Identification and Characterization of an 8-kDa Light Chain Associated with *Dictyostelium discoideum* MyoB, a Class I Myosin

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*Dictyostelium discoideum* MyoB is a single-headed class I myosin. Analysis of purified MyoB by SDS-PAGE indicated the presence of an ∼9-kDa light chain. A tryptic digest of MyoB yielded a partial sequence for the light chain that exactly matched a sequence in a 73-amino-acid, 8,296-Da protein (dictybase number DDB0188713). This protein, termed MlcB, contains two EF-hand motifs and shares ∼30% sequence identity with the N- and C-terminal lobes of calmodulin. FLAG-MlcB expressed in *Dictyostelium* co-immunoprecipitated with MyoB but not with the related class I myosins MyoC and MyoD. Recombinant MlcB bound Ca\(^{2+}\) with a \(K_d\) value of 0.2 \(\mu\)M and underwent a Ca\(^{2+}\)-induced change in conformation that increased \(\alpha\)-helical content and surface hydrophobicity. Mutational analysis showed that the first EF-hand was responsible for Ca\(^{2+}\) binding. In the presence and absence of Ca\(^{2+}\), MlcB was a monomer in solution and bound to a MyoB IQ motif peptide with a \(K_d\) value of ∼0.5 \(\mu\)M. A MyoB head-neck construct with a Ser to Glu mutation at the T65 site bound MlcB and displayed an actin-activated Mg\(^{2+}\)-ATPase activity that was insensitive to Ca\(^{2+}\). We conclude that MlcB represents a novel type of small myosin light chain that binds to IQ motifs in a manner comparable with a single lobe of a typical four-EF-hand protein.

The class I myosins are ubiquitously expressed, single-headed, non-filament forming myosins composed of a heavy chain and one or more light chains (1–3). Myosin I heavy chains consist of three domains: an N-terminal motor domain that drives movement along actin filaments, an \(\alpha\)-helical neck region to which the light chains bind and a C-terminal tail. Seven different myosin heavy chain genes (*myoA–F* and *myoK*) are present in the genome of the highly motile social amoeba *Dictyostelium discoideum* (4). MyoA, MyoE, and MyoF have relatively short tails that consist solely of a tail homology 1 (TH1) domain that binds membranes. MyoB, MyoC, and MyoD have longer tails that consist of a TH1 domain, a TH2 domain that binds actin filaments in a nucleotide-independent manner and an SH3 domain. MyoK is an atypical myosin I (MyoK) that has no tail but that contains a TH2 domain-like insert in the motor domain.

The *Dictyostelium* myosin I isozymes localize to dynamic, actin-rich cortical regions of the cell, including macropinocytic cups and the leading edge of migrating cells (5, 6). Disruption of one or more myosin I heavy chain genes can produce defects in cortical tension, the uptake of fluids and particles, endosome recycling, and pseudopod retraction (7–12). Although there is a substantial degree of overlap in the functions of the myosin isozymes, individual isozymes apparently carry out unique tasks. MyoB, for example, plays a role in cellular translocation, the suppression of lateral pseudopod formation, and rapid intracellular particle motility, all of which are not functionally substituted for by other myosin I isozymes (13).

Although the cellular functions of the *Dictyostelium* myosin I isozymes have the subject of extensive investigation, only the 16-kDa light chain associated with MyoD, termed MlcD, has been identified (14). Like the myosin II essential and regulatory light chains (ELC and RLCs, respectively) and calmodulin (CaM), which acts as the light chain for many types of unconventional myosins, MlcD consists of four helix-loop-helix EF-hand Ca\(^{2+}\)-binding motifs. In contrast to CaM, but similar to most ELCs and RLCs, MlcD has lost the ability to bind Ca\(^{2+}\) with high affinity due to mutations and deletions within the EF-hand motifs (14).

In this paper we report the identification of a novel light chain associated with *Dictyostelium* MyoB. The MyoB light chain, which we have termed MlcB, has a molecular mass of 8 kDa and consists of two EF-hands. MlcB is thus half the size of previously described myosin light chains. MlcB is shown to be a monomer in solution and to contain a single functional EF-hand that binds Ca\(^{2+}\) with submicromolar affinity. MlcB represents the first example of a two-EF-hand protein that functions as a myosin light chain.

**EXPERIMENTAL PROCEDURES**

**Sequence Analysis of the MyoB Light Chain**—MyoB and MyoD were purified from *Dictyostelium* AX3 cells as described previously (15). MyoB (170 \(\mu\)g) was digested overnight with a 1:50 (w/w) ratio of trypsin at room temperature in 50 mM ammonium bicarbonate, pH 8.0. The digest was lyophilized, resuspended in 0.1% trifluoroacetic acid, and applied to a high performance liquid chromatography Superpac Pep-S 5-mm reverse phase column (Amersham Biosciences). The column was eluted with a gradient to 50% acetonitrile. Selected peptides were sequenced at the Alberta Peptide Institute (Edmonton, Canada).

**Cloning of MlcB and MyoB**—DNA sequences encoding MlcB and the head-neck region of MyoB (amino acids 1–731) were obtained by reverse transcription-PCR using as a template poly(A) mRNA extracted from growth-phase AX3 cells using the GeneElute Direct mRNA mini-prep kit (Sigma). PCR products were ligated into the pCR2.1 cloning vector and sequenced.
vector (Invitrogen). MlcB with alanine in place of Asp-16 (MlcB-D16A) and MyoB with the serine at the TEDS site changed to glutamic acid (MyoB-S332E) were created using the QuikChange site-directed mutagenesis kit (Stratagene). The fidelity of all constructs was verified by sequencing.

**Bacterial Expression of MlcB**—The MlcB and MlcB-D16A coding sequences were cloned into the pET28a vector (Novagen) to add an N-terminal hexahistidine (His6)-tag followed by a thrombin cleavage site. Constructs were transformed into Escherichia coli strain BL21(DE3). Bacteria were grown in LB to an A600 of 0.6, and expression was then induced by addition of isopropyl β-D-thiogalactopyranoside to a final concentration of 1 mM. After 8 h at 37 °C, cells were harvested, resuspended in ice-cold TBS (50 mM Tris-HCl, pH 7.4, 150 mM NaCl) containing 0.2 mg/ml lysozyme, lysed by sonication, and centrifuged at 15,000 × g for 1 h. MlcB was recovered in the supernatant and purified over a nickel-nitriotriacetic acid His-Bind column (Novagen). Fractions containing MlcB were pooled, diluted with 20 mM Tris-HCl, pH 7.4, and loaded onto a DEAE anion exchange column equilibrated in 20 mM Tris-HCl, pH 7.4, 50 mM NaCl. The column was washed with Tris-HCl, pH 7.4, 150 mM NaCl and eluted stepwise with 175, 200, and 300 mM NaCl. Fractions containing MlcB were pooled and chromatographed over a Sephacryl S-100 gel filtration column (Amersham Biosciences) to remove thrombin and re-chromatographed over a Sephacryl S-100 column. MlcB uniformly labeled with 15N or 13C/15N was prepared from bacteria grown in M9 minimal medium supplemented with 1 g/liter 15NH4Cl and 10 ml/liter 15N-BioExpress-1000 medium or 1 g/liter 15NH4Cl, 2 g/liter [13C]glucose, and 10 ml/liter of 15C/15N-BioExpress-1000 medium, respectively (Cambridge Isotope Laboratories).

A bicistronic MlcB construct was created as follows. DNA encoding MlcB, with a stop codon, was inserted into the BamHI/XhoI site of pGEX-4T3 to generate pGEX-4T3-MlcB. A second copy of MlcB with a 5′-ribosome-binding site and an N-terminal Myc epitope tag was generated by PCR and cloned into the XhoI site of pGEX-4T3-MlcB to generate a vector that expresses both GST-MlcB and Myc-MlcB. Following expression, the GST-MlcB was visualized by immunoblot analysis using an anti-Myc antibody (Santa Cruz Biotechnology) and enhanced chemiluminescence (Amersham Biosciences).

**Expression of MlcB and MyoB-S332E ΔTail in Dictyostelium**—N-terminal FLAG epitope-tagged versions of MlcB and MyoB-S332E ΔTail were produced by cloning into the pTX-FLAG plasmid (16). The pBsrH vector, which contains a blasticidin resistance cassette, was used to create a vector expressing MlcB fused at the N terminus to maltose-binding protein (MBP) (17). The MBP coding sequence was obtained from the pMAL-C2X vector (New England Biolabs). AX3 cells were transformed by electroporation and selected for growth in HL5 medium containing 20 μg/ml G-418 and/or 10 μg/ml blasticidin. Clonal cell lines were obtained by plating at limiting dilution and checked for protein expression by immunoblot analysis with the anti-FLAG M5 anti-body (Sigma) and/or a monoclonal anti-MBP antibody (New England Biolabs). For immunoprecipitation experiments, cells were lysed in 0.3% Triton, 50 mM Tris-HCl, pH 7.4, 150 mM KCl, and 1× protease inhibitor mixture containing either 5 mM EGTA or 5 mM CaCl2, and centrifuged at 15,000 × g for 15 min. Anti-FLAG M2 agarose beads (Sigma) were added to the supernatant, incubated for 1 h at 4 °C on a rocking platform, and collected by centrifugation. The beads were washed 5× with lysis buffer and eluted with 2× SDS sample buffer (12% sucrose, 2% SDS) or 200 μg/ml of FLAG peptide. Immunoblot analysis was performed using anti-FLAG M5 antibody and rabbit polyclonal antibodies to MyoB, MyoD (15), and MyoC (a gift from Dr. John Hammer III, National Institutes of Health, Bethesda, MD) (6).

**Isothermal Titration Calorimetry (ITC)**—ITC experiments were performed using a MicroCal VP-ITC instrument. All ITC experiments were carried out using MlcB and MlcB-D16A from which the His-tag had been removed. For Ca2+ titration experiments, proteins were diluted against 10 mM HEPES, pH 7.4, 50 mM NaCl, 2 mM EDTA, and 2 mM EGTA and then extensively dialyzed against 10 mM HEPES, pH 7.4, 50 mM NaCl in the presence of Chelex-100 resin (Bio-Rad). Titrations were performed at 30 °C with 2 ml of 50 μM MlcB or MlcB-D16A in the calorimetric cell and a 500 μM solution of CaCl2 in the injection syringe. Typical experiments involved 29 injections of 10 μl, with a pressure power of 15 μcal/s. ITC was also used to monitor the binding of MlcB and MlcD to a 24-residue peptide (Ac-KDFDCTAKIQAFRNWKAKKHSLNE-H2) corresponding to the IQ motif sequence in the MyoB neck. Recombinant MlcD used in these studies was purified from E. coli as described previously (14). MlcB, MlcD, and the MyoB IQ motif peptide were dialyzed against 10 mM HEPES, pH 7.4, 50 mM NaCl containing either 2 mM EDTA and 2 mM EGTA or 2 mM CaCl2. Titrations were performed as described above, using a 50 μM solution of MlcB or MlcD in the calorimetric cell and injection of 10 μl of a 625 μM solution of the MyoB IQ motif peptide. The time-dependent differential power signal was integrated to obtain the total heat evolved after each injection of ligand and corrected for the heat of dilution of the ligand alone. Data analysis and curve fitting were performed using the MicroCal ORIGIN software package.

**CD and Fluorescence Spectroscopy**—Far- and near-UV CD spectra were taken from 180 to 250 nm and from 250 to 320 nm, respectively, on an Olis RSM 1000 spectrophotometer. Far-UV spectra were collected using 150 μM MlcB and a 0.1-mm path length cuvette, with a step size of 0.2 nm, a scan speed of 100 nm/min, a response time of 2.0 s, and a bandwidth of 2 nm. Near-UV spectra were collected in 10-μm cells using 500 μM MlcB and the same scanning parameters. The far- and near-UV spectra reported represent the average of 15 and 32 scans, respectively, with background signal from the buffer subtracted. Secondary structure prediction based on the far-UV CD spectra was performed using the CDNN 2.1 neural network program (18). Fluorescence experiments with 8-anilino-1-naphthalene-sulfonic acid (ANS; Sigma) were performed on a PerkinElmer Life Sciences LS50B luminescence spectrometer at room temperature using 30 μM MlcB and 70 μM ANS. Excitation was at 388 nm, and fluorescence emission was monitored between 400 and 600 nm. Ca2+-free and Ca2+-bound forms of MlcB were prepared for CD experiments by dialysis against 5 mM Tris-HCl, pH 7.4, containing either 2 mM EDTA and 2 mM EGTA or 2 mM CaCl2. For the fluorescence experiments the buffers contained 10 mM HEPES, pH 7.4, 50 mM NaCl and either 2 mM EDTA and 2 mM EGTA or 2 mM CaCl2.

**NMR Spectroscopy**—1H-15N HSQC NMR spectra of 0.37 mM uniformly 15N-labeled MlcB were recorded on a Varian INOVA 600 MHz spectrometer equipped with a pulse field gradient triple resonance cryo-
TABLE 1. SDS-PAGE analysis of MyoB and MyoD purified from Dictyostelium. The Coomassie Blue-stained SDS gels show MyoD electrophoresed on a 12% gel (A), MyoB on an 8% gel (B), and MyoB on a 20% gel. Bands corresponding to the myosin heavy chains (HC) and putative light chains (LC) are indicated. MyoB contains a prominent 16-kDa light chain, whereas MyoD contains a faint band that electrophoreses ahead of the dye front on the 8% gel and with a size of ~9 kDa on the 20% gel. The locations of molecular mass standards are shown.

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probe at 25 °C. The experiments used the enhanced sensitivity pulsed-field gradient approach (19) and comprised a 1024 × 128 raw data matrix, which were zero-filled once in each dimension. The proton chemical shifts were referenced to 0.0 ppm using the trimethylsilyle resonance of the DSS signal in the one-dimensional spectrum. Initial buffer conditions were 10 mM Tris-HCl, pH 6.8, 10 mM NaCl, 90% H2O/10% D2O. Incremental additions of a 500 mM CaCl2 stock solution were made to a final calcium concentration of 3.7 mM. Spectra were collected after each addition and processed and analyzed using NMRPipe (20) and NMRRview (21).

Analytical Ultracentrifugation—Experiments were performed using a Beckman Optima XL-1 instrument equipped with an AN60-Ti rotor. Ca2+-free and Ca2+-bound MlcB were prepared by dialysis against 5 mM Tris-HCl, pH 7.4, 50 mM NaCl containing either 2 mM EDTA and 2 mM EGTA or 2 mM CaCl2. Sedimentation equilibrium experiments were performed at MlcB concentration of 0.4, 0.8, and 1.2 mg/ml and at rotor speeds of 22,000, 25,000, and 28,000 rpm. Data were collected at 280 nm and molecular masses calculated using the Beckman XL-1 data analysis software. Sedimentation velocity experiments were performed at MlcB concentrations of 0.4, 0.8, and 1.2 mg/ml and a rotor speed of 55,000 rpm. Between 200 and 250 scans were taken for each experiment. Data were analyzed using the SEDFIT program.

Actin-activated Mg2+-ATPase Assays—To purify MyoB-S332E-DTail, cells were lysed in 0.3% Triton X-100 and incubated on ice for 1.5 h to induce formation of an actin-mycosin rigor complex (22). The cytoskeleton was collected by centrifugation and washed twice. ATP (10 mM) was then added to release the MyoB-S332E-DTail. Following centrifugation the supernatant was loaded onto an anti-FLAG M2 agarose affinity column and MyoB-S332E-DTail eluted using 200 μg/ml FLAG peptide. ATPase activities were measured by the release of 32P from [γ-32P]ATP as described (23). Actin prepared from rabbit skeletal muscle (24) was added to the assays at a concentration of 30 μM. Reactions were supplemented with recombinant MlcB to ensure that the MyoB necks were fully occupied by the light chain.

RESULTS

SDS-PAGE Analysis of MyoB—MyoB and MyoD were purified from Dictyostelium using established methods and analyzed by SDS-PAGE (15). As shown previously, MyoD consists of a 125-kDa heavy chain and a 16-kDa light chain (Fig. 1A) (14). In contrast, no band with a molecular mass in the range corresponding to that of known myosin light chains (16–30 kDa) was detected in preparations of MyoB (Fig. 1, B and C). MyoB was not denatured or inactive, since it displayed good levels of K+ EDTA ATPase activity and bound to actin filaments in an ATP-dependent manner (15). On closer examination the MyoB preparations were seen to contain a weakly staining band that electrophoresed ahead of the dye front on an 8% SDS gel and close to the 9-kDa molecular size marker on a 20% SDS gel (Fig. 1, B and C). These results raised the possibility that MyoB contains a low molecular mass light chain.

Identification of a Putative MyoB Light Chain by Sequence Analysis of Tryptic Peptides—In an attempt to obtain sequence information for the putative MyoB light chain, MyoB was digested with trypsin, and the resulting peptides separated by reverse phase high performance liquid chromatography. Eight peptides that appeared to be well resolved from neighboring peptides were chosen for automated Edman sequence analysis (Fig. 2). Five peptides yielded amino acid sequences from the MyoB heavy chain, two peptides provided ambiguous sequences, and one peptide (number 8) yielded an amino acid sequence (DGLLMTE-AEITEFFEAADPNNTGF) that was not present in the MyoB heavy chain. The initial yield of peptide number 8 was 140 pmol, which was within the range of initial yields (70–520 pmol) obtained for the five peptides that were derived from the MyoB heavy chain. Peptide number 8 is therefore not derived from a minor protein contaminant of the MyoB preparation but must originate from a protein that is present in molar amounts roughly equivalent to the MyoB heavy chain.

A search of the Dictyostelium sequence data base (www.dictybase.org) with the sequence of peptide number 8 revealed an exact match to a single protein (DDB0188713) that is 73 amino acids in length and has a calculated molecular mass of 8,296 Da (Fig. 3A). The size of this protein is consistent with the presence of a small light chain associated with MyoB. We have named this previously uncharacterized protein MlcB, for myosin light chain B. The sequence of MlcB was confirmed by analysis of reverse transcription PCR products prepared using RNA isolated from growth-phase Dictyostelium as a template. The Dictyostelium gene sequence shows that the mlcB gene is located on chromosome 5 and is interrupted by two introns. Exon 1 encodes the N-terminal methionine, exon 2 encodes residues 2–42, and exon 3 encodes the remainder of the protein.

MlcB Contains Two Putative EF-hand Motifs—MlcB is a highly acidic protein (pI = 3.53) containing 17 aspartic acid and glutamic acid residues and only 4 arginine and lysine residues. Analysis of the MlcB sequence shows the presence of two putative helix-loop-helix EF-hand motifs (Fig. 3A). The classical EF-hand motif is a linear sequence of about 36 amino acids composed of two α-helices surrounding a 12-residue Ca2+-binding loop. Within the 12-residue loop segment the residues at positions 1, 3, and 5 coordinate Ca2+ directly by side chain carboxylate oxygen atoms, the residue at position 7 coordinates Ca2+ via its backbone carbonyl oxygen atom, the residue at position 9 hydro-
to the CaM family, such as S100A2 and calbindin D(9k). The close relative relationship between MlcB and the subgroup of EF-hand proteins that function as myosin light chains further supports the view that MlcB is a MyoB light chain.

**Co-immunoprecipitation of FLAG-MlcB with MyoB**—To obtain additional evidence for the association of MlcB with MyoB, a construct encoding MlcB with an N-terminal FLAG tag was transduced into *Dictyostelium*. Cells were selected for growth in the presence of G418 and examined for the expression of FLAG-MlcB by immunoblot analysis. A single band with a size close to that expected for FLAG-MlcB (~8 kDa) was detected using an anti-FLAG antibody. A band with a size close to that expected for FLAG-MlcB (~8 kDa) was detected using an anti-FLAG antibody. The overexpression of FLAG-MlcB did not have a noticeable effect on the growth rates of cells in suspension or on surfaces, the organization of the actin cytoskeleton, or multicellular development (data not shown). Immunoprecipitation of FLAG-MlcB using anti-FLAG M2 antibody beads resulted in the co-immunoprecipitation of the MyoB heavy chain but not the MyoC or MyoD heavy chains (Fig. 4B). This result confirms that MlcB is bound to MyoB and further reveals that MlcB is not a light chain for the closely related long tailed myosin I isoforms MyoC or MyoD.

**Purification and Ca**\(^{2+}\)**-binding Properties of Recombinant MlcB**—MlcB was purified from *E. coli* as a His-tagged protein. His-MlcB is 93 residues in length, has a calculated mass of 10.4 kDa, and electrophoresed on a 20% SDS gel with an apparent size of ~9 kDa (Fig. 5A). For some studies, the N-terminal His-tag was removed from MlcB by thrombin cleavage, yielding a 76-residue-long protein with a calculated mass of 8.6 kDa (Fig. 5B). After removal of the His-tag, recombinant MlcB exhibited a mobility on SDS gels very similar to that of the light chain observed in preparations of MyoB (compare Figs. 5B and 1C). MlcB electrophoresed as a fairly diffuse band on 20% SDS gels, which in part may explain the difficulty in detecting the light chain in preparations of MyoB.

**ITC experiments** showed that MlcB bound Ca\(^{2+}\) in an exothermic reaction, with an enthalpy change (ΔH) of −7.7 kcal/mol of injectant (Fig. 5C). A one-site model yielded an excellent fit to the Ca\(^{2+}\) binding data with a K\(_{d}\) of 0.2 ± 0.06 μM. To test whether Ca\(^{2+}\) binding is mediated by the first EF-hand, the aspartic acid at position 1 of the first EF-hand (Asp-16) was mutated to alanine (Fig. 5A). ITC experiments showed that MlcB-D16A was unable to bind Ca\(^{2+}\) (Fig. 5C). These results show that a functional N-terminal EF-hand is essential for MlcB to bind Ca\(^{2+}\) with high affinity.
MlcB Undergoes a Conformational Change upon Binding Ca\(^{2+}\) — The effect of Ca\(^{2+}\) on the secondary structure of MlcB was investigated using far-UV CD spectroscopy. In the absence of Ca\(^{2+}\) the far-UV CD spectrum of MlcB exhibited a large positive peak of ellipticity at 196 nm and large negative peaks of ellipticity near 208 and 222 nm, typical of proteins that have a significant amount of \(\alpha\)-helical secondary structure (Fig. 6A). In the presence of Ca\(^{2+}\) the intensity of each of the 196, 208, and 222 nm bands increased, suggesting the formation of additional \(\alpha\)-helical structure. Analysis of the spectra using the neural net program CDNN showed that upon addition of Ca\(^{2+}\) the \(\alpha\)-helical content of MlcB increased from 29 to 42% and the random coil content decreased from 36 to 27%.

MlcB contains no tryptophan residues but has 8 phenylalanine and 4 tyrosine residues. The CD spectrum in the 250–270 nm and 270–290 nm range can provide information on the chirality of the phenylalanine and tyrosine side chains, respectively. The near-UV CD signal of MlcB in the absence of Ca\(^{2+}\) shows a strong positive signal from 275 to 290 nm that is converted in the presence of Ca\(^{2+}\) to a broad slightly negative signal (Fig. 6B). This result shows that the packing around the tyrosine side chains is disrupted in the presence of Ca\(^{2+}\), which may indicate that the tyrosine residues adopt a more surface-exposed, flexible conformation in Ca\(^{2+}\)-bound MlcB.

To further examine the effects of Ca\(^{2+}\) on the hydrophobicity of MlcB, studies were performed using ANS, a hydrophobic probe that alters its fluorescence emission properties when it is bound to non-polar sites on proteins. The fluorescence emission maximum of ANS increased 1.8-fold in intensity and shifted from 519 to 488 nm in the presence of Ca\(^{2+}\)-free MlcB (Fig. 6C). These changes are consistent with an interaction between ANS and hydrophobic sites on the surface of MlcB. Upon addition of a saturating level of Ca\(^{2+}\), the fluorescence emission maximum of ANS exhibited an additional 2.3-fold increase in intensity and was further blue-shifted to 480.5 nm (Fig. 6C). These results indicate that the binding of Ca\(^{2+}\) enhances the number of hydrophobic residues exposed on the surface of MlcB.

To obtain greater insight into the extent of the Ca\(^{2+}\)-induced structural changes in MlcB, uniformly \(\mathrm{^{15}N}\) labeled MlcB was examined using two-dimensional \(\mathrm{^{1}H-^{15}N}\) heteronuclear NMR spectroscopy. The \(\mathrm{^{1}H-^{15}N}\) HSQC spectrum of Ca\(^{2+}\)-free MlcB displayed good overall chemical shift dispersion of the \(\mathrm{^{1}H-^{15}N}\) correlation resonances, and the number of resonances detected was close to that expected for a protein of the size of MlcB (Fig. 7A). These data are indicative of a well ordered protein with a significant amount of helical and random coil structure and a small amount of extended structure. The addition of saturating concentrations of Ca\(^{2+}\) significantly changed the \(\mathrm{^{1}H-^{15}N}\) correlation spectrum of MlcB (Fig. 7B). The dispersion of backbone \(\mathrm{^{1}H}\) resonances increased, particularly in the 8.0–8.5 ppm region, and additional resonances appeared at greater than 8.5 ppm in the \(\mathrm{^{1}H}\) dimension. The extent of the Ca\(^{2+}\)-induced spectral change is comparable with that...
observed for EF-hand proteins, such as CaM, troponin C, and S100 family members (28, 29), and is consistent with a Ca$^{2+}$-mediated stabilization or reorientation of the MlcB secondary structure.

The residues giving rise to the peaks in the $^1$H-$^{15}$N correlation spectrum of MlcB were assigned using standard triple resonance NMR experiments and uniformly labeled $^{13}$C/$^{15}$N MlcB. Resonances corresponding to Gly-17 and Gly-21 within the Ca$^{2+}$-binding loop of the first EF-hand underwent chemical shift changes upon addition of Ca$^{2+}$, while resonances corresponding to Thr-55 and Gly-56 of the second EF-hand Ca$^{2+}$-binding loop greatly increased in intensity but maintained their $^1$H and $^{15}$N chemical shifts (Fig. 7). These observations are consistent with Ca$^{2+}$ binding to the N-terminal EF-hand which in turn affects the structural integrity of the C-terminal loop via a short anti-parallel β-sheet that we have identified in MlcB (data not shown) and that is typical for EF-hand pairs. These long range effects appear to decrease the flexibility and/or surface exposure of the region of the C-terminal loop containing Thr-55 and Gly-56.

MlcB Is a Monomer—Sedimentation experiments performed in the analytical ultracentrifuge over a range of MlcB concentrations (0.4–1.2 mg/ml) yielded a molecular mass for MlcB of 9.4 ± 0.7 kDa in the presence and absence of Ca$^{2+}$ (Fig. 8A, B). This value shows that MlcB is a monomer in solution. Sedimentation velocity experiments yielded a sedimentation coefficient for Ca$^{2+}$-free MlcB of 1.44 ± 0.028. In the presence of Ca$^{2+}$ the sedimentation coefficient increased slightly to 1.50 ± 0.06, suggesting that the binding of Ca$^{2+}$ causes MlcB to adopt a slightly more compact conformation.

To further examine whether MlcB forms dimers, a bicistronic vector was developed that allowed for the co-expression of GST-MlcB and Myc-MlcB in the same bacterial cell. Purification of GST-MlcB over a glutathione-Sepharose column in both the presence and absence of Ca$^{2+}$ failed to result in the co-purification of Myc-MlcB (data not shown). This result confirms that MlcB displays little or no tendency to self-associate.

Interaction of MlcB with the MyoB IQ Motif—The neck region of MyoB contains a single IQ motif with the sequence: IQKAFRNWKAK (30). A 24-residue peptide encompassing the MyoB IQ motif was synthesized and tested for its ability to bind MlcB. Titration of the peptide into solutions of MlcB in the absence and presence of Ca$^{2+}$ yielded $K_d$ values of 0.50 ± 0.01 μM and 0.60 ± 0.01 μM, respectively (Fig. 9, A and B). In both cases the binding interaction proceeded with a large negative enthalpy change (20–25 kcal/mol of injectant) and reached a binding stoichiometry close to 1. Apparently, the conformational change induced in MlcB by Ca$^{2+}$ does not appreciably alter the affinity of MlcB for the MyoB IQ motif. Similarly, the presence of the IQ motif peptide did not have a significant effect on the Ca$^{2+}$ binding properties of MlcB (data not shown). ITC titrations were also performed using the MyoD light chain, MlcD. No interaction between MlcD and the MyoB IQ motif peptide was detected (Fig. 9, A and B). This result is consistent with the finding that FLAG-MlcD expressed in Dictyostelium does not co-immunoprecipitate with MyoB (14) and supports the view that MlcB and MlcD exhibit a high degree of specificity for myosin heavy chain IQ motifs.

Actin-activated Mg$^{2+}$-ATPase Activity of MyoB—The ability of MlcB to bind Ca$^{2+}$ suggested the possibility that the motor activity of MyoB is regulated by Ca$^{2+}$. Purified MyoB displayed only a low level of actin-activated MgATPase activity, most likely because the TEDS site was not phosphorylated. In agreement with a previous report, MyoB could not be phosphorylated and activated using either Dictyostelium PakB or human Pak1 (23). A recombinant MyoB head-neck construct (MyoB-ΔTail) was therefore generated that contained a serine to glutamic acid mutation at the TEDS site (S332E) (Fig. 10A). The incorporation of a negative charge at the TEDS site is expected to constitutively activate MyoB (31). Co-immunoprecipitation studies performed using a Dictyostelium cell line expressing both FLAG-MyoB-S332E-ΔTail and a MBP-tagged MlcB showed that the two proteins formed a complex in the presence and absence of Ca$^{2+}$ (Fig. 10B). MyoB-S332E-ΔTail was purified to homogeneity and on SDS gels electrophoresed with a molecular mass close to its expected size of 83

**FIGURE 7.** Two-dimensional NMR spectrum of MlcB. $^1$H-$^{15}$N HSQC spectrum of His-MlcB in the absence of Ca$^{2+}$ (A) and the presence of 2 mM Ca$^{2+}$ (B). Resonances arising from Gly-17 and Gly-21, in the Ca$^{2+}$-binding loop of the first EF-hand, and Thr-55 and Gly-56, in the Ca$^{2+}$-binding loop of the second EF-hand, are indicated. Numerous peaks shift position or are strengthened upon Ca$^{2+}$ binding, providing evidence for a significant change in the conformation of MlcB. Residues are numbered as for native MlcB (Fig. 3A).

**FIGURE 8.** Sedimentation equilibrium analysis of MlcB. His-MlcB at a concentration of 100 μM was centrifuged at a rotor speed of 28,000 rpm in the absence of Ca$^{2+}$ (A) or the presence of 2 mM Ca$^{2+}$ (B). In both cases a fit to the data using a single species model yielded a molecular mass of 9,400 Da. This model shows a good fit to the data as judged by the randomness of the residual from the curve fit (top panels). Equivalent results were obtained using His-MlcB at concentrations of 50 and 150 μM and rotor speeds of 22,000 and 25,000 rpm.
kDa (Fig. 10C). A small amount of a protein migrating at the expected size of MlcB could be visualized by silver staining when SDS-PAGE was performed on a 20% gel (data not shown). MyoB-S332E-ΔTail displayed a K^+EDTA-ATPase activity of 6.2 μmol/min/mg, close to the value of 5.3 μmol/min/mg reported for MyoB (15). The Mg^{2+}-ATPase activity of MyoB-S332E-ΔTail was activated 8.5-fold by the addition of F-actin. No change in the actin-activated Mg^{2+}-ATPase activity was observed upon addition of Ca^{2+} (Fig. 10D).

**DISCUSSION**

In this report we show that MyoB, a long tailed *Dictyostelium* myosin I isoform, is associated with a novel 8-kDa light chain, which we have termed MlcB. The evidence that MlcB is a MyoB light chain may be summarized as follows: (i) purified MyoB examined by SDS-PAGE does not contain a "normal sized" (16–30 kDa) light chain but does contain a protein the size of MlcB; (ii) a peptide derived from MlcB was isolated with good yield from a digest of MyoB, showing that MlcB co-purifies with MyoB; (iii) sequence comparisons show that MlcB is closely related to known myosin light chains; (iv) MlcB binds to a peptide corresponding to the IQ motif in the MyoB neck region; (v) FLAG-MlcB expressed in *Dictyostelium* co-immunoprecipitates with the MyoB heavy chain; and (vi) a MyoB head-neck construct (MyoB-S332E-ΔTail) co-immunoprecipitates with MBP-tagged MlcB. We believe that, taken together, these results provide convincing evidence that MlcB is a light chain that binds to the neck region of MyoB.

Light chains bind to sequences in the myosin neck region known as IQ motifs that contain the generalized consensus sequence IQXxxRGGxR, where x is any amino acid (30). The essential role of light chains is to stabilize the α-helical myosin neck, so that it can act as a rigid lever arm to amplify and transmit conformational changes that occur in the head domain (32). Light chains can also be critical sites for regulation of myosin activity, either through phosphorylation or by the direct binding of Ca^{2+} (2,33). The studies reported here show that MlcB displays an apparent K_d for Ca^{2+} of 0.2 μM, an affinity that is sufficiently high to allow MlcB to sense physiological changes in Ca^{2+} concentration. In contrast, MlcD, the MyoD light chain, exhibits a low affinity for Ca^{2+} (K_d ~50 μM) and is unlikely to be responsible for changes in cellular Ca^{2+} levels (14). Interestingly, MyoB and MyoD have distinct cellular functions. In stationary stage cells MyoB and MyoD co-localize to the leading edge of lamellipodia and at sites of cell-cell contact, but in starved, aggregated cells, MyoB remains at these sites, whereas MyoD relocates to the cytoplasm (34). Cells lacking MyoD exhibit no obvious behavioral defects, but cells lacking MyoB are unable to suppress lateral pseudopod formation and are impaired in phagocytosis, pinocytosis, and intracellular particle movement (9,12,13,35,36). The possibility that some of these functional differences may reflect the divergent Ca^{2+}-binding properties of the light chains, rather than variations in the heavy chains, must now be taken into consideration.

The binding of Ca^{2+} produced significant changes in the secondary structure of MlcB and resulted in the exposure of additional surface hydrophobic sites, raising the possibility that Ca^{2+} alters the mode of binding of MlcB to the MyoB neck region and perhaps regulates the functional properties of MyoB. The results presented here show that Ca^{2+} does not alter the affinity of MlcB for the MyoB IQ motif (Fig. 9) or the ability of MlcB to bind the MyoB-S332E-ΔTail construct (Fig. 10B). Moreover, Ca^{2+} did not modulate the actin-activated MgATPase activity of MyoB-S332E-ΔTail (Fig. 10D). Further studies are required to examine the possibility that Ca^{2+} may regulate the cellular localization of MyoB or the motor activity of MyoB as measured using actin filament gliding assays.

Phylogenetic analyses of the head and neck/tail domains show that *Dictyostelium* MyoB and MyoD are the counterparts of *Acanthamoeba castellanii* myosin IB and myosin IC (AMIB and AMIC), respectively (37). If the heavy and light chains of myosin co-evolve, it would be expected that MyoB/AMIB and MyoD/AMIC would be associated with comparable light chains. This relationship holds for MyoD/AMIC,
since AMIC has a 16.8-kDa CaM-like light chain (MICLC) that shares 43% sequence identity with MlcD (14, 38). On the other hand, AMIB co-purifies with one subunit of a 27-kDa protein and variable, and always less than equimolar amounts, of a 14-kDa protein (39, 40). The discrepancy in the size and nature of the light chains associated with AMIB and MyoB is surprising, especially in light of the fact that they have IQ motifs that share 61% sequence identity. If the light chains for MyoB and AMIB are dissimilar, it could indicate that the cellular functions of the two myosins have diverged. Alternatively, the possibility that a light chain equivalent to MlcB is associated with AMIB but has gone undetected due to its small size cannot be discounted.

Previously described myosin light chains include CaM, RLCs, ELCs, MICLC, MlcD, and unique CaM-like proteins associated with human myosin X (41) and the class XIV Toxoplasma gondii myosin A (42). All of these light chains have four EF-hands and belong to the CTER (CaM, troponin C, ELC, and RLC) subfamily of EF-hand proteins (43). MlcB is half the size of other myosin light chains, but sequence alignments place it firmly within the CTER branch of the EF-hand superfamily. Other small (~ 8 kDa) proteins with two EF-hands that belong to the CTER subfamily include human calibrin (27), the Arabidopsis proteins CML28 and CML29 (44), a second Dictyostelium protein (dictyBase number DDB0219456), and an E. histolytica protein (GenBank™ accession number EAL49262). The Dictyostelium DDB0219456 protein and the Entamoeba two-EF-hand protein share a particularly high degree of sequence identity with MlcB (Fig. 3, A and B). Although no functional information on these proteins is available, their similarity to MlcB suggests the possibility that they may be myosin light chains. In this regard, it is noteworthy that Entamoeba contains a myosin I with 54% identity and 70% similarity to MyoB (37, 45).

The x-ray structure of the yeast four-EF-hand protein Mlc1p in a complex with IQ2 and IQ4 of the class V myosin Myo2p has been solved (46). Mlc1p binds to IQ2, a “complete” IQ motif that conforms to the IQxxRxxxx consensus sequence, via both its N- and C-terminal domains. The C-terminal lobe, which consists of EF-hands 3 and 4, interacts with the more variable GxxxR part of the motif and the N-terminal lobe, which consists of EF-hands 1 and 2, interacts with the more conserved IQxxxR consensus sequence, via both its N- and C-terminal domains. The C-terminal lobe, which consists of EF-hands 3 and 4, interacts with the more variable GxxxR part of the motif and the N-terminal lobe, which consists of EF-hands 1 and 2, interacts with the more conserved IQxxxR consensus sequence, via both its N- and C-terminal domains.

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REFERENCES
1. Osherov, N., and May, G. S. (2000) Cell Motil. Cytoskeleton 47, 163–173
2. Barylko, B., Binns, D. D., and Albanesi, I. P. (2000) Biochim. Biophys. Acta 1496, 23–35
3. Coluccio, L. M. (1997) Am. J. Physiol. 273, C547–C559
4. De La Roche, M. A., and Côté, G. P. (2001) Biochim. Biophys. Acta 1525, 245–261
5. Fukui, Y., Lynch, T. J., Brzeska, H., and Korn, E. D. (1989) Nature 341, 328–331
6. Jung, G., Remmert, K., Wu, X., Volosky, J. M., and Hammer, J. A., III (2001) J. Cell Biol. 153, 1479–1498
7. Neuhauß, E. M., and Soldati, T. (2000) J. Cell Biol. 150, 1013–1026
8. Schwarz, E. C., Neuhauß, E. M., Kistler, C., Henskel, A. W., and Soldati, T. (2000) J. Cell Sci. 113, 621–633
9. Fark, D. L., Wessels, D., Jenkins, L., Pham, T., Kuhl, S., Titus, M. A., and Soll, D. R. (2003) J. Cell Sci. 116, 3985–3999
10. Dai, J., Ting-Beall, H. P., Hochmuth, R. M., Sheetz, M. P., and Titus, M. A. (1999) Am. J. Physiol. 277, 1168–1176
11. Novak, K. D., Peterson, M. D., Reedy, M. C., and Titus, M. A. (1995) J. Cell Biol. 131, 1207–1221
12. Jung, G., Wu, X. F., and Hammer, J. A., III (1996) J. Cell Biol. 133, 305–323
13. Wessels, D., Murray, J., Jung, G., Hammer, J. A., III, and Soll, D. R. (1991) J. Cell Biol. Cytoskeleton 20, 301–315
14. De La Roche, M. A., Lee, S.-F., and Cote, G. P. (2003) Biochem. J. 374, 697–705
15. Lee, S.-F., and Côté, G. P. (1993) J. Biol. Chem. 268, 20923–20929
16. Levi, S., Polyaakov, M., and Egelhofer, T. T. (2000) Plasmid 44, 231–238
17. Fischer, M., Haase, I., Simmell, E., Gericke, G., and Müller-Taubenberger, A. (2004) FEBS Lett. 577, 227–232
18. Bohm, G., Muhr, R., and Janicke, R. (1992) Protein Eng. 5, 191–195
19. Kay, L. E., Keiffer, P., and Saarinen, T. (1992) J. Am. Chem. Soc. 114, 10663–10665
20. Delaglio, F., Grzesiek, S., Vuister, G. W., Zhu, G., Pfeifer, J., and Bax, A. (1995) J. Biomol. NMR 6, 277–293
21. Johnson, B. A., and Blevis, R. A. (1994) J. Biomol. NMR 4, 603–614
22. Manstein, D. J., and Hunt, D. M. (1995) J. Muscle Res. Cell Motil. 16, 325–332
23. Lee, S.-F., and Côté, G. P. (1995) J. Biol. Chem. 270, 11776–11782
24. Spudich, J. A., and Watt, S. (1971) J. Biol. Chem. 246, 4866–4871
25. Lewitt-Bentley, A., and Rety, S. (2000) Curr. Opin. Struct. Biol. 10, 637–643
26. Mandenian, B. J., Shaw, G. S., and Sykes, B. D. (1990) Biochem. Cell Biol. 68, 587–601
27. Yamaguchi, K., Yamaguchi, F., Miyamoto, O., Sugimoto, K., Konishi, R., Hatase, O., and Tokuda, M. (1999) J. Biol. Chem. 274, 3610–3616
28. Smith, S. P., and Shaw, G. S. (1998) Biochim. Biophys. Acta 76, 324–333
29. Slupszy, C. M., Reinhach, F. C., Smillie, L. B., and Sykes, B. D. (1995) Protein Sci. 4, 1279–1289
30. Bahler, M., and Rhoade, A. (2002) FEBS Lett. 513, 107–113
Identification and Characterization of an 8-kDa Light Chain Associated with Dictyostelium discoideum MyoB, a Class I Myosin

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