Structure of the Small Dictyostelium discoideum Myosin Light Chain MlcB Provides Insights into MyoB IQ Motif Recognition*

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Background: MlcB is a MyoB-specific light chain.

Results: MlcB adopts a unique fold among EF-hand calcium-binding proteins and binds the MyoB IQ motif via a hydrophobic surface that is maintained in the holo state.

Conclusion: The MlcB structure and mode of IQ recognition is unique among myosin light chains.

Significance: ApoMlcB structure provides a basis for MyoB IQ-motif recognition.

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‡ The atomic coordinates and structure factors (code 2M1U) have been deposited in the Protein Data Bank (http://wwpdb.org/).
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Dictyostelium discoideum MyoB is a class I myosin involved in the formation and retraction of membrane projections, cortical tension generation, membrane recycling, and phagosome maturation. The MyoB-specific, single-lobe EF-hand light chain MlcB binds the sole IQ motif of MyoB with submicromolar affinity in the absence and presence of Ca2+. However, the structural features of this novel myosin light chain and its interaction with its cognate IQ motif remain uncharacterized. Here, we describe the NMR-derived solution structure of apoMlcB, which displays a globular four-helix bundle. Helix 1 adopts a unique orientation when compared with the apo states of the EF-hand calcium-binding proteins calmodulin, S100B, and calbindin D9k. NMR-based chemical shift perturbation mapping identified a hydrophobic MyoB IQ binding surface that involves amino acid residues in helices I and IV and the functional N-terminal Ca2+ binding loop, a site that appears to be maintained when MlcB adopts the holo state. Complementary mutagenesis and binding studies indicated that residues Ile-701, Phe-705, and Trp-708 of the MyoB IQ motif are critical for recognition of MlcB, which together allowed the generation of a structural model of the apoMlcB–MyoB IQ complex. We conclude that the mode of IQ motif recognition by the novel single-lobe MlcB differs considerably from that of stereotypical bilobal light chains such as calmodulin.

The class I myosins represent a group of widely expressed, single-headed, non filament-forming myosins (1–3). The myosin-I heavy chain comprises a conserved N-terminal motor domain that drives ATP-dependent movement along actin filaments, an IQ-motif containing helical neck region that recruits light chains (LCs), and a C-terminal tail. Class I myosins can have a short tail consisting solely of an acid phospholipid binding tail homology 1 (TH1) domain or a long tail containing a TH1 domain, a glycine/proline/alanine/glutamine (GPAQ) domain that binds actin filaments, and a Src homology 3 (SH3) domain (1, 2).

The highly motile social amoeba Dictyostelium discoideum, which is a commonly used model system for studying mechanisms of cell motility, expresses seven class I myosin isoforms. MyoA, MyoE, and MyoF have short tails, and MyoB, MyoC, and MyoD possess long tails, whereas MyoK has neither a neck region nor tail but does contain a GPR-rich insert within its motor domain that binds actin filaments (4–8). These motor proteins localize to dynamic, actin-rich regions of the cell, including pseudopodia and the leading edge of migrating cells. Cellular defects in cortical tension, endosome recycling, phagocytosis, and pseudopod retraction result from the disruption of the class I myosins (9–16). The severity of these phenotypes is enhanced when combinations of these myosins are knocked out, illustrating functional redundancy among the class I myosins (17–19). However, individual class I isoforms also display unique properties, such as the role of MyoB in rapid movement of intracellular particles and the suppression of lateral pseudopod extension during chemotaxis (9, 15, 20).

Despite significant advances in our understanding of the structures and cellular functions of D. discoideum class I myosins over the past two decades, only recently has detailed information began to emerge regarding the LCs that bind to the 20–25 residue IQ motifs within the neck region of the heavy chains (21). The number of IQ motifs among the class I isoforms, which conform in varying degrees to an IQMXXRGXXXR consensus sequence, range from none in...
MyoK to three in MyoC (5). The short-tailed MyoA and MyoE employ calmodulin (CaM) as a light chain, whereas the long-tailed MyoB, MyoC, and MyoD each bind a unique LC (22–24). MlcD, the cognate LC for MyoD, is 16.5 kDa in size and comprises four EF-hand motifs, all of which have lost the ability to bind calcium (Ca$^{2+}$) with high affinity due to mutations in key Ca$^{2+}$-coordinating residues (24). A MlcB-specific LC, termed MlcB, was identified as a small (8.3 kDa) single-lobe protein comprising two EF-hand motifs, of which only the N-terminal motif is capable of binding Ca$^{2+}$ (22). Although MlcB undergoes a Ca$^{2+}$-dependent conformational change, it binds to the single MyoB IQ motif with submicromolar affinity in both the absence and presence of Ca$^{2+}$. Another small (8.6 kDa) LC referred to as MlcC is closely related to MlcB and binds to the first two IQ motifs of MyoC (23). In contrast to MlcB, neither of the MlcC EF-hand motifs possesses the ability to bind Ca$^{2+}$.

Here we present the NMR-derived solution structure of apoMlcB, the first of a single-lobe myosin LC. A comparison of this structure with other EF-hand-containing proteins indicates that apoMlcB represents a novel member of the EF-hand superfamily of Ca$^{2+}$-binding proteins. NMR-based chemical shift perturbation studies, site-directed mutagenesis, and computational docking identified the MyoB IQ motif binding site on the MlcB structure and showed that it involved both hydrophobic and electrostatic interactions.

**EXPERIMENTAL PROCEDURES**

**Cloning of Bicistronic GST-MyoBIQ and Myc-MlcB**—The MyoB IQ motif (MyoB-IQ) was cloned into the BamHI/XhoI sites of the pGEX-4T-3 vector (GE Healthcare) using annealed oligomers comprising residues 693–716 of MyoB (KDFDCTAIQKAFRNNWKAKKHSLE) to generate GST-MyoBIQ. PCR was used to amplify the GST-MyoBIQ construct with NcoI and BamHI restriction sites at the 5′ and 3′ ends, respectively. An MlcB-encoding plasmid with a 5′-ribosome-binding site and an N-terminal Myc epitope tag was also generated by PCR with BglII and XhoI restriction sites flanking the construct. Ligation of GST-MyoBIQ and Myc-MlcB encoding fragments into the pET-28a vector (Novagen) was performed simultaneously to generate a vector that expressed both GST-MyoBIQ and Myc-MlcB. A control vector encoding GST and Myc-MlcB was similarly constructed. For isothermal titration calorimetric experiments, the MyoBIQ sequence was cloned into a modified pET-21b vector containing an N-terminal GST tag and a C-terminal hexahistidine tag. Alanine mutations of Cys-697, Ile-701, Phe-705, and Trp-708 in both MyoBIQ and Myc-MlcB were performed using the QuikChange site-directed mutagenesis kit (Stratagene). All vectors were transformed into BL21 (DE3) cells for protein expression.

**Protein Expression and Purification and Peptide Synthesis**—Bacterial expression and purification of uniformly $^{13}$C,$^{15}$N-labeled apomlcB in 10 mM HEPES, pH 7.4, 50 mM NaCl. Fractions eluting at 60 – 80 ml were pooled and concentrated at 4 °C using Amicon Ultra centrifugal filters (Millipore Corp.) with a molecular mass cut off of 3 kDa. A synthetic 24-residue MyoBIQ peptide comprising residues 693–716 of MyoB (Ac-KDFDCTAIQKAFRNNWKAKKHSLE-NH$_2$) was generated for NMR mapping studies (22).

Expression of the plasmidic constructs involved induction of transformed Escherichia coli BL21 (DE3) cells with 1 mM isopropyl β-D-thiogalactopyranoside after growth on Luria-Bertani (LB) medium at 37 °C reached an A$_{600}$ of 0.6. Subsequent growth at 30 °C was continued for an additional 6 h. Cells were then resuspended in 1 × phosphate-buffered saline (PBS; 10 mM Na$_2$HPO$_4$, 1.8 mM KH$_2$PO$_4$, pH 7.3, 140 mM NaCl, 2.7 mM KCl) containing 0.5 mg/ml lysozyme, 0.1 mg/ml DNase I, and 1 mM phenylmethylsulfonyl fluoride. Cell lysis by sonication and clarification of the cell lysate by centrifugation were performed as described previously (22). GST fusion proteins were purified at 4 °C using GST-Sepharose 4B resin (GE Healthcare). Extensive washing with 1 × PBS was followed by elution in 5 × 1-ml fractions with 50 mM Tris–HCl, pH 8.0, 10 mM reduced glutathione. The GST–MyoBIQ–His wild-type and mutant proteins were purified using Ni$^{2+}$-charged chelating Sepharose fast flow resin (GE Healthcare). Cells were resuspended in 25 mM Tris–HCl, pH 8.0, 250 mM NaCl. The column was washed with resuspension buffer containing 35 mM imidazole, and proteins were eluted with the resuspension buffer containing 500 mM imidazole.

**NMR Spectroscopy and Structure Calculation**—A sample of 0.95 mM uniformly $^{13}$C,$^{15}$N-labeled apomlcB in 10 mM HEPES, pH 6.8, 50 mM NaCl, 5 mM EDTA, 5 mM EGTA in 90% H$_2$O, 10% D$_2$O was used in NMR experiments collected at 25 °C on Varian 500 and 800 MHz spectrometers equipped with pulse field-gradient triple-resonance cryoprobe. Sequential backbone- and side-chain chemical shift assignments were completed using $^1$H,$^15$N HSQC, HNCACB, CBCACONH, CCONH, HCCONH, and HCHC-TOCSY (two-dimensional total correlation spectroscopy) experiments. Interproton distances were determined from three-dimensional $^{15}$N NOESY-HSQC and aliphatic $^{13}$C NOE-SY-HSQC datasets collected in 90% H$_2$O, 10% D$_2$O and in 100% D$_2$O and an aromatic $^{13}$C NOE-SY-HSQC dataset collected in 100% D$_2$O. All dipolar coupling experiments were collected with mixing times of 100 ms. Datasets were processed using NMRPipe (25) and analyzed using NMRView (26).

A combination of 480 sequential, 508 medium ($1 < |i − j| ≤ 5$), and 186 long range ($|i − j| > 5$) NOE-derived distance restraints interpolated from peak intensities and calibrated to known distances found in α-helical regions ($d_{a,a+3}$, $d_{a,a+4}$) were included as inputs to generate 200 structures of apomlcB using a simulated annealing protocol in CNS 1.2 (27) with 20,000 steps in the first cooling stage and followed by 10 cycles of 1000 minimization steps. Backbone dihedral angle restraints were obtained from the analysis of $^{13}$C$_N$, $^{13}$C$_B$, $^{13}$C$_z$, $^{1}H_N$, and $^{15}$N chemical shifts using TALOS (28) with restraints restricted to ±30° or the error from the TALOS output, whichever was greater. The final ensemble included 20 low-energy structures that possessed no distance violations greater than 0.3 Å and no angle violations greater than 5°. PROCHECK was used to assess
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the quality of the structures (29). Chemical shift assignments, NMR restraints, and the structure coordinates for apoMlcB have been deposited to the BioMagResBank (accession no. 18880) and the Protein Data Bank (accession no. 2M1U), respectively. Structure figures were made using MOLMOL (30) and PyMOL (31). The Dali web server was used to perform structural homology searches (32).

Steady-state {1H}-15N heteronuclear NOE data were collected at 25 °C on a Bruker Avance 800 MHz spectrometer equipped with a cryoprobe. The pulse sequence was based on that of Farrow et al. (33), wherein spectra were collected in an interleaved fashion, with an on/off saturation train during the recycling delay, which was 3 s. {1H}-15N NOE values were calculated from the ratio of peak intensities with and without proton saturation for 47 well resolved resonances. Datasets were 10 mM HEPES, pH 6.8, 50 mM NaCl, 5 mM EDTA, 5 mM EGTA at 90% H2O, 10% D2O. Datasets were processed and analyzed using NMRPipe (25) and analyzed using NMRView (26).

NMR-based MyoB IQ Motif Binding Experiments— Backbone amide proton and nitrogen chemical shifts were assigned for 0.95 mM 13C,15N apoMlcB in the presence of 2.4 mM backbone amide proton and nitrogen chemical shifts were assigned and PyMOL (31). The Dali web server was used to perform structural homology searches (32).

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NMR-based MyoB IQ Motif Binding Experiments— Backbone amide proton and nitrogen chemical shifts were assigned for 0.95 mM 13C,15N apoMlcB in the presence of 2.4 mM concentrations of synthetic 24-residue MyoBIQ peptide using 1H,15N HSQC, HNCACB, and CBCACONH datasets recorded at 25 °C on a Varian INOVA 500 MHz spectrometer equipped with a triple-resonance cryoprobe. Sample buffer conditions were 10 mM HEPES, pH 6.8, 50 mM NaCl, 5 mM EDTA, 5 mM EGTA in 90% H2O, 10% D2O. Datasets were processed and analyzed using NMRPipe (25) and NMRView (26), respectively. Sequential backbone chemical shift assignments of calcium-saturated MlcB (Ca2+–MlcB) in the absence and presence of the MyoBIQ synthetic peptide were also completed in a manner and under conditions similar to those described above for apoMlcB with the exception of the presence of 5 mM CaCl2 instead of the divalent chelators.

Co-purification Experiments—Bacterial cells expressing wild-type and mutant GST-MyoBIQ and Mbc-MlcB constructs were harvested and reconstituted in 1× PBS buffer and applied to a column containing GST-Sepharose 4B resin. After elution, the two most concentrated fractions, as assessed using the Bradford assay (Bio-Rad), were collected and used in subsequent analyses. To quantify the amount of GST-MyoBIQ present in each fraction, samples were diluted 5–10-fold and assayed for GST activity using the Novagen GST-Tag Assay kit. The 20 lowest-energy structures were selected and involved a 12-min equilibration time between injections. For the wild-type MyoBIQ titration, a 9-min equilibration time was sufficient to restore base line between the injections.

For Ca2+ titration experiments of MlcB in the absence and presence of MyoBIQ, the above MlcB sample was also extensively dialyzed against 10 mM HEPES, pH 7.4, 50 mM NaCl in the presence of Chelex-100 resin (Bio-Rad). Titrations were performed similarly to that described above, with the exception that 2 ml of 50 μM MlcB in the absence or presence of 1 mM MyoBIQ synthetic peptide in the calorimetric cell was titrated with a 625 μM solution of CaCl2 in the syringe.

Modeling of the MlcB–MyoB IQ Complex—A model of the MlcB–MyoBIQ complex was generated using a previously published protocol in HADDOCK 2.0 (34) with the following alterations. Amino acids residues in apoMlcB that exhibited backbone-normalized 1H,15N chemical shift perturbations greater than the mean plus half a standard deviation were deemed active and were subsequently refined on the basis of having a solvent accessibility of >50%. Neighboring or passive residues were also assessed using this criterion and were included in the docking of the MyoBIQ peptide. Residues Lys-693–Ala-719 of the MyoBIQ peptide were modeled as an α-helix in MODELLER (35) using the MyoV IQ1 x-ray crystal structure (PDB accession code 2IX7; Ref. 36) as a template. Additional MlcB amino acid residues used as restraints for the docking of MyoBIQ on the apoMlcB structure included Ile-701, Phe-705, and Trp-708, which when substituted for Ala were found to reduce binding to apoMlcB in pulldown experiments. An ensemble of 2000 rigid body docking models was generated in the first iteration, of which the lowest 200 energy models were subjected to semi-flexible simulated annealing and refinement in explicit water. Subsequently, 20 low energy models with the lowest HADDOCK score and highest buried surface area were selected from the most populated cluster for further analysis.

RESULTS

Structure of ApoMlcB—The solution NMR structure of apoMlcB was determined using 1174 distance restraints and 103 dihedral restraints. Of the 200 generated structures, 196 were accepted with no NOE violations >0.3 Å and no dihedral violations >5°. The 20 lowest-energy structures were selected for further analysis, and an energy-minimized average structure was calculated from the ensemble (Fig. 1). Structural statistics of the ensemble are summarized in Table 1.

ApoMlcB formed a globular four-helix bundle with overall dimensions of 22 × 27 × 24 Å comprising two EF-hand motifs connected by a short linker (Asp-34–Thr-39). EF-hand 1 included helix 1 (α1: Ser-2–Phe-15), a canonical 12-residue EF-hand Ca2+ binding loop (Asp-16–Glu-27) previously shown to bind Ca2+ (22), and helix 2 (α2: Val-25–Arg-33), whereas EF-hand 2 consisted of helix 3 (α3: Glu-40–Ala-50), a non-functional Ca2+ binding loop (Asp-51–Ala-62), and helix 4 (α4: Tyr-60–Ser-69) (Fig. 1). Two short strands in the two Ca2+ binding loops (Phe-22–Ser-24; Phe-57–Asp-59) formed an antiparallel β-sheet, which is a common structural component in EF-hand Ca2+-binding proteins.
FIGURE 1. ApoMlcB structure. A, the backbone superposition (N, C\(^\alpha\), C\(^\gamma\) atoms) of the 20 lowest energy NMR-derived conformers of apoMlcB. B, backbone ribbon representation of the energy-minimized average structure of apoMlcB. The N and C termini are labeled accordingly, and the four \(\alpha\)-helices are indicated in sequential order. C, the amino acid sequence of MlcB displayed in one-letter code. Helices are depicted as cylinders, whereas functional and non-functional Ca\(^{2+}\) binding loops are represented by open semicircles.

### TABLE 1

| Structural statistics for apoMlcB |
|----------------------------------|
| **Restraints used for structure calculations** |  |
| Sequential | 480 |  |
| Medium range (1 \(\leq |i-j| \leq 5\)) | 508 |  |
| Long range (|i-j| > 5) | 186 |  |
| Dihedral restraints (\(\Phi, \Psi\)) | 103 |  |
| **Energies (kcal mol\(^{-1}\))** |  |
| \(E_{\text{overall}}\) | 160 \(\pm\) 15 |  |
| \(E_{\text{NOE}}\) | 80.5 \(\pm\) 1.2 |  |
| \(E_{\text{bond}}\) | 16.4 \(\pm\) 0.4 |  |
| \(E_{\text{dihedral}}\) | 70.2 \(\pm\) 1.6 |  |
| \(E_{\text{improper}}\) | 9.8 \(\pm\) 0.9 |  |
| \(E_{\text{other}}\) | 2.1 \(\pm\) 0.4 |  |
| **r.m.s.d. from experimental restraints** |  |
| Dihedral angles (\(^\circ\)) | 0.58 \(\pm\) 0.05 |  |
| Distances (Å) | 0.030 \(\pm\) 0.001 |  |
| **r.m.s.d. from idealized geometry** |  |
| Angle (\(^\circ\)) | 0.43 \(\pm\) 0.01 |  |
| Bond (Å) | 0.003 \(\pm\) 0.0002 |  |
| **Ramachandran statistics for residues 2–69 (%)** |  |
| Residues in most favored regions | 82.6 |  |
| Residues in additionally allowed regions | 14.4 |  |
| Residues in generously allowed regions | 2.8 |  |
| Residues in disallowed regions | 0.2 |  |
| **r.m.s.d. to mean structure (Å)** |  |
| Residues 2–69 | 0.77 \(\pm\) 0.16 |  |
| Backbone atoms | 1.41 \(\pm\) 0.14 |  |
| Residues 2–15, 25–69 | 0.51 \(\pm\) 0.10 |  |
| Backbone atoms | 1.01 \(\pm\) 0.10 |  |
| Residues 25–69 | 0.34 \(\pm\) 0.05 |  |
| Heavy atoms | 0.38 \(\pm\) 0.06 |  |

*Force constants of 150 kcal mol\(^{-1}\) \(\text{rad}^{-2}\) for NOE energy and 100 kcal mol\(^{-1}\) \(\text{rad}^{-2}\) for dihedral energy were used in the high temperature annealing step and 150 kcal mol\(^{-1}\) \(\text{rad}^{-2}\) for NOE energy and 20 kcal mol\(^{-1}\) \(\text{rad}^{-2}\) for dihedral energy in the slow cooling step. Final minimization involving the Powell method used 75 kcal mol\(^{-1}\) \(\text{rad}^{-2}\) for NOE energy and 400 kcal mol\(^{-1}\) \(\text{rad}^{-2}\) for dihedral energy. To maintain covalent geometry close to ideality quadratic harmonic potential terms with force constants of 1000 kcal mol\(^{-1}\) \(\text{rad}^{-2}\), 500 kcal mol\(^{-1}\) \(\text{rad}^{-2}\) for bond, angular, and improper terms, respectively were used. Regions of MlcB used for calculating Ramachandran statistics and r.m.s.d. values were defined based on distance and dihedral restraints and \(^{1}H\)-\(^{15}N\) NOE data.*

An overlay of 20 the lowest energy structures showed apoMlcB to be well defined by the NMR-derived distance and dihedral restraints, with the exception of the region encompassing Asp-16–Ser-24 of the first Ca\(^{2+}\) binding loop and Val-70–Ser-73 at the extreme C terminus (Fig. 1A). These observations were supported by \(^{1}H\)-\(^{15}N\) NOE analysis of apoMlcB (Fig. 2), which revealed significantly reduced \(^{1}H\)-\(^{15}N\) NOE values in these regions (average values of 0.65 and –0.078, respectively) relative to the 0.77 average value for the protein core (Ser-2–Ser-69). Residues in helix 1 also displayed reduced \(^{1}H\)-\(^{15}N\) NOE values (average value of 0.65) relative to the core average suggestive of it also being less well defined relative to the other helices (Fig. 2). Indeed, the root mean square deviation (r.m.s.d.) value for the backbone atoms encompassing Ser-2–Ser-69 for the ensemble relative to the minimized average structure was 0.77 \(\pm\) 0.16, whereas the corresponding r.m.s.d. values for the protein core excluding either the N terminus of the functional Ca\(^{2+}\) binding loop (Asp-16–Ser-24) or helix 1 and the N terminus of the functional Ca\(^{2+}\) binding loop (Ser-2–Glu-25) were 0.51 \(\pm\) 0.10 and 0.34 \(\pm\) 0.04, respectively. The lack of convergence in the canonical Ca\(^{2+}\) binding loop of the first MlcB EF-hand has previously been observed for the EF-hands of other Ca\(^{2+}\)-binding proteins, including CaM, troponin C, members of the S100 protein family, when in their apo state (37–39), whereas a loosely packed helix has been reported for human S100B when in its apo state (40, 41).

**Structural Similarities to Other Ca\(^{2+}\)-binding Proteins**—The uniqueness of MlcB as a single-lobe myosin LC led us to search for structural homologs using the Dali web server (32). Of the identified structural homologs, apoMlcB displayed the highest degree of structural similarity to the C-terminal lobe of the myosin tail interacting protein (MTIP) from *Plasmodium knowlesi* (42) and rat apoCaM (43) (Z scores of 4.5). A high degree of structural similarity was also found with Ca\(^{2+}\)-bound troponin C and to the N- and C-terminal lobes of CaM bound to Ca\(^{2+}\), Zn\(^{2+}\), and Mg\(^{2+}\) and IQ motifs (Table 2).

As a Ca\(^{2+}\) sensor and myosin LC, CaM adopts a closed conformation in the absence of Ca\(^{2+}\) and protein target, a semi-open conformation in the absence of Ca\(^{2+}\) but presence of protein target, and an open conformation in presence of Ca\(^{2+}\) and
protein target (44). To further characterize apoMlcB within the CaM superfamily and assess its conformation as a myosin LC, interhelical angles were measured and compared with the various conformational states of CaM (36, 43, 45), S100B (46, 47), and the single lobe EF-hand protein calbindin D9k (48, 49) (Table 3). When considering the full complement of apoMlcB interhelical angles, the highest degree of similarity was to the C-terminal lobe of apoCaM, which suggested that the apoMlcB structure is most reminiscent to the closed conformation of CaM. When the interhelical angles associated with the two isolated helix-loop-helix EF-hand motifs of apoMlcB were used (120° and 126°, respectively), the highest degree of similarity was to that of the N- and C-terminal lobes of apo- and Ca2+/H11001-calbindin D9k (N-terminal: 120° and 130°, respectively; C-terminal: 121° and 118°, respectively). Interestingly, both apo- and Ca2+/calbindin D9k adopt a closed conformation and display only subtle, local Ca2+/induced conformational changes (48, 49) in contrast to CaM, which undergoes a global conformational change that exposes a hydrophobic target binding surface (44). Despite these observed conformational similarities, structural overlays showed that helix 1 of apoMlcB adopted a unique orientation relative to the other three helices when compared with the C-terminal lobe of apoCaM (45) or calbindin D9k (49) (Fig. 3; Table 3), which to the best of our knowledge represents a novel fold within the EF-hand Ca2+/binding protein superfamily.

MlcB Binds MyoBIQ at a Surface Involving Its Functional Ca2+/Binding Loop and Helices I and IV—To assess the impact of MyoBIQ binding on the structure of MlcB and to identify the MyoBIQ-binding site, two-dimensional heteronuclear NMR-based chemical shift perturbation studies were performed. An overlay of the 1H,15N HSQC spectra of apoMlcB in the absence (Fig. 4A, black) and presence of the MyoBIQ peptide (Fig. 4A, red) revealed an overall increase in the dispersion of apoMlcB 1H,15N resonances in the presence of MyoBIQ. Notably, the addition of the peptide caused a subset of resonances to display large changes in chemical shift.

Quantitation of the MyoBIQ-induced backbone HN and 15N chemical shift changes in apoMlcB, using our backbone HN and 15N resonance assignments in the absence and presence of MyoBIQ, allowed us to identify those residues whose chemical environment was altered by the interaction with MyoBIQ (Fig. 4B). Tyr-13, Asn-14, and Phe-15 of helix 1, Asp-18, Asp-20, Gly-21, and Val-23 in the first Ca2+/binding loop, Val-25 and Arg-33 of helix 2, Asp-34 and Leu-36 of the linker region, and Tyr-60, Lys-61, and Phe-63 of helix 4 exhibited chemical shift changes >0.5 S.D. above the mean chemical shift change (0.87 ppm) (Fig. 4, B and C, red). Additional apoMlcB residues displaying notable chemical shift changes (i.e. greater than the mean but less than 0.5 S.D. above the mean) included Glu-10 and Phe-12 of helix 1, Asp-16, Gly-17, Tyr-19, and Ser-24 of the first Ca2+/binding loop, Gly-30 and Ile-31 of helix 2, Gly-35 of the linker region, Phe-46 of helix 3, Ile-58, Asp-59, and Ala-62 of the non-functional Ca2+/binding loop, and Tyr-68 of helix 4 (Fig. 4, B and C, blue). When these residues were mapped onto the surface of the energy-minimized average apoMlcB structure (Fig. 5A), the MyoBIQ-binding site was found to be com-
prised of a planar hydrophobic surface with electronegative character formed by the first canonical Ca\(^{2+}\) binding loop, the N termini of helices 2 and 4, and the C terminus of helix 1 (Fig. 5).

Both the apo and holo states of MlcB were previously shown to bind MyoBIQ with very similar affinities (22). However, the influence of MyoBIQ on Ca\(^{2+}\) binding to MlcB was not assessed. Isothermal titration calorimetric experiments indicated that the affinity for Ca\(^{2+}\) displayed by MlcB was not affected by the presence of MyoBIQ (Fig. 6; 0.34 ± 0.09 μM in the absence of MyoBIQ, 0.37 ± 0.07 μM in the presence of MyoBIQ). Together, these observations suggest that Ca\(^{2+}\) binding does not substantially impact the MyoBIQ-binding site on MlcB. We subsequently used NMR-based chemical perturbation analysis to identify residues of MlcB whose 1H,15N resonances were altered by the presence of MyoBIQ (Fig. 7). Binding of Ca\(^{2+}\) to MlcB caused significant perturbations for residues in helix 1 (Phe-12), the first Ca\(^{2+}\) binding loop (Phe-22, Val-25), helix 2 (Arg-29, Ile-32), and helix 4 (Lys-61, Ala-64). Additional residues in these regions as well as the non-functional Ca\(^{2+}\) binding loop and helix 3 displayed chemical shift changes between the mean and 1 S.D. above the mean (Fig. 7, A and B). Of note, many of these residues (Phe-12, Phe-22, Val-23, Val-25, Asp-59, Lys-61, Phe-63, Val-23, Ala-50, Ala-64, Lys-61, Leu-67, Tyr-68) participated in the MyoBIQ-binding site (Figs. 4 and 5), whereas others (Phe-28, Ile-32, Phe-43, Phe-63) contact the former directly or indirectly through the hydrophobic core. In an attempt to assess whether the MyoBIQ-binding site on MlcB was affected by Ca\(^{2+}\), spectra of apoMlcB and holo-MlcB in the presence MyoBIQ were compared.

Overall, there is a notable degree of similarity in the position of numerous residues (Fig. 7C). Quantitation of the MyoBIQ-induced backbone H\(^{\alpha}\) and 15N chemical shift changes in holo-MlcB revealed significant chemical shift changes for Gly-21 and Val-25 in the first Ca\(^{2+}\) binding loop, Leu-36 of the linker region, and Lys-61 of helix 4 (Fig. 7D, red). A subset of residues throughout holo-MlcB also displayed MyoBIQ-induced chemical shift changes above the mean (Fig. 7D, blue). Indeed, the overall trend of MyoBIQ-induced chemical shift perturbations observed for apoMlcB (Fig. 4C) and holo-MlcB (Fig. 7D) was very similar, and when combined with similarities of the spectra shown in Fig. 7C, the mode of MyoBIQ recognition by MlcB did not appear to be significantly altered in the holo state.

**Identification of Residues in MyoBIQ Involved in Binding MlcB—MyoBIQ and MlcB were co-expressed in bacteria as GST and Myc fusion proteins, respectively. Purification of GST-MyoBIQ on glutathione-Sepharose resin resulted in the co-purification of Myc-MlcB (Fig. 8). We used this co-purification assay to define the key amino acid residues in MyoBIQ responsible for binding to MlcB. The combination of sequential and structural alignments of MyoBIQ with the IQ1 and IQ2 motifs of myosin V (MyoV) from the x-ray crystal structure of the apoCaM-MyoV complex (36) helped guide our decision as to which amino acid residues in MyoBIQ to mutate (Fig. 8A).

Specifically, Ile-701, Phe-705, and Trp-708 of MyoBIQ were shown to be important MlcB-interacting amino acid residues based on the observation that the I701A, F705A, and T708A MyoBIQ mutants failed to co-purify with MlcB (Fig. 8, B and C).
Substitution of Cys-697 and Thr-698 for alanine had no effect on MlcB binding (Fig. 8), which was expected given that the corresponding wild-type sequences in MyoV-IQ1 and MyoV-IQ2 contain alanine at these positions (Ala-769 and Ala-793, respectively). Isothermal titration calorimetric analysis indicated that the binding of apoMlcB to the I701A (Kd = 27 ± 4.2 μM), F705A (Kd = 43 ± 4.1 μM), and T708A (Kd = 4.9 ± 0.14 μM) MyoBIQ mutants was reduced by ~39-, 61-, and 7-fold, respectively, when compared with the affinity of the native apoMlcB-MyoBIQ interaction (Kd = 0.7 ± 0.02 μM) (Fig. 9), whereas the C697A MyoBIQ mutant displayed a similar affinity (Kd = 0.5 ± 0.06 μM; data not shown).

Toward providing additional molecular level insight into MyoBIQ recognition by apoMlcB, the NMR-based chemical shift perturbations and mutagenesis data were used as experimental inputs to dock a helical model of the MyoBIQ peptide, derived from the MyoV-IQ1 x-ray crystal structure (36), onto the apoMlcB structure. In the generated models, the helical MyoBIQ peptide was consistently positioned on the surface of apoMlcB in a single orientation, a representative model of which is shown in Fig. 10, A and B. The side chains of Ile-701, Phe-705, and Trp-708 from MyoBIQ participate in hydrophobic interactions with the aliphatic side chain of Lys-61 and the aromatic side chains of Phe-12, Tyr-13, Phe-15, and Tyr-60 in MlcB (Fig 10, C and D).

**DISCUSSION**

Substantial progress has been made toward characterizing the structure, function, and regulation of the *D. discoideum* class I long-tailed myosins, including the identification of novel myosin LCs associated with MyoB, MyoC, and MyoD, specifically MlcB, MlcC, and MlcD, respectively (22–24). All three of
**FIGURE 6.** *MyoBIQ does not influence MlcB affinity for calcium.* Raw heat measurements (**upper panel**) and integrated heats after correction for heats of dilution (**lower panel**) at 30 °C are displayed for the interaction of calcium with apoMlcB (A) and apoMlcB (B) in the presence of MyoBIQ. The calculated dissociation constant ($K_d$) and associated S.D. from duplicate experiments are reported accordingly.

**FIGURE 7.** *MyoBIQ binding affect similar regions of apo- and holo-MlcB.* A, plot of normalized chemical shift changes ($\Delta\delta$) along the MlcB sequence induced by the binding of calcium. The red bars represent residues displaying chemical shift changes, 0.5 S.D. greater than the mean chemical shift difference (0.14 ppm), which is shown as a horizontal dashed line. Blue bars indicate residues exhibiting chemical shift changes between 0.5 S.D. and the mean. B, backbone ribbon representation of apoMlcB on which residues whose HN resonances were affected Ca$^{2+}$ are mapped using the same color code described in A. C, overlay of $^1H,^15N$ HSQC spectra of $^{13}C,^{15}N$ apoMlcB (black) and holo-MlcB (red) and presence of MyoBIQ. D, backbone ribbon representation of apoMlcB on which residues whose H$^n$ resonances were affected by MyoBIQ in the presence of Ca$^{2+}$, with coloring the same as described in B. The normalized chemical shift changes were calculated using the formula described in Fig. 4.
these myosin LCs display features and properties consistent with being members of the CaM superfamily of EF-hand Ca$^{2+}$-binding proteins and display the ability to specifically recognize their cognate IQ motifs (21). However, MlcB and MlcC are only half the size (8.3 and 8.6 kDa, respectively) of conventional LCs and consist of a single globular lobe comprising two EF-hands. MlcC does not bind Ca$^{2+}$, whereas MlcB has a single functional EF-hand Ca$^{2+}$ binding motif (22, 23). Isothermal titration calorimetric experiments showed that MlcB binds to MyoBIQ with the same submicromolar affinity in the absence and presence of Ca$^{2+}$ (22). The fact that MlcB and MlcC do not require Ca$^{2+}$ for IQ motif recognition suggests that they may exhibit unique modes of binding to their respective myosin IQ motifs, a concept supported by the apoMlcB structure and MyoBIQ binding studies described here.

The ability of apoMlcB to bind MyoBIQ with submicromolar affinity (22) and its structural similarity to the C-terminal lobe of CaM suggest that its mode of IQ recognition involves a ligand-induced conformational change from a closed to semi-open conformation (44). This type of conformational change would be expected to result in significant chemical shifts for residues spanning apoMlcB in the same submicromolar affinity in the absence and presence of Ca$^{2+}$ (22). The fact that MlcB and MlcC do not require Ca$^{2+}$ for IQ motif recognition suggests that they may exhibit unique modes of binding to their respective myosin IQ motifs, a concept supported by the apoMlcB structure and MyoBIQ binding studies described here.

MlcB contains 12 aromatic residues, several of which are located within (Tyr-19, Phe-22, Tyr-60) and directly adjacent to (Phe-12, Tyr-13, Phe-15, Phe-63, Tyr-68) the two EF-hand motifs positioned at one end of the apoMlcB structure, where they interact and form a partially exposed hydrophobic surface (Fig. 5B). The MyoBIQ-binding site predicted by the chemical shift data relies on this exposed hydrophobic surface (Phe-12, Tyr-13, Phe-15, Val-23, Tyr-60, Phe-63, Tyr-68), which is surrounded by several acidic residues (Asp-16, Asp-18, Asp-20, Asp-59; Fig. 5B). HADDOCK-based modeling of the apoMlcB-MyoBIQ complex shows that the predicted binding surface is able to accommodate a helical MyoBIQ peptide (Fig. 10). The finding that apoMlcB has an exposed hydrophobic surface is also consistent with previous work showing that 8-anilino-1-naphthalenesulfonic acid (ANS), a hydrophobic probe, alters its fluorescence emission properties in the presence of apoMlcB (22). The addition of Ca$^{2+}$ induces a large change in the near-UV CD spectrum of MlcB and further enhances the fluorescence emission of 8-anilino-1-naphthalenesulfonic acid, consistent with the view that hydrophobic residues, including tyrosine side chains, became more surface-exposed and dynamic (22).

The observation that Ca$^{2+}$ and MyoBIQ independently affects the same residues in apoMlcB (Figs. 4 and 7) relates to its ability to bind MyoBIQ in the absence and presence of Ca$^{2+}$ with the same affinity (22). Although the N-terminal canonical EF-hand Ca$^{2+}$ binding loop is not well defined in the apoMlcB structural ensemble (Fig. 1A), it is probable that Ca$^{2+}$ coordi-
nation would alter the conformation of this region and lead to a reorientation of at least some of these aromatic side chains. Indeed, we have observed significant Ca\textsuperscript{2+}-induced chemical shift changes for all of these residues (Figs. 4 and 7). However, the observed chemical shift changes are, for the most part, localized to residues within or near the MyoBIQ-binding site or to residues that directly or indirectly bind to the region via the protein core, indicating that the ability for MlcB to bind MyoBIQ does not appear significantly influenced by a Ca\textsuperscript{2+}-induced conformational change typically observed for Ca\textsuperscript{2+} sensors such as CaM (44, 50, 51). This is in agreement with our chemical shift perturbation studies (Fig. 7), where Ca\textsuperscript{2+} does not appear to significantly alter the MyoBIQ-binding site. Therefore, the computational model of the apoMlcB-MyoBIQ complex (Fig. 10) presents a reasonable representation of the Ca\textsuperscript{2+}-MlcB-MyoBIQ complex.

We conclude that the MyoBIQ-binding site on MlcB is surface-accessible and at least partially formed to accommodate the ligand, thereby obviating the need for Ca\textsuperscript{2+} or MyoBIQ to induce a substantial structural rearrangement.
EF-hand Ca\(^{2+}\) binding loop appears to provide a degree of conformational flexibility, at least in the apo state, to accommodate MyoBIQ. However, the observation that in the presence of Ca\(^{2+}\) MyoBIQ binds to the same region with the same affinity suggests that this flexibility is not critical. The location of the MyoBIQ-binding site on the MlcB surface and the orientation of MyoBIQ peptide in our modeled complex is similar to an NMR-derived structural model of a complex between the IQ motif and the C-terminal EF-hand domain of the human cardiac sodium channel Nav1.5 (52), which also maps to the \(\alpha_1\) and \(\alpha_4\) helical plane. Thus, although the mode of IQ recognition displayed by apoMlcB is not unprecedented, it is unique among those currently reported for myosin LCs.

The LCs stabilize the \(\alpha\)-helical myosin neck region, allowing it to function as a rigid lever arm to amplify small changes within the myosin motor domain (53). The crystal structure of apoCaM bound to the first two IQ motifs of murine MyoV shows that interactions with both the N- and C-terminal lobe of CaM stabilizes a helical neck region approximately 20 residues in length (36). In contrast, the structural data presented here indicate that MlcB stabilizes at most an 11-residue stretch of the MyoB IQ motif (from Ile-701 to Lys-711). That the myosin step size and consequently the sliding velocity is linearly related to the length of the lever arm, suggests that MyoB is likely to be a slow-moving motor (54). In many cases the LCs also play an important role in regulating myosin function. Calcium promotes the dissociation of CaM from the IQ motifs of some myosin I family members, resulting in a severe loss in motility, whereas for mammalian Myo1d and Myo1e, a calcium-induced rearrangement of CaM on the IQ motif causes a 2–3-fold decrease in activity (55). The results presented here show that calcium does not alter the affinity of MlcB for the MyoB IQ motif nor does it significantly change the binding interface between MlcB and the IQ motif. Thus, it seems unlikely that the motile properties of MyoB will be significantly affected by calcium binding to MlcB. Further studies will be required to understand the biological significance of the functional calcium-binding site in MlcB.

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