that native M9b is a rho GAP. This is comparable to results from the Bähler laboratory obtained with bacterially expressed fusion proteins of the myr5 GAP domain and Sf9 insect cell lysates containing baculovirus-expressed myr5 (Reinhard et al., 1995; Müller et al., 1997). In comparison with equimolar concentrations of another rho GAP, p190, native M9b has a similar specific activity (Fig. 9; Settleman and Foster, 1995). A critical next step in our research will be to determine whether there is molecular ‘crosstalk’ between the motor (head) domain and the GAP (tail) domain.

Cellular function of M9b

The cellular function of the class IX myosins is currently unknown. Given the relatively high level of expression of M9b in leukocytes and the unique linkage of an active myosin motor domain with an active GAP domain, it is likely that the class IX myosins are involved in rho-mediated signalling pathways, presumably including remodeling of the leukocyte actin cytoskeleton.

The effects of rho on the actin cytoskeleton have been well characterized in fibroblasts, where rho regulates stress fiber and focal contact formation (reviewed in Ridley, 1996; Tapon and Hall, 1997). It has been known for several years that nonhydrolyzable GTPyS stimulates actin polymerization in permeabilized neutrophils (Therrien and Naccache, 1989; Bengtsson et al., 1990). Much evidence is now accumulating that it is rho that affects actin polymerization in leukocytes as well as actin-dependent leukocyte processes (reviewed in Dharmawardhane and Bokoch, 1997). For instance, as in fibroblasts, rho appears to help regulate levels of polymerized actin in leukocytes (Koch et al., 1994; Allen et al., 1997), possibly through activation of phospholipase C (Bourgoin et al., 1995; Kwak et al., 1995). Treatment of leukocytes with Clostridium botulinum C3 transferase (which ADP ribosylates and inactivates rho) produces different effects on several actin-mediated processes; inactivation of rho hinders cytokinesis (Aepfelbacher et al., 1995), increases cell spreading (Aepfelbacher et al., 1996) and inhibits T-lymphocyte cell-mediated cytotoxicity (Lang et al., 1992). Rho, possibly through its ability to cluster integrins (reviewed in Ridley, 1996), has also been shown to transmit signals from external stimuli, such as from chemotactrant receptors, to affect leukocyte adhesion (Laudanna et al., 1996), motility (Stasia et al., 1991) and aggregation (Tominaga et al., 1993).

It is possible that class IX myosins act to modulate one or more of the above described leukocyte functions for rho. The M9b motor domain may serve to localize it to the site of leukocyte rho functions, i.e. on actin. For instance, Aepfelbacher et al. (1996) have shown that rho is a negative regulator of human monocyte cell spreading. Our previous localization studies in a leukocyte cell line suggest a model where M9b inactivates rho to allow monocyte spreading. HL60 cell M9b is colocalized with F-actin in the cortex (where
activated GDP-bound rho would exist) of rounded, undifferentiated cells (Wirth et al., 1996). This pattern changes to a more diffuse, cytoplasmic localization (where inactivated GDP-bound rho would exist) in spread, macrophage-differentiated cells and is no longer colocalized with F-actin (Wirth et al., 1996). M9b may serve to inactivate rho (causing depolymerization of actin) in order to allow actin remodeling and macrophase spreading to occur.

Alternatively, we have also previously demonstrated that the expression of HL60 M9b increases upon HL60 cell differentiation into macrophase-like cells (Wirth et al., 1996). This up-regulation of protein expression may be necessary if M9b is required to participate in the rho-actin dependent processes (i.e. chemotaxis, cytotoxicity, phagocytosis) of mature leukocytes.

We gratefully acknowledge Kari Jensen for preparation of M9b antibodies. P. L. P. was supported by a NIH Postdoctoral Fellowship (DK 09407). P. L. P. and M. S. M. were supported by NIH grant DK 58979, NIH program project grant DK 38979, a Yale Liver Center (DK 09407). P. L. P. and M. S. M. were supported by NIH grant DK 5070-5075. P. L. P. was supported by a NIH Postdoctoral Fellowship.

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