Structure of the Single-lobe Myosin Light Chain C in Complex with the Light Chain-binding Domains of Myosin-1C Provides Insights into Divergent IQ Motif Recognition*

Received for publication, June 30, 2016, and in revised form, July 19, 2016 Published, JBC Papers in Press, July 27, 2016, DOI 10.1074/jbc.M116.746313

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Myosin light chains are key regulators of class 1 myosins and typically comprise two domains, with calmodulin being the archetypal example. They bind IQ motifs within the myosin neck region and amplify conformational changes in the motor domain. A single lobe light chain, myosin light chain C (MlcC), was recently identified and shown to specifically bind to two sequentially divergent IQ motifs of the Dictyostelium myosin-1C. To provide a molecular basis of this interaction, the structures of apo-MlcC and a 2:1 MlcC-myosin-1C neck complex were determined. The two non-functional EF-hand motifs of MlcC pack together to form a globular four-helix bundle that opens up to expose a central hydrophobic groove, which interacts with the N-terminal portion of the divergent IQ1 and IQ2 motifs. The N- and C-terminal regions of MlcC make critical contacts that contribute to its specific interactions with the myosin-1C divergent IQ motifs, which are contacts that deviate from the traditional mode of calmodulin-IQ recognition.

The class 1 myosins (myosin-1) are a widely expressed family of single-headed, non-filament-forming, membrane-binding myosins (1–3). The myosin-1 heavy chain consists of a highly conserved N-terminal motor domain with sites for binding actin and for ATP hydrolysis, a light chain-binding domain (LCBD) (4) that acts as the motor’s lever arm, and a C-terminal tail. The myosin-1 tail contains a tail homology 1 (TH1) domain that interacts with acidic phospholipids and, in some cases, also contains a glycine- and proline-rich TH2 domain that binds filamentous actin and an Src homology 3 domain that forms complexes with proteins that stimulate actin filament assembly (4–10). Myosin-1 is able to cross-link the plasma membrane to the underlying actin cytoskeleton and plays a direct role in the control of cortical and membrane tension (11, 12). These motor proteins can also drive vesicle trafficking, pseudopod retraction, and the uptake of particles and fluids by phagocytosis and micropinocytosis (13–16).

The myosin-1 LCBD includes one or more IQ motifs, which are α-helical sequences between 18 and 25 residues in length that terminate with a loosely conserved IQXXRGXXXR consensus sequence (17, 18). IQ motifs bind calmodulin (CaM) or CaM-related light chains (LCs) in the absence of Ca²⁺. The LCs stabilize the α-helical structure of the LCBD, allowing it to function as a rigid swinging lever arm to amplify ATP-dependent conformational changes that originate within the motor domain (19). The LCs are also important regulatory sites that interact with the motor domain to influence mechanochemical properties such as step size, motility rate, and force sensing (1, 20, 21).

The social amoeba Dictyostelium discoideum has been used extensively to study myosin-1 structure, function, and regulation. The seven Dictyostelium myosin-1 isoforms include three with short tails that contain only a TH1 domain (myosin-1A, -1E, and -1F), three with long tails that have TH1, TH2, and Src homology 3 domains (myosin-1B, -1C, and -1D), and one with no tail (myosin-1K) (22). A variety of different LCs are associated with the Dictyostelium myosin-1 isoforms. Myosin-1A and myosin-1E use CaM as a LC, whereas myosin-1D binds MlcD, a 17-kDa CaM-related protein that lacks the ability to bind Ca²⁺ (23, 24). The LCBDs of myosin-1B and myosin-1C contain one and three IQ motifs, respectively, with the IQ motif of the former displaying specific binding to the small (i.e. ~8.5 kDa) LC MlcB (25). The first two IQ motifs of myosin-1C are divergent from the consensus IQ sequences in that they are 18 amino acid residues in length, and the conserved glutamine is substituted for a lysine and is specifically recognized by the LC MlcC (23). The identity of the LC that binds IQ3 of myosin-1C is not currently known.

MlcB and MlcC consist of two helix-loop-helix EF-hand motifs connected by a short linker sequence, and particularly relevant to the biological function are monomers in solution (23, 25, 26). The first EF-hand of MlcB is functional and binds Ca²⁺ with a $K_i$ of 0.2 μM, whereas both EF-hands of MlcC have lost the ability to bind Ca²⁺. NMR studies of MlcB show that it adopts a conformation in solution that differs from that of other...
Ca\textsuperscript{2+}-binding proteins and that it binds to the myosin-1B IQ motif using a surface distinct from that employed by either the N- or C-terminal lobe of apo-CaM (26).

In this report, we describe the NMR-derived solution structure of free MlcC and the x-ray crystal structure of a complex between MlcC and the first two IQ motifs of myosin-1C. The results show that MlcC undergoes a transition from a closed to a semi-open state upon binding the IQ motif and that its structure in both states is very similar to that of the C-terminal lobe of apo-CaM. The binding of MlcC to IQ1 and IQ2 induces a distinct bend into the myosin-1C LCBD. The two MlcC molecules in the complex are rotated by ~20° relative to the helix axis, which leaves the opposite, concave, side of the LCBD largely exposed. Although MlcC and the C-terminal lobe of apo-CaM are structurally very similar, the myosin-1C IQ1 and IQ2 motifs exhibit a very high degree of specificity for MlcC. The specificity is likely due to hydrophobic interactions between the last residue in the IQ motif and residues at the extreme N and C termini of MlcC.

Results

Structure of MlcC—Toward assessing the structural properties of MlcC in solution, the NMR-based assignment of 98 and 96% of the backbone and side chain \(^1\)H, \(^13\)C, and \(^15\)N chemical shifts was completed. The water-refined NMR ensemble of the 20 lowest energy MlcC structures was determined using a total of 1279 NOE-based distance and 120 TALOS-derived dihedral angle restraints (Fig. 1, A and B). With the exception of Met-1–Thr-7 and Trp-73–Asp-74, the MlcC structure was well defined by the NMR data and was consistent with \(^1\)H-\(^15\)N NOE data (Fig. 1C). The root mean square deviation (r.m.s.d.) of the structured core of MlcC from the minimized average structure was 0.69 ± 0.13 and 1.16 ± 0.12 Å for backbone and heavy atoms, respectively. Structural statistics and related validation scores, including recall precision factors and discriminating power coefficients, are summarized in Table 1.

The structure shows that MlcC comprises two EF-hand helix-loop-helix motifs connected by a five-residue linker region (Fig. 1, A and B). The four helices (labeled A to D) run roughly parallel or antiparallel to one another to produce a compact four-helix bundle with dimensions of 19 \times 18 \times 32 Å. The two non-functional Ca\textsuperscript{2+}-binding loops interact via two short \(\beta\)-strands (Val-25–Thr-26; Tyr-60–Val-61) that form an antiparallel \(\beta\)-sheet. MlcC contains five basic and 13 acid residues throughout the MlcC structure, including the flexible N- and C-terminal segments, the central hydrophobic core, and the inter-EF-hand linker (Fig. 1E).

Structure database searches using the DALI server indicated that the MlcC structure most closely resembles that of the C-terminal lobe of apo-CaM, with a Z-score of 8.9, a backbone r.m.s.d. of 1.2 Å, and similar interhelical angles (Fig. 1F; Table 2) (27, 28). The structural similarity is consistent with the 43% primary sequence identity shared by MlcC and the C-lobe of CaM.

Binding of MlcC to the Myosin-1C IQ1 and IQ2 Motifs—To initially probe the interaction of MlcC with the individual myosin-1C IQ motifs, a GB1 fusion protein containing the myosin-1C IQ2 motif (GB1-M1C-IQ2) was expressed in Esche-
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TABLE 1
NMR structural statistics for MlcC

Regions of MlcC used for calculating Ramachandran statistics and r.m.s.d. values were defined based on distance and dihedral restraints and [1H]-31N NOE data.

| Restraints used for structure calculations | Total NOE | Short range (|i-j| < 10) | Medium range (|i-j| < 5) | Long range (|i-j| > 5) | Dihedral restraints (ψ, ϕ) |
|-------------------------------------------|----------|------------------------|------------------------|------------------------|----------------------------|
| Total NOE                                  | 1,279    | 661                    | 337                    | 281                    | 120                        |
| Van der Waals                              | -323.4 ± 77.8 | 534.7 ± 9.9          | 533.2 ± 11.4          |                         |                            |
| r.m.s.d. from experimental restraints      | 0.016 ± 0.003 | 0.0036                | 0.0114                |                         |                            |
| r.m.s.d. from idealized geometry           | 0.0140 ± 0.0003 | 0.087 ± 0.0018       |                         |                         |                            |
| Global quality scores (raw/Z-score)        | 0.39/1.12 | -0.01/0.28             | -0.05/-0.30           |                         |                            |
| Veriﬁ3D                                    |          |                        |                        |                         |                            |
| PROCHECK(ϕ, ψ)                             |          |                        |                        |                         |                            |
| PROCHECK(all)                              |          |                        |                        |                         |                            |
| MollPridey clash score                     | 12.27/-0.58 |                      |                        |                         |                            |
| Recall precision factors validation       | Recall   | Precision              | F-score                | Discriminating power   | 0.91                        |
|                                          | 0.91     | 0.87                   | 0.89                   | 0.76                   |                            |
| Ramachandran statistics (%)               | Residues in most favored regions | 90.8                  |                         |                         |                            |
|                                          | Residues in additionally allowed regions | 9.2                   |                         |                         |                            |
| r.m.s.d. to mean structure (Å)             | Backbone atoms (residues 7-70) | 0.69 ± 0.13           |                         |                         |                            |
|                                          | Heavy atoms (residues 7-70)   | 1.16 ± 0.12           |                         |                         |                            |

Consistent with a ligand-induced conformational change as opposed to a ligand binding to a localized region on the MlcC structure.

A recombinant GB1 fusion protein containing the tandem IQ1 and IQ2 motifs of myosin-1C (GB1-M1C-IQ1.2) was also produced. Circular dichroism spectroscopy showed that M1C-IQ1.2 had an α-helical content of ~50%, whereas the individual M1C-IQ1 or M1C-IQ2 motif peptides had less than 10% helical content (Fig. 3). When the GB1-M1C-IQ1.2 peptide was incubated with MlcC and assessed by size exclusion chromatography, the GB1-M1C-IQ1.2-MlcC complex eluted with an apparent molecular mass of ~30 kDa, and SDS-PAGE confirmed that the complex contained both MlcC and GB1-M1C-IQ1.2 (Fig. 3B). The apparent size of the complex was consistent with a trimer in which two molecules of MlcC (~8 kDa) bind to the GB1-M1C-IQ1.2 fusion protein (~14 kDa).

The GB1-M1C-IQ1.2 fusion protein was digested with TEV protease to liberate the M1C-IQ1.2 fragment, which was then used in ITC experiments with MlcC. Titration of MlcC into a solution containing M1C-IQ1.2 resulted in a distinct biphasic binding curve (Fig. 4B). M1C-IQ1.2 bound one molecule of MlcC with a dissociation constant (Kd) of 0.25 ± 0.03 mM and a second molecule with an ~200-fold lower affinity (Kd = 55 ± 12 mM). No binding interaction was detected when apo-CaM was titrated into M1C-IQ1.2, demonstrating that the myosin-1C IQ motifs exhibit a high degree of specificity for MlcC (Fig. 4B).

Structure of the 2:1 MlcC-M1C-IQ1.2 Complex—The complex of MlcC and M1C-IQ1.2 readily crystallized, and its structure was determined to a resolution of 1.9 Å by molecular replacement using the myosin V IQ motif–apo-CaM complex as a search model (PDB accession 2IX7; Table 3). The x-ray crystal structure shows two molecules of MlcC, one per M1C-IQ motif, bound to M1C-IQ1.2 (Fig. 5A). M1C-IQ1.2 folds into a single 11-turn α-helix that is uniformly bent with a radius of curvature of ~80 Å. The two molecules of MlcC are attached to the convex surface of the α-helix and are rotated by ~20° relative to one another. There is a small region of contact (buried surface area of 300 Å²) between the two MlcC molecules involving Met-69 and Trp-73 at the C terminus of the M1C-IQ1-bound MlcC and Val-16 and Arg-19 of the M1C-IQ2-bound MlcC. The interaction with MlcC leaves a large portion of the bottom concave surface of the M1C-IQ1.2 helix exposed, including the side chains of Arg-699, Asp-703, Ser-706, Asn-710, Ala-717, Glu-721, Asn-724, Asn-728, Asn-732, and Leu-735 (Fig. 5).

With the exception of the extreme N- and C-terminal regions, the MlcC molecules bound to the IQ1 and IQ2 motifs have nearly identical structures (r.m.s.d. of 0.46 Å for 65 Ca atoms). Comparison with the NMR-derived structure of MlcC shows that MlcC undergoes a significant conformational change upon binding to the IQ motif (r.m.s.d. of 3.04 Å for 66 Ca atoms). The bottom portions of helices A, C, and D are displaced outward between 4.5 and 6 Å, which opens up a central V-shaped hydrophobic groove that allows MlcC to grasp the helical IQ motif (Fig. 6; Table 2). The structure of the M1C-IQ motif-bound MlcC closely resembles the semi-open conformation adopted by the C-lobe of apo-CaM upon binding to an IQ motif (r.m.s.d. of 0.93 Å for 65 Ca atoms) (Fig. 6B; Table 2) (29).

N-terminal Region of MlcC Binds to the IQ1 but not the IQ2 Motif of Myosin-1C—The M1C-IQ1 and M1C-IQ2 motifs are each 18 residues in length (Fig. 7A), and their register is such that equivalent residues in each motif are positioned on the same face of the helical myosin neck region. Despite this similarity, they do not interact in an equivalent manner with MlcC because of the ~20° rotation of the second MlcC molecule relative to the helix axis (Fig. 5). The MlcC-binding site within M1C-IQ1 extends from Trp-701 to Phe-718, resulting in a buried surface area of 2,302 Å² and a solvent-free energy gain (ΔG') of ~14.2 kcal/mol (p value = 0.218) as calculated by the PISA server (30). The interface with M1C-IQ2 extends from Gln-719 to Arg-737, yielding a buried surface area of 2,190 Å² and a ΔG' of ~10.2 kcal/mol (p value = 0.324). These values are suggestive of highly specific interactions, and the difference in ΔG'}
values for M1C-IQ1 and M1C-IQ2 reflects the more hydrophobic nature of the M1C-IQ1 motif. Several of the hydrophobic residues in M1C-IQ1 (Trp-701, Met-704, Ala-705, and Phe-718) that interact with MlcC are substituted by more polar residues in M1C-IQ2 (Gln-719, Cys-722, Ser-723, and Tyr-736, respectively) (Fig. 7B).

The N terminus of MlcC, which is disordered in the NMR structure, is tightly anchored to the M1C-IQ1 motif but not to the M1C-IQ2 motif (Fig. 7C). Examination of the M1C-IQ1 motif shows that the side chain of Met-1 in MlcC binds into a hydrophobic pocket formed by the side chains of Phe-718 in the M1C-IQ1 motif and Trp-73 at the C terminus of MlcC. The side chain of Val-16 in the adjacent M1C-IQ2-bound MlcC stabilizes the formation of the hydrophobic cluster through interactions with both the Phe-718 and Trp-73 side chains. In the M1C-IQ2 motif, the formation of the hydrophobic cluster is disrupted. Phe-718 in the M1C-IQ1 motif is replaced in the M1C-IQ2 motif by the more polar Tyr-736 residue (Fig. 7A). Instead of interacting with Trp-73, the side chain of Tyr-736 is solvent-exposed where it can hydrogen bond to a water molecule. It is also likely that the absence of an adjacent MlcC molecule that could help orient the Tyr-736 and Trp-73 side chains contributes to the disruption of the hydrophobic Met-1 binding pocket.

**Interactions between MlcC and Lysine Residues in the Myosin-1C IQ Motifs**—The glutamine residue that is characteristic of canonical IQ motifs is replaced in both of the myosin-1C IQ motifs by a lysine residue (Lys-709 and Lys-727) (Fig. 7A). In the complex with MlcC, the side chains of Lys-709 and Lys-727 are completely buried by the loop formed by the inter-EF-hand linker sequence (Fig. 8A). The lysine side chain makes three hydrogen bond interactions with the main chain carbonyl groups of Leu-34, Leu-37, and Asp-39 in MlcC (Fig. 8A). The conserved Arg-713 and Arg-731 residues of the myosin-1C IQ motifs help to anchor the inter-EF-hand linker to the IQ motif through a hydrogen bond interaction with the main chain carbonyl of Met-40 of MlcC. The side chain of Arg-713, but not Arg-731, is involved in a salt bridge interaction with Asp-39 in the inter-EF-hand linker of MlcC.

The C-terminal region of the M1C-IQ1 and M1C-IQ2 motifs contains a second lysine residue (Lys-716 and Lys-734, respectively). The side chain of Lys-716 forms a salt bridge with Glu-52 in the acidic helix C of MlcC, whereas Lys-734 in M1C-IQ2 forms salt bridges with Glu-45 and Glu-48 closer to the beginning of helix C (Fig. 8B). This difference is likely caused by the replacement of Gln-719 in M1C-IQ1 with Arg-737 in M1C-IQ2. The Arg-737 side chain displaces the Lys-734 side chain toward Glu-45/48 of MlcC by charge repulsion interaction and provides an alternative electrostatic binding partner for Glu-52.

**Discussion**

Most myosin LCs are composed of two globular lobes, each of which is made up of a pair of helix-loop-helix EF-hand motifs. Dictyostelium MlcC is an atypical small light chain composed of two EF-hand motifs, neither of which is able to bind Ca²⁺ (23). MlcC functions as the light chain for myosin-1C, a long-tailed class-1 myosin. Here, we report the NMR-derived...
solution structure of MlcC as well as the x-ray crystal structure of a complex containing MlcC bound to both the IQ1 and IQ2 motifs of myosin-1C. In solution, the two EF-hand motifs of MlcC pack together to form a globular four-helix bundle flanked by short disordered N- and C-terminal segments. To bind to the helical myosin-1C IQ motifs, the four-helix bundle opens up to expose a central hydrophobic groove that grasps the N-terminal portion of the IQ motif. Hydrophobic residues in the flexible N- and C-terminal segments anchor MlcC to the C-terminal portion of each of the IQ motifs and to the adjacent MlcC molecule and are predicted to be responsible for high affinity specific binding to the myosin-1C IQ motifs.

M1C-IQ1.2, a fragment containing both the myosin-1C IQ1 and IQ2 motifs, bound one molecule of MlcC with a $K_d$ of $0.25 \pm 0.03$ nM and a second MlcC molecule with a $K_d$ of $55 \pm 12$ nM. When compared with the affinity of MlcC for peptides that correspond to the individual M1C-IQ1 ($K_d = 71 \pm 6$ nM) and M1C-IQ2 motifs ($K_d = 460 \pm 4$ nM) previously reported...
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(23), it is apparent that the tandem arrangement of the myosin-1C IQ motifs greatly enhanced the affinity for MlcC. This may be due in part to the helical conformation of M1C-IQ1.2 in solution (Fig. 3). The presence of a pre-formed α-helical binding site in the M1C-IQ1.2 fragment would significantly lower the energetic cost for the binding of the MlcC.

An increase in α-helical content does not explain why the two IQ motifs in the M1C-IQ1.2 fragment bind MlcC with a 200-fold difference in binding affinity, whereas the individual IQ1 and IQ2 motif peptides bind MlcC with a 6-fold difference in affinity. The results show that the tandem arrangement of the IQ motifs in M1C-IQ1.2 produces one very high affinity (0.25 nM) binding site for MlcC. We propose that the high affinity binding site is the M1C-IQ1 motif and arises from interactions between the flexible N- and C-terminal segments of MlcC and the C-terminal portion of the M1C-IQ1 motif. The key residues involved in this interaction are Met-1 and Trp-73 of MlcC and Phe-718 of the M1C-IQ1 motif, which are brought together to form a hydrophobic cluster than anchors MlcC to the M1C-IQ1 motif. The Val-16 residue of the adjacent M1C-IQ2-bound MlcC contacts both Phe-718 and Trp-73 and so may play a critical role in stabilizing the hydrophobic cluster (Fig. 7C). Additional contacts, including a π-π stacking interaction between His-3 and Phe-71 of MlcC and a hydrophobic interaction between His-3 and Ala-711 of M1C-IQ1, further strengthen the connections between the three structural elements. Together with the central hydrophobic groove, the flexible N- and C-terminal segments allow the small single-lobed MlcC to contact residues along the entire length of the M1C-IQ1 motif, from Trp-701 to Phe-718. Interestingly, Trp-701 interacts with Val-16 in MlcC and so mimics the interactions between Trp-73 and Val-16 at the interface between the two MlcC molecules.

The N-terminal segment of MlcC does not bind to the M1C-IQ2 motif. We propose that the hydrophobic interactions required for this binding interaction are destabilized for the M1C-IQ2 motif in which the Phe-718 has been substituted by a tyrosine (Tyr-736). The more polar side chain of Tyr-736 is required for this binding interaction are destabilized for the M1C-IQ2 motif. We propose that the hydrophobic interactions between Trp-718 and Trp-73 of MlcC and Phe-718 of the M1C-IQ2 motif, which are brought together to form a hydrophobic cluster than anchors MlcC to the M1C-IQ2 motif. The Val-16 residue of the adjacent M1C-IQ2-bound MlcC contacts both Phe-718 and Trp-73 and so may play a critical role in stabilizing the hydrophobic cluster (Fig. 7C). Additional contacts, including a π-π stacking interaction between His-3 and Phe-71 of MlcC and a hydrophobic interaction between His-3 and Ala-711 of M1C-IQ1, further strengthen the connections between the three structural elements. Together with the central hydrophobic groove, the flexible N- and C-terminal segments allow the small single-lobed MlcC to contact residues along the entire length of the M1C-IQ1 motif, from Trp-701 to Phe-718. Interestingly, Trp-701 interacts with Val-16 in MlcC and so mimics the interactions between Trp-73 and Val-16 at the interface between the two MlcC molecules.

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### TABLE 3

| Data collection | Data collection and refinement statistics for the 2:1 MlcC:M1C-IQ1.2 complex |
|-----------------|--------------------------------------------------------------------------------|
| Beamline        | CLS CMCF-ID                                                                   |
| Wavelength (Å)  | 1.7712                                                                       |
| Temperature (K) | 100                                                                           |
| Resolution (Å)  | 28.2–1.90 (1.97–1.90)                                                         |
| Space group     | P1                                                                            |
| Unit cell dimensions | a, b, c (Å) 28.7, 31.4, 56.4 α, β, γ (°) 75.8, 84.6, 67.1                 |
| R/Factors (%)   | 4.3 (13.3)                                                                   |
| Completeness (%)| 90.9 (69.5)                                                                   |
| Redundancy (%)  | 3.8 (3.6)                                                                    |
| No. of reflections | 48,431                                                                          |

| Refinement       | Refinement statistics for the 2:1 MlcC:M1C-IQ1.2 complex                     |
|------------------|--------------------------------------------------------------------------------|
| Resolution (Å)   | 28.2–1.90 (2.05–1.90)                                                         |
| R/Factors (%)     | 0.14/0.19 (0.12/0.19)                                                         |
| No. of atoms      | 1606                                                                          |
| Protein          | 15.0                                                                          |
| Water            | 238                                                                           |
| B-factors (Å²)   | 0.007                                                                         |
| r.m.s.d.         | 28.5                                                                          |
| Bond lengths (Å) | 0.90                                                                          |
| Ramachandran statistics | Favored (%) 98.9, Allowed (%) 1.1, Outliers (%) 0                         |

| Numbers in parentheses refer to the highest resolution shell. |
It is interesting to note that the myosin-1C LCBD does have a third IQ motif (23). Unfortunately, the light chain that binds to the myosin-1C IQ3 (M1C-IQ3) motif has not been identified, and so it was not incorporated into the constructs studied here. Further work will be required to identify the M1C-IQ3 light chain and to determine whether its presence enhances the binding of MlcC to the M1C-IQ2 motif. It is apparent, however, that the affinity of MlcC for an IQ motif and thus its mode of binding are strongly influenced by the presence of an adjacent MlcC. As a result, binding studies using peptides that span only a single IQ motif greatly underestimate the affinity of MlcC for the myosin-1C LCBD.

The two MlcC molecules do not bind in a symmetrical manner to the M1C-IQ1 and M1C-IQ2 motifs but instead are rotated by $\theta_{110} = 20^\circ$. Whether this is caused by variations in the IQ motif sequence or by contacts between the MlcC molecules is not clear. As a result, equivalent residues in the M1C-IQ1 and M1C-IQ2 motifs interact in a slightly different manner with MlcC. The sum of the altered binding interactions may further contribute to the difference in binding affinity exhibited by MlcC for the IQ1 and IQ2 motifs in the M1C-IQ1.2 fragment.

The inability of the M1C-IQ1.2 fragment to bind apo-CaM is consistent with previous work, which showed that only MlcC could rescue the expression of a myosin-1C-motor-LCBD construct in *Dictyostelium* (23). The M1C-IQ1 and M1C-IQ2 motifs are each 18 residues in length and thus are considerably shorter than conventional IQ motifs that bind apo-CaM or CaM-related LCs, which typically include 23–25 amino acids (31). When the C-lobe of apo-CaM is modeled in place of MlcC on the M1C-IQ.1.2 fragment, there are substantial steric clashes between the two N-lobes that could preclude their binding to the IQ motifs (Fig. 9A).

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also lack the sequence features necessary to bind the N-lobe of apo-CaM. The glycine and arginine residues in the C-terminal portion of conventional IQ motifs play important roles in binding the N-lobe of CaM, and both are missing in the myosin-1C IQ motifs (31, 32).

The inability of the C-lobe of apo-CaM to bind to the myosin-1C IQ motifs is surprising, especially given its close relationship, both in terms of primary sequence and structure, to MlcC (Fig. 6B). A sequence alignment shows that the hydrophobic residues that line the central groove of MlcC and contact the myosin-1C IQ motifs are well conserved in the C-lobe of CaM (Fig. 9B). The interactions mediated by the central groove therefore cannot be sufficient for tight binding to the myosin-1C IQ motifs. The glutamine residue that is one of the most strongly conserved features of the IQ motif is replaced in both of the myosin-1C IQ motifs by a lysine residue (Lys-709 and Lys-727). Although this substitution might be expected to play a key role in the binding specificity displayed by MlcC, a comparison of the structures of MlcC and the C-lobe of apo-CaM bound to their respective IQ motifs does not support this view. The glutamine and lysine side chains are both buried within the loop formed by the inter-EF hand linker sequence and in both cases make three hydrogen bond interactions with main-chain atoms in the linker. The inter-EF-hand linkers of MlcC and the C-lobe of apo-CaM vary in terms of primary sequence but fold into nearly identical loop conformations when bound to the IQ motif. Because the glutamine to lysine substitution does not induce a conformational change or result in the formation of no new bonds, it is unlikely to confer specificity to the binding interaction. The C-terminal portion of the myosin-1C IQ motifs contains a second conserved lysine residue (Lys-716 and Lys-734). Lys-716 forms a salt bridge with Glu-45 in MlcC, whereas Lys-734 forms salt bridges with Glu-48 and Glu-52 in MlcC. All three glutamic acid residues are conserved in the C-lobe of CaM, which again rules out a role for Lys-716 or Lys-734 in the specific recognition of MlcC (Fig. 9B).

This analysis leads us to conclude that specific recognition of MlcC by the myosin-1C IQ motifs is largely dependent on the C-terminal hydrophobic residue (Phe-718 and Tyr-736), which replaces the arginine residue found in conventional IQ motifs (Fig. 7A). As discussed earlier, high affinity binding of MlcC to the M1C-IQ1 motif requires the formation of a hydrophobic cluster that brings together Phe-718, Met-1, and Trp-73 of the M1C-IQ1-bound MlcC and Val-16 of the M1C-IQ2-bound MlcC. This mode of binding is not possible for the C-lobe of apo-CaM due to the presence of a lysine residue in place of the key C-terminal Trp-73 residue of MlcC (Fig. 9B). Moreover, the Met-79 residue of CaM, which corresponds to Met-1 of MlcC, is part of the last N-lobe helix and so lacks the conformational flexibility to fold back along the IQ motif in a manner comparable with Met-1. The myosin-1C IQ motifs are therefore uniquely designed to bind the single-lobed MlcC, because they lack an N-lobe-binding site and depend on specific interactions with hydrophobic residues on the short flexible N- and C-terminal segments for high affinity binding.

Light chains enhance the mechanical stiffness of the myosin LCBD so that it can act as a lever arm to amplify conformational changes in the motor domain. The N- and C-lobes of apo-CaM bind to opposite sides of the helical LCBD and together stabilize a segment spanning ~30 residues, whereas MlcC contacts only a single side of the LCBD and stabilizes a region ~18 residues in length. This suggests that the myosin-1C LCBD may be less rigid than those of other myosins. Indeed, the LCBD of myosin-1C is distinctly curved in the r-x crystal structure, whereas LCBDs of myosin-1 and myosin-V that bind apo-CaM are relatively straight (29, 31, 33). Further studies will be required to understand how the unique LCBD of myosin-1C affects its mechanical and force-sensing properties.

MlcC leaves a contiguous ~54 Å long segment of the myosin-1C LCBD exposed. This surface, which has a net positive charge and is relatively polar, is available for potential binding interactions with other cellular components. Mammalian myosin-1C is targeted to the tips of hair-cell stereocilia via its CaM-binding IQ motifs, which are exposed by the Ca$^{2+}$-dependent release of CaM (34, 35). It will be interesting to determine whether the LCBD plays a role in the localization of Dictyostelium myosin-1C. Dictyostelium myosin-1C is the only myosin-1 that has been shown to redistribute from the cell periphery to the spindle apparatus during mitosis (36). When localized to the actin-rich cell cortex, myosin-1C is involved in the generation of cortical tension and the extension and retraction of actin-rich cellular protrusions required for migration, endocytosis, micropinocytosis, and phagocytosis (11, 14, 15, 37). During mitosis, myosin-1C binds to and stabilizes spindle microtubules via its tail domain, a function that is important for normal spindle function. It will be exciting to determine how the unique LCBD of myosin-1C contributes to its cellular functions and to the diversity of myosin-1 isoform function in Dictyostelium.
Experimental Procedures

**Plasmid Construction**—A pET28a (+) plasmid encoding MlcC was constructed as described previously (23). DNA encoding the myosin-1C IQ2 motif (M1C-IQ2; residues 719–739 of myosin-1C) or the IQ1 and IQ2 motifs (M1C-IQ1,2; residues 699–739 of myosin-1C) was inserted into the BamHI and XhoI restriction sites of a pET21a (+)-derived plasmid containing upstream DNA coding for a hexahistidine tag, the protein G B1 domain (GB1), and a TEV protease recognition sequence. The fidelity of the constructs were verified by DNA sequencing.

**Protein Expression and Purification**—Recombinant MlcC was expressed in *E. coli* strain BL21(DE3) and purified as described previously (23). Uniformly $^{13}$C/$^{15}$N-labeled recombinant MlcC for NMR structural studies was obtained by growing *E. coli* at 37 °C with shaking in M9 minimal medium supplemented with 1 g/liter $^{15}$NH$_4$Cl, 2 g/liter [1$^{13}$C]glucose, and 10 ml/liter $^{13}$C/$^{15}$N-BioExpress-1000 medium (Cambridge Isotope Laboratories). Once bacterial cell growth reached an absorbance ($A_{600}$) of 0.6, protein expression was induced by the addition of isopropyl β-D-thiogalactopyranoside to a final concentration of 1 mM. Cells were harvested after an additional 6 h of growth with shaking at 37 °C.

*E. coli* strain BL21(DE3) harboring the plasmid encoding GB1-M1C-IQ2 or GB1-M1C-IQ1.2 was grown at 37 °C in LB medium supplemented with 100 mg/liter ampicillin at 37 °C to an $A_{600}$ of 0.6. Protein expression was induced as described above. Cells were allowed to grow for an additional 4 h and were then harvested by centrifugation, resuspended in 25 mM Tris-HCl, pH 8.0, 250 mM NaCl, and heated to 80 °C for 15 min. After centrifugation at 15,000 × g for 1 h, the majority of GB1-M1C-IQ2, but only a small fraction of GB1-M1C-IQ1.2, was recovered in the supernatant. The supernatant fractions were chromatographed over a Ni$^{2+}$-nitriloacetic acid column (Novagen) pre-equilibrated with 10 mM HEPES, pH 7.0, 150 mM NaCl (Buffer A), 25 mM imidazole. Fractions eluted with Buffer A containing 500 mM imidazole were pooled and applied to a Hi-Load 16/60 Superdex 75 size exclusion column (GE Healthcare) pre-equilibrated with 10 mM HEPES, pH 6.8, 50 mM NaCl, 1 mM β-mercaptoethanol (Buffer B). After centrifugation at 160,000 × g for 30 min, the supernatant was loaded onto a Ni$^{2+}$-nitriloacetic acid column equilibrated with Buffer B and then eluted with Buffer B containing 300 mM imidazole. An excess of TEV protease was added to the eluted fractions to cleave GB1 from M1C-IQ1.2, and the mixture was dialyzed overnight against 20 mM HEPES, pH 7.0, 50 mM NaCl, 5 mM dithiothreitol (DTT) at 4 °C. The resulting precipitate was solubilized by addition of 6 M urea and applied to a Resource S cation exchange column (GE Healthcare) equilibrated with 20 mM HEPES, pH 7.0, 6 M urea, and 5 mM DTT. M1C-IQ1.2 was eluted using a gradient of NaCl and remained soluble following dialysis against 20 mM HEPES, pH 7.0, 50 mM NaCl, 5 mM β-mercaptoethanol (Buffer C).

**Isothermal Titration Calorimetry**—Experiments were performed using a MicroCal VP calorimeter at 30 °C with all components dialyzed against Buffer C containing 2 mM EGTA. The syringe was loaded with MlcC or CaM (100–200 μM), and 10-μl aliquots were injected into the calorimetric cell containing 10–20 μM M1C-IQ1.2. Data were analyzed using MicroCal ORIGIN software.

**Circular Dichroism Spectroscopy**—Far-UV circular dichroism spectra were obtained using a Chirascan spectrophotometer (Applied Photophysics) and a 0.1-mm path length cuvette containing either 24 μM myosin-1C IQ1, 70 μM myosin-1C IQ2, or 45 μM myosin-1C IQ1-IQ2. Each recombinant peptide was dialyzed extensively against 15 mM Tris-HCl, pH 7.5, 50 mM NaF, 2 mM β-mercaptoethanol. Spectra were recorded from 190 to 240 nm at 20 °C and were averaged for 10 scans. Secondary structure was predicted using the program Spectral Works (Olis Inc.) with the CONTIN/LL algorithm (50).

**NMR Spectroscopy and Structure Calculation**—NMR spectra were recorded on a single protein sample containing 1 mM uniformly $^{13}$C/$^{15}$N-labeled MlcC in 10 mM HEPES, pH 6.8, 50 mM NaCl, 1 mM β-mercaptoethanol, 90% H$_2$O, 10% D$_2$O at 30 °C on a Varian 500 and 800 MHz spectrometers equipped with pulse field-gradient triple-resonance cryoprobes. Assignment of backbone and side-chain chemical shift resonances was made using the following experiments: $^1$H-$^{15}$N HSQC, HNCA, CBCA(CO)NH, H(CO)NH, H(C)CH-, and H(C)CH-TOCSY. Inter-proton distance restraints were derived from NOE cross-peak intensities observed in $^{15}$N-edited NOESY-HSQC (mixing time = 100 ms) and $^{13}$C-edited aliphatic and aromatic NOESY-HSQC (mixing time = 100 ms) datasets. Backbone dihedral (ϕ, ψ) torsion angles for 60 amino acid residues, obtained from $^1$H, $^{13}$C$^\wedge$, $^{13}$C$^\beta$, $^{13}$C$^\gamma$, and $^{15}$N chemical shifts using TALOS (38) with a minimum range of ±30°, were used for the final structural calculations. Datasets were processed using NMRPipe (39) and analyzed using NMR-View (40). Backbone and side chain chemical shift values and NOE-derived unassigned peak lists were used as inputs for automated NOE assignment and structure calculation in CYANA version 2.1 (41). In total 85% of NOE peaks were assigned after seven interactive cycles of NOE assignment and structure calculation, in which 100 conformers were generated per cycle using random torsion angle values with 10,000 steps of torsion angle dynamics-driven simulated annealing. The 20 structures with the lowest target function from the last iterative cycle were subjected to water refinement with CNS 1.3 using the ABACUS approach (42). The structural quality of the ensemble of conformers was assessed using PROCHECK-NMR and RFP web server (43, 44). The chemical shift assignments and final ensemble were deposited into the Biological Magnetic Resonance Bank and Protein Data Bank (accession numbers 19274 and 2MBU, respectively).

A sample containing 400 μM $^{13}$C/$^{15}$N MlcC and 450 μM GB1-M1C-IQ2 in 10 mM HEPES, pH 6.8, 50 mM NaCl, 1 mM β-mercaptoethanol, 90% H$_2$O, 10% D$_2$O was subjected to the following experiments at 30 °C on a 600 MHz Varian INOVA JOURNAL OF BIOLOGICAL CHEMISTRY 19615
spectrometer equipped with a cryprobe: $^{1}$H-$^{15}$N HSQC, HNCA, HN(CO)CA, HNCO, HN(CA)CO, and $^{15}$N-edited NOESY-HSQC (100-ms mixing time). CCPNMR analysis version 2.1 was used for backbone resonance assignment (45). The weighted backbone amide proton and $^{15}$N chemical shift changes of MlcC in the absence and presence of GB1-M1C-IQ2 were calculated using the formula $\Delta \delta = ((0.17\Delta \delta_p)^2 + (\Delta \delta_N)^2)^{1/2}$.

**Crystallization, Data Collection, and Refinement**—A 2.5-fold excess of MlcC was combined with M1C-IQ1.2, and the resulting ternary complex was isolated by size exclusion chromatography in buffer containing 20 mM HEPES, pH 7.0, 50 mM NaCl, 5 mM DTT. The complex was crystallized via hanging drop vapor diffusion at 22 °C. The drop contained 1 μl of protein complex solution (10.4 mg/ml) and 2 μl of reservoir solution (0.7 mM NaHPO$_4$/K$_2$PO$_4$, pH 5.6), with crystals forming within 24 h of drop setup. The crystals were cryoprotected using the well solution containing 30% glycerol. Data collection at 100 K was carried out at the Canadian Light Source CMCF-ID beamline. The data were indexed, scaled, and merged using the XDS package (46). The Phaser-MR module of the PHENIX software package (47) was used to find a molecular replacement solution to the data using two copies of the myosin V IQ apo-CaM complex as a search model (PDB accession 2I7X (29)). From the initial solution, the remaining helix of the M1C-IQ1.2 molecule as well as the termini of MlcC were built and refined using PHENIX (47) and Coot (48). The final refined structure and data files were deposited in the Protein Data Bank with the accession number 5IBW. The PyMOL Molecular Graphics System (Version 1.7.4 Schrödinger, LLC) was used for visualization and figure generation.

**Author Contributions**—D. N. L., J. L., S. W. C., G. P. C., and S. P. S. conceived and coordinated the study; D. N. L., J. L., Y. Y., E. M., S. C., S. W. C., G. P. C., and S. P. S. designed, performed, and analyzed the experiments; D. N. L., J. L., S. C., G. P. C., and S. P. S. wrote and reviewed the paper.

**Acknowledgment**—We thank Kim Munro of the Protein Function Discovery Facility for technical assistance with the ITC experiments.

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