Biochemical and Motile Properties of Myo1b Splice Isoforms*

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Myo1b is a widely expressed myosin-I isoform that concentrates on endosomal and ruffling membranes and is thought to play roles in membrane trafficking and dynamics. Myo1b is alternatively spliced within the regulatory domain of the molecule, yielding isoforms with six (myo1b\(^a\)), five (myo1b\(^b\)), or four (myo1b\(^c\)) non-identical IQ motifs. The calmodulin binding properties of the myo1b IQ motifs have not been investigated, and the mechanical and cell biological consequences of alternative splicing are not known. Therefore, we expressed the alternatively spliced myo1b isoforms truncated after the final IQ motif and included a sequence at their C termini that is a substrate for bacterial biotin ligase. Site-specific biotinylation allows us to specifically attach the myosin to motility surfaces via a biotin-streptavidin linkage. We measured the ATPase and motile properties of the recombinant myo1b splice isoforms, and we correlated these properties with calmodulin binding. We confirmed that calcium-dependent changes in the ATPase activity are due to calcium binding to the calmodulin closest to the motor. We found that calmodulin binds tightly to some of the IQ motifs (\(K_d < 0.2 \mu M\)) and very weakly to the others (\(K_d > 5 \mu M\)), suggesting that a subset of the IQ motifs are not calmodulin bound under physiological conditions. Finally, we found the in vitro motility rate to be dependent on the myo1b isoform and the calmodulin concentration and that the myo1b regulatory domain acts as a rigid lever arm upon calmodulin binding to the high affinity and low affinity IQ motifs.

Myosin-I isoforms are single-headed members of the myosin superfamily that link lipid membranes to the actin cytoskeleton. Eight myosin-I genes are present in humans (1), and at least two of these genes are alternatively spliced (2, 3). The molecular roles of most myosin-I isoforms are not yet known. However, it is well established that myosin-I motors function in endocytosis, membrane trafficking, membrane retraction, and mechano-signal transduction (4–8).

Like most members of the myosin superfamily, myosin-I isoforms have a conserved motor domain followed by a regulatory domain that acts as the lever arm. It is the rotation of this lever arm that drives motility and force generation (9). The regulatory domains of myosin-I isoforms are composed of one or more IQ motifs that are sequences of \(\sim 25\) amino acids that bind calmodulin and calmodulin-like proteins (10, 11). The sequences of IQ motifs are not well conserved, and the sequences of motifs within a single myosin are different. The conformational state of the bound calmodulin and the IQ-calmodulin affinity depend on the sequence of the IQ motif. This diversity of binding results in a range of IQ-calmodulin affinities. Additionally, calcium binding to calmodulin can change the affinity and conformation of the IQ-bound calmodulin, which can result in the regulation of myosin-I activity. Calcium binding to vertebrate isoforms affects the ATPase and motile properties of the motors, which is due to calcium-dependent changes in the conformation state of the regulatory domains (12–16).

Myo1b is a widely expressed isoform that concentrates on endosomal and ruffling membranes and is thought to play roles in membrane trafficking and dynamics (3, 5). Myo1b is alternatively spliced within the regulatory domain of the molecule, yielding isoforms with six (myo1b\(^a\)), five (myo1b\(^b\)), or four (myo1b\(^c\)) non-identical IQ motifs (Fig. 1) (3). The first three IQ motifs (IQs 1–3) are the same for each splice isoform. Splicing occurs in IQs 4–6 as follows: myo1b\(^a\) contains IQ4, IQ5, and IQ6; myo1b\(^b\) contains IQ6 and is spliced within IQ4 and IQ5, resulting in an IQ4/5 hybrid; and myo1b\(^c\) is spliced within IQ4 and IQ6, resulting in the creation of an IQ4/6 hybrid and the loss of IQ5 (Fig. 2).

Calmodulin is thought to be the regulatory domain-associated light chain for myo1b and all vertebrate myosin-I isoforms (3, 10, 11, 17). However, the calmodulin binding properties of the myo1b IQ motifs have not been investigated, and the mechanical and cell biological consequences of alternative splicing are not known. Therefore, we measured the ATPase and motile properties of the recombinant myo1b splice isoforms and correlated these properties with calmodulin binding. We confirmed that calcium-dependent changes in the ATPase activity are due to calcium binding to the calmodulin closest to the motor (15), and we found that calmodulin binds very weakly to a subset of the alternatively spliced IQ motifs. Additionally, we found the in vitro motility rate to be dependent on the myo1b isoform and the calmodulin concentration.

**EXPERIMENTAL PROCEDURES**

*Proteins and Reagents—*All experiments were performed in KMg25 (10 mM Mops, \(pH 7.0, 25\) mM KCl, 1 mM MgCl\(_2\), 1 mM EGTA, 1 mM dithiothreitol). Calcium concentrations were adjusted by adding CaCl\(_2\) to KMg25 and are reported as free calcium. Actin (18), pyrene-actin (19), and calmodulin (20) were purified as described. The concentration of calmodulin was determined by absorbance (\(\epsilon_{275} = 3300\) \text{ M}^{-1} \text{ cm}^{-1}\)).

The cDNA for rat myo1b (splice isoform b) was provided by Dr. Martin Bähr. Splice isoforms a and c were generated with PCR primers of appropriate sequences. Splice isoforms were truncated after IQ6, generating constructs that contained the motor and IQ motifs (Fig. 2). A FLAG sequence for purification and a 15-amino acid (AviTag) sequence for site-specific biotinylation (21) were inserted at the C termini, and the constructs were subcloned into a baculovirus transfer vector (pBlue-Bac4.5). A construct consisting of the motor domain and IQ1 was also prepared, but this construct does not contain the AviTag sequence and was not used for motility experiments (Fig. 2). Recombinant baculoviruses were generated using standard procedures and screened by plaque assays.

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2 The abbreviation used is: Mops, 4-morpholineethanesulfonic acid.
Truncated myo1b splice isoforms and myo1bIQ were purified from S9 cells that were co-infected with virus containing recombinant myo1b and calmodulin as described (22). Calmodulin (2 μM) was included in column elution buffers. Myo1b splice isoforms were biotinylated with 20 μg/ml BirA (Avidity), a bacterial biotin ligase, in KMg25, 2 μM calmodulin, 10 mM MgATP, and 50 μM biotin at 30 °C for 1 h. Free biotin and ATP were removed by dialysis versus KMg25, and the protein was concentrated using spin concentrators (Millipore).

The concentration of myo1b was determined using the Coomassie Plus reagent (Pierce). Final stock concentrations were typically 5 mg/ml. Preparations of 4-liter cultures yielded 4–15 mg of protein. Preparations of myo1bIQ and myo1b had higher yields than myo1b and myo1bIQ.

Calmodulin Stoichiometry—Myo1b (1 μM) splice isoforms were mixed with 4 μM phallodin-stabilized actin and 1–50 μM calmodulin in a total volume of 100 μl. The mixture was dialyzed overnight in KMg25 in the absence or presence of 100 μM free calcium. The acmyo1b was sedimented at 25 or 37 °C, and the pellets were washed with 50 μl of KMg25 to remove free calmodulin. The proteins in the pellets were resolved by SDS-PAGE. The amounts of calmodulin in the pellets were determined by monitoring the fluorescence intensity of calmodulin resolved by SDS-PAGE. The amounts of calmodulin in the pellets were determined by comparing the fluorescence with calmodulin standards on the same gels. Incompletely denatured calmodulin is resolved as a doublet by SDS-PAGE. The amounts of calmodulin in the pellets were determined by monitoring the fluorescence intensity of calmodulin resolved by SDS-PAGE. The amounts of calmodulin in the pellets were determined by monitoring the fluorescence intensity of calmodulin resolved by SDS-PAGE. The amounts of calmodulin in the pellets were determined by monitoring the fluorescence intensity of calmodulin resolved by SDS-PAGE.

Steady-state ATPase Assays and ADP Release—Steady-state ATPase activities were measured in KMg25 at 37 °C using the NADH-coupled assay as described (25, 26). Unless stated otherwise, the final protein concentrations after mixing were 0.4 μM myo1b, 2 μM calmodulin, 0–200 μM phallodin-stabilized actin. The rate of ADP release from actin-bound myo1b isoforms was determined by stopped-flow fluorescence at 37 °C as described (27). Experiments were performed on different days, with different pyrene-actin preparations. Variation in the signal-to-noise ratios of the transients (Fig. 5B) is due to differences in the fraction of actin labeled with pyrene.

RESULTS

Myo1b Splice Isoforms Have Identical Steady-state ATPase Properties—We determined the actin-activated ATPase activities of the myo1b splice constructs (Fig. 2) in the absence and presence of 100 μM free calcium (Fig. 3A). The activities of the splice isoforms and myo1bIQ are the same in the absence of calcium at 37 °C (TABLE ONE). Our ATPase assays yield significantly higher Vmax rates (~10-fold) than determined previously for myo1b under similar conditions (15), which may be the result of the assay system used (26).

The addition of 100 μM calcium increases the Vmax ~4-fold for the three splice constructs and myo1bIQ, but it does not significantly change the KATPase Value (TABLE ONE). The concentration at which calcium activates the ATPase activity of the splice isoforms is also the same as for myo1bIQ (Fig. 3B; TABLE ONE). Therefore, the calmodulin bound to the first IQ motif is responsible for calcium regulation of the ATPase activity, confirming the previous studies (15).

Calmodulin Binds Weakly to a Subset of the IQ Motifs—We determined the number of calmodulins bound to each isoform by sedimentation assays. Myo1b (1 μM) was mixed with 1–50 μM calmodulin and was incubated in the presence of 4 μM phallodin-stabilized actin. The actomyosin complexes were pelletized, and the proteins were resolved by SDS-PAGE along with calmodulin standards. Because 2 μM calmodulin was always present during the preparation of the myosin (see “Experimental Procedures”), the IQ motifs that bind with dissociation constants (Kd) <0.2 μM should have calmodulin bound at the start of our experiments.

In the absence of calcium and in presence of 1 μM calmodulin, the myo1b IQ motifs are not saturated with calmodulin, i.e. there is not
stoichiometric binding of calmodulin to the splice isoforms (Fig. 4; TABLE TWO). Increasing the calmodulin concentration to 50 μM resulted in approximately stoichiometric levels bound. The requirement for high concentrations of free calmodulin to achieve stoichiometric calmodulin binding suggests that a subset of the IQ motifs bind calmodulin weakly. We cannot determine the precise affinities, but the weakest appear to have $K_d$ values of 5 μM (TABLE TWO). We also measured the binding of calmodulin to myo1ba at 37 °C and did not find a significant difference (Fig. 4A).

Calmodulin binding to myo1b$^a$ and myo1b$^b$ in the presence of 100 μM calcium is similar to binding in the absence of calcium (Fig. 4; TABLE TWO). Therefore, calcium does not greatly affect the calmodulin-concentration binding stoichiometry under the conditions of the experiment. In the presence of calcium, fewer calmodulins are bound to myo1b$^b$ at 1 μM calmodulin, indicating that the affinity of at least one of the IQ motifs for calmodulin is calcium dependent (Fig. 4B).

To determine whether the steady-state ATPase activities were affected by the binding of calmodulin to the IQ motifs with weak calmodulin affinities, we measured the actin-activated ATPase activity in the presence of 1–50 μM calmodulin and in the presence and absence of 100 μM calcium (Fig. 3C). We found the ATPase activities to be independent of the calmodulin concentration.

The Motile Activity of Myo1b Depends on the Calmodulin Concentration—We determined the motile activity of myo1b using the in vitro motility assay (24). Because we are interested in determining how differences in the regulatory domain affect motility, it is essential that our motor constructs are site-specifically attached to the motility surface. Therefore, we constructed myo1b splice isoforms to contain a C-terminal tag that can be biotinylated by bacterial biotin ligase (Fig. 2).

Standard motility chambers were prepared, but instead of attaching myo1b directly to the nitrocellulose-coated coverslip, the slide was first coated with streptavidin and then blocked with bovine serum albumin. Biotinylated myo1b was added to the coverslip and allowed to bind to the immobilized streptavidin. All three isoforms supported high quality motility (smooth gliding without stops) of actin filaments. We did not detect motility if the bovine serum albumin blocking step preceded the addition of streptavidin or if we used non-biotinylated myo1b. There-
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Steady-state ATPase Properties of myo1b

Experiments were performed at 37 °C in KMg25 (10 mM Mops, pH 7.0, 25 mM KCl, 1 mM MgCl2, 1 mM EGTA, 1 mM dithiothreitol).

| Splice isoform | −Calcium* | + Calcium* | Calmodulin/myo1b | Kd b |
|----------------|-----------|------------|-----------------|------|
|                | Vmax μM s−1 | KATPase μM | Vmax μM s−1 | KATPase μM |
| Myo1b a        | 0.5 ± 0.1 | 40 ± 20 | 2.2 ± 0.24 | 24 ± 6.0 | 0.6 ± 0.04 |
| Myo1b b        | 0.6 ± 0.1 | 80 ± 40 | 2.3 ± 0.28 | 50 ± 18 | 0.8 ± 0.04 |
| Myo1b c        | 0.7 ± 0.1 | 60 ± 20 | 2.3 ± 0.43 | 40 ± 20 | 0.7 ± 0.2 |
| Myo1b IQ       | 0.6 ± 0.1 | 50 ± 20 | 2.2 ± 0.23 | 33 ± 12 | 0.5 ± 0.2 |

* Steady-state ATPase properties of myo1b from hyperbolic fits of the data in Fig. 3. The uncertainties are the S.E. of the fits.

Calcium concentration required to produce half-maximal activation of the steady-state ATPase activity obtained from fits of the data in Fig. 3B to the Hill equation.

The presence of 10 μM calcium completely abolished motility of the three splice isoforms, and motility was not rescued by the addition of 1–50 μM calmodulin in the presence of calcium. However, upon washout of calcium, motility was recovered. Therefore, the loss of motility was not the result of a calcium-dependent denaturation of myosin but was most likely due to calmodulin-dependent conformational changes within the regulatory domain.

**DISCUSSION**

**Alternative Splicing Does Not Affect Myo1b ATPase Properties**—The steady-state ATPase properties of myo1b are not affected by splicing within the IQ motifs (Fig. 3A; TABLE ONE). The calcium concentration dependence of the activation of the ATPase is also not affected by splicing (Fig. 3B; TABLE ONE), confirming that the regulatory effect is
mediated by calcium binding to the calmodulin associated with the IQ motif adjacent to the motor domain (15). Additionally, there is not a calmodulin concentration dependence on the ATPase rate, again confirming that IQs 2–6 do not directly affect the ATPase cycle. Therefore, alternative splicing of myo1b is not a mechanism to regulate the basic biochemical properties of the motor.

Myo1b IQ Motifs Bind Calmodulin with a Range of Affinities—Our results indicate that the myo1b IQ motifs have >25-fold range in calmodulin affinities. In the presence of 1 μM calmodulin and in the absence of calcium, the myo1b has three calmodulins bound, and myo1b and myo1b have four calmodulins bound (Fig. 4). Therefore, we deduce that the IQ motifs not involved in alternative splicing (IQs 1–3), as well as IQ4 and IQ 4/5, bind calmodulin tightly ($K_d < 0.2 \mu M$), whereas IQ4/6, IQ5, and IQ6 have weaker calmodulin affinities.

It has been reported for other myosin-I isoforms that one or more calmodulins dissociate from the regulatory domain in the presence of calcium (12–14). We found that the affinity of calmodulin binding to myo1b and myo1b does not significantly change in the presence of 100 μM calcium. However, calcium clearly weakens the interaction between a calmodulin and a myo1b IQ motif (Fig. 4B). We propose that IQ4/5 is the calcium-sensitive IQ motif of myo1b because it is unique to the isoform.

Differences in calmodulin affinities are most likely due to differences in the sequences of the IQ motifs. Notably, the sequences of the alternatively spliced IQ motifs clearly diverge from the consensus sequence (IQXXXRGXGXXX) (10, 11). IQ5 and IQ6 do not contain the “isoleucine-glutamine” pair of residues that are thought to interact with the C-terminal lobe of calmodulin (Fig. 1). Rather, glutamine is replaced with a tryptophan residue. Additionally, IQ5 and IQ6 do not contain the arginine of the “arginine-glycine” residue pair that interacts with the N-terminal lobe of calmodulin (Fig. 1). The sequences of IQ2 and IQ4 also diverge from the consensus IQ sequences, but calmodulin binding does not appear to be as greatly weakened. The tighter binding may be due to better conservation of side-chain charge and size of the substituted residues. High resolution structural studies are required to determine the relationship of IQ motif sequence to binding affinity.

In the native myo1b molecule, it is possible that the tail domain, which is missing from our expression constructs, interacts with calmodulins that bind weakly to the C-terminal IQ motifs. Additionally, it is possible that the AviTag and FLAG sequences at the end of the expression constructs interfere with calmodulin binding. Either of these possibilities could result in our overestimation of the effective calmodulin dissociation constants (TABLE TWO).

The Regulatory Domain Acts as a Rigid Lever Arm upon Calmodulin Binding—The velocity (V) of actin gliding in the in vitro motility assay can be described by $V = \frac{d}{t_{mot}}$, where $t_{mot}$ is the time that myosin spends strongly bound to actin and d is the step size (29, 30). The step size is determined by the length of the lever arm and angle through which the lever arm swings. Thus, if the regulatory domain acts as a rigid lever arm and $t_{mot}$ remains constant, the velocity of motility should be linearly related to the number of IQ motifs. Because the myo1b splice isoforms have the same steady-state ATPase and ADP release rates, it is likely that they have the same $t_{mot}$ values. Therefore, we can use the in vitro motility assay to determine whether the IQ motifs function within the regulatory domains of the splice isoforms as rigid lever arms.

A plot of motility rate versus the number of IQ motifs present in the splice isoforms is not linear for the range of calmodulin concentrations tested (Fig. 6A). The motility rate is the same for isoforms with 5 and 6 IQ motifs in the presence of 1 μM calmodulin and increases slightly for both in the presence of 50 μM calmodulin. This non-linear relationship is likely due to flexibility of IQ motifs that do not have bound calmodulin. However, when we plot the motility rate versus the number of calmodulins bound to myo1b, we find a linear relationship (Fig. 6B). Therefore, the IQ motifs within the regulatory domains of the splice isoforms act as rigid lever arms only when calmodulin is bound. The linear relationship also suggests that the weakly associating calmodulins bind to the IQ motifs in order from IQ4 to IQ6, i.e. calmodulin binds to IQ4 before IQ5 and IQ6. If calmodulin bound to the more C-terminal IQ motifs first, then the flexibility within the intervening IQ motif would decrease the effective step size and motility rate.

A linear fit of the data in Fig. 6A yields a slope of 22 nm/s/calmodulin. If we divide this number by the rate of ADP release ($1/t_{mot} = 5.5 \text{ s}^{-1}$), we get a step size of 4 nm/calmodulin. This value is larger than the ~2-nm
step/IQ motif for the myo1b molecule determined by single molecule studies (31). This discrepancy may be accounted for by differences in calmodulin concentration and method of attachment to the motility surface.

Cellular Implications—The cellular concentration of calmodulin in most cells is <10 μM (for review, see Ref. 32). Unless myo1b is sequestered at locally high concentrations (>20 μM), it is unlikely that the IQ motifs with weak calmodulin affinities are occupied by calmodulin in the cell. Rather, other proteins, or light chains, might associate with these sites. For example, myosin-II binds essential and regulatory light chains, and binding of non-calmodulin proteins has been observed for unconventional myosins, including myosin-I (33), myosin-V (34, 35), and myosin-X (36). We propose that unidentified proteins may regulate myo1b localization and activity via these sites, as suggested for myo1c (37) and myosin-V (34).

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