The *Dictyostelium* class I myosin, MyoD, contains a novel light chain that lacks high-affinity calcium-binding sites

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Dictyostelium discoideum MyoD, a long-tailed class I myosin, co-purified with two copies of a 16 kDa light chain. Sequence analysis of the MyoD light chain showed it to be a unique protein, termed MlcD, that shares 44 % sequence identity with *Dictyostelium* calmodulin and 43 % sequence identity with *Acanthamoeba castellanii* myosin IC light chain. MlcD comprises four EF-hands; however, EF-hands 2–4 contain mutations in key Ca²⁺-co-ordinating residues that would be predicted to impair Ca²⁺ binding. Electrospray ionization MS of MlcD in the presence of Ca²⁺ and La³⁺ showed the presence of one major and one minor metal-binding site. MlcD contains a single tryptophan residue (Trp³⁹), the fluorescence intensity of which was quenched upon addition of Ca²⁺ or Mg²⁺, yielding apparent dissociation constants (K_d') of 52 μ M for Ca²⁺ and 450 μ M for Mg²⁺. The

low affinity of MlcD for Ca²⁺ indicates that it cannot function as a sensor of physiological Ca²⁺. Ca²⁺ did not affect the binding of MlcD to MyoD or to either of the two MyoD IQ (Ile-Gln) motifs. FLAG–MlcD expressed in *Dictyostelium* formed a complex with MyoD, but not with the two other long-tailed *Dictyostelium* myosin I isoenzymes, MyoB and MyoC. Through its specific association with the Ca²⁺-insensitive MlcD, MyoD may exhibit distinct regulatory properties that distinguish it from myosin I isoenzymes with calmodulin light chains.

Key words: calmodulin, cell motility, cytoskeleton, *Dictyostelium discoideum*, myosin light chain.

INTRODUCTION

The lower eukaryote *Dictyostelium discoideum* expresses 12 different myosin heavy chains, including seven non-filamentous, single-headed class I myosins (MyoA–MyoF and MyoK) (reviewed in [1,2]). Each myosin I heavy chain consists of a conserved motor domain that is responsible for ATP-dependent movement along actin filaments, an α -helical neck region that binds light chains by means of sequences known as IQ (Ile-Gln) motifs and a C-terminal tail. Light chains have an essential role in stabilizing the myosin neck region, so that it can function as a rigid lever arm that swings relative to the motor domain to generate movement, and, in addition, can be important sites for regulation, either through phosphorylation or by binding Ca²⁺ [3,4]. Although the neck regions of all *Dictyostelium* myosins contain one or more IQ motifs, to date, only the essential and regulatory light chains associated with *Dictyostelium* myosin II have been identified [5,6]. In the present paper, we describe the characterization of a novel calmodulin-like protein that functions as the light chain for *Dictyostelium* MyoD.

Dictyostelium MyoA and MyoE (and probably also MyoF, for which a full sequence is not available) have short tails consisting of a basic TH1 domain that binds anionic phospholipids, whereas MyoB, MyoC and MyoD have longer tails comprising a TH1 domain, a TH2 domain that binds actin filaments in a nucleotide-insensitive manner and an SH3 domain (reviewed in [7,8]). MyoK is an atypical myosin I that almost completely lacks a neck and tail region [9]. Short-tailed myosins I can power motile processes by translocating actin filaments relative to membranes, whereas the long-tailed myosins I can also contract and collapse actin

filament networks [10,11] and interact via their SH3 domain with the CARMIL (capping protein, Arp2/3 and myosin I linker) family of scaffolding proteins [12]. *Dictyostelium* cells lacking one or more myosin I isoenzymes exhibit defects in a wide range of actin-based motile processes, including pseudopod formation, phagocytosis, chemotactic streaming, fluid-phase pinocytosis and the maintenance of cortical tension [9,13–17]. The different *Dictyostelium* myosin I isoforms do not have identical cellular roles. For example, cells lacking MyoD exhibit no obvious behavioural defects, MyoC null cells are impaired in phagocytosis, and MyoB null cells have defects in pseudopod formation, phagocytosis, motility and chemotactic streaming [13,14,16,18]. The molecular basis for these distinct functions is not clear, but may relate to differences in the structure of the tail domain or in the regulatory properties of each myosin.

Myosins I from lower eukaryotes are activated by the phosphorylation of a serine/threonine residue in the motor domain of the heavy chain at a position known as the TEDS (Thr-Glu-Asp-Ser) rule site [19,20]. Phosphorylation of the TEDS rule site is catalysed by members of the p21-activated kinase (PAK) family, whose activity is controlled by the small GTPases Cdc42 and Rac [21–23]. In contrast, vertebrate myosins I have an aspartic or glutamic residue at the TEDS rule site and so do not require heavy chain phosphorylation for activity. The vertebrate myosins I employ the ubiquitous Ca²⁺-sensor protein calmodulin as a light chain, and their motor activity can be modulated by the binding of Ca²⁺ to the calmodulin light chains [24–27].

Despite the fact that the *Dictyostelium* myosins I have been the subject of extensive investigation, the identity of their light chains remains unknown. In the present study, we show that the

Abbreviations used: CTER, calmodulin, troponin C, myosin II ELC and RLC; ECL[®], enhanced chemiluminescence; ELC, essential light chain; ESI, electrospray ionization; GST, glutathione S-transferase; MICLC, myosin IC light chain; MlcD, myosin light chain D; RLC, regulatory light chain.

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light chain associated with MyoD is a unique calmodulin-related protein that has lost the ability to bind Ca^{2+} with high affinity. The MyoD light chain, termed MlcD, is not associated with MyoB or MyoC, indicating that differences in both the heavy and light chains may contribute to the distinct physiological functions of the long-tailed *Dictyostelium* myosin I isoenzymes.

EXPERIMENTAL

MyoD purification and sequence analysis

Lysates of *Dictyostelium* AX3 cells were chromatographed over a column of phosphocellulose P11 (Whatman) to resolve MyoB, MyoC and MyoD as previously described [28]. MyoD was purified further by precipitation with skeletal-muscle actin and by chromatography over a MonoQ HR 5/5 column. The stoichiometric ratio of the MyoD heavy and light chains was analysed by densitometry of Coomassie-Blue-stained SDS gels using a FluorChem Imaging System (Alpha Innotech Corp., San Leandro, CA, U.S.A.).

For sequence analysis, 150 μg of MyoD was subjected to SDS/PAGE on a 16 % gel and was transferred electrophoretically on to HyBond nitrocellulose (Amersham Biosciences). Following staining with Amido Black, pieces of nitrocellulose containing the 16 kDa light chain (MlcD) were excised, blocked with 0.5 % polyvinylpyrrolidone (PVP-40; Sigma) in 100 mM ethanoic acid for 30 min at 37 °C, suspended in 100 mM NaHCO_3 and incubated at 37 °C for 12 h with trypsin [29]. Peptides were resolved on a Superpac Pep-S (Amersham Biosciences) column using an acetonitrile gradient in 0.1 % (v/v) trifluoroacetic acid. Automated Edman sequence analysis was performed using an Applied Biosystems 470A Sequencer (Alberta Peptide Institute, Edmonton, Canada).

Cloning and bacterial expression of MlcD and the MyoD IQ motifs

Genomic DNA for PCR analysis was prepared from growth-phase *Dictyostelium* AX3 cells as described [30]. Total RNA was extracted from growth-phase cells with TRIzol® Reagent (Invitrogen) and was subsequently used as the substrate for cDNA synthesis using mMLV reverse transcriptase (Promega). MlcD and the MyoD IQ motifs were created by PCR with Expand High Fidelity polymerase (Roche Diagnostics) using cDNA as a template. All IQ motif constructs contained a C-terminal His₆ tag. Constructs were subcloned into pGEX-4T3, transformed into *Escherichia coli* strain DH5 α and sequenced to confirm correct amplification. The expression of GST (glutathione S-transferase) fusion proteins was induced by adding IPTG (isopropyl β -D-thiogalactoside) for 4 h at 37 °C. Bacteria were harvested, resuspended in TBS (50 mM Tris/HCl, pH 7.4, and 150 mM NaCl) containing 0.2 mg/ml lysozyme, sonicated on ice and centrifuged at 15 000 g for 20 min. GST-MlcD and GST-IQb were expressed as soluble proteins, but GST-IQa and GST-IQab were recovered in the pellet. Attempts to solubilize GST-IQa and GST-IQab were unsuccessful, although extensive washes of the pellet fraction with TBS containing 2 M urea resulted in the removal of the majority of bacterial proteins. GST-MlcD and GST-IQb were purified using glutathione-Sepharose 4 Fast Flow (Amersham Biosciences). GST was cleaved from GST-MlcD by a 30 min incubation at room temperature (21 °C) with thrombin (a gift from Dr M. Nesheim, Queen's University). Thrombin and GST were removed either by heating the sample at 90 °C for 4 min in the presence of 5 mM CaCl_2 or through the use of successive columns of glutathione-Sepharose and benzamidine-Sepharose 4 Fast Flow (Amersham Biosciences). MlcD isolated by both procedures behaved identically.

MS analysis

MlcD was stripped of bivalent cations by dialysis against 10 mM ammonium acetate, pH 5.0, and 5 mM EDTA. EDTA was then removed by extensive dialysis against 10 mM ammonium acetate and 250 mM KCl, followed by 10 mM ammonium acetate. Electrospray ionization (ESI) mass spectra were acquired on a Micromass Q-ToF Ultima spectrometer (Protein Function Discovery Centre, Queen's University) in positive-ion mode. MlcD (10 μM) in the absence or presence of 10 μM CaCl_2 or 5 μM LaCl_3 was infused into the spectrometer at a rate of 10 $\mu\text{l}/\text{min}$ using 10 mM ammonium acetate, pH 5.0, as solvent. Scans were typically collected from a mass-to-charge ratio of 550–2900 over 2.4 s, averaged out over 40 scans and deconvoluted to parental masses using the MassLynx software package.

Fluorescence measurements

MlcD was dialysed against 100 mM Hepes, pH 7.0, and 5 mM EDTA, followed by extensive dialysis against 100 mM Hepes, pH 7.0, and 250 mM KCl and then 100 mM Hepes, pH 7.0. The intrinsic tryptophan fluorescence of MlcD was analysed using a PerkinElmer LS50B luminescence spectrometer. Excitation was at 280 nm and fluorescence emission was monitored at 345 nm. During experiments, solutions were kept at 21 °C and were continuously mixed using a small magnetic stirring bar. Titrations were performed by adding 1–2 μl aliquots of stock CaCl_2 and MgCl_2 solutions. The final added volume of CaCl_2 or MgCl_2 was always less than 5 % of the total volume. Titrations were carried out at three different MlcD concentrations (1, 5 and 10 μM) with virtually identical results. Results were fitted to the equation $y = ax^h / (k^h + x^h)$, where y is the fraction of maximum fluorescence change, a is the maximum change in fluorescence, k is the Ca^{2+} concentration required for half-maximal fluorescence change, h is the Hill constant, and x is the Ca^{2+} or Mg^{2+} concentration. Quantum yields were normalized to a value of 0.13 for free tryptophan [31].

Actin-binding and activity assays

ATPase activities were measured by the release of ^{32}P from [γ - ^{32}P]ATP. K^+ /EDTA-ATPase activity assays were performed in 0.5 M KCl, 35 mM EDTA, 1 mM ATP and 25 mM Tris, pH 7.5. MgATPase activity assays were carried out in 10 mM MgCl_2 , 1 mM ATP, 1 mM dithiothreitol, 10 mM Tes, pH 7.5, and 0.25 mM EGTA, with or without 1 mM CaCl_2 , and with or without 100 $\mu\text{g}/\text{ml}$ of rabbit skeletal muscle actin [32]. Actin-pelleting experiments were performed by incubating MyoD (30 $\mu\text{g}/\text{ml}$) with actin (100 $\mu\text{g}/\text{ml}$) in 100 mM KCl, 1 mM dithiothreitol and 10 mM Tes, pH 7.5, containing either 1 mM EGTA or 1 mM CaCl_2 , for 1 h at room temperature followed by centrifugation in a Beckman TLA 100 rotor at 100 000 g for 20 min. Pellets were resuspended in the assay buffer to the original volume and were analysed, along with equal amounts of the supernatants, by SDS/PAGE.

Expression and analysis of FLAG-MlcD

The MlcD construct was cloned into the pDXA-FLAG plasmid to add an N-terminal FLAG epitope [33]. Plasmids were transformed into *Dictyostelium* AX3 cells and clonal cell lines were selected using 20 $\mu\text{g}/\text{ml}$ G418. Lysates from cells (30 g wet mass) expressing FLAG-MlcD were chromatographed over phosphocellulose to resolve MyoB, MyoC and MyoD [28]. Extracts for immunoprecipitation experiments were prepared by lysing cells in TBS containing 0.1 % (v/v) Triton X-100 and a protease inhibitor

Figure 1 Identification of a light chain associated with *Dictyostelium* MyoD

(A) A Coomassie-Blue-stained SDS polyacrylamide gel of MyoD shows the 125 kDa heavy chain (HC) and an apparent light chain (LC) of approx. 16 kDa. The locations of molecular mass standards are shown. (B) The full-length MlcD coding region was amplified by PCR using either genomic DNA or cDNA as template. The larger size of the amplified product obtained from genomic DNA as compared with cDNA is due to the presence of a single 133 bp intron inserted following the codon for Ser⁵⁸. The location of DNA size standards is shown. (C) Alignment of the deduced amino-acid sequence of MlcD with *Dictyostelium* calmodulin (Cal; accession number AAA33172), calmodulin-like protein (CalB; accession number AAB60882) and *Acanthamoeba* myosin IC light chain (MICLC; accession number AAB17871). The solid line denotes the sequences of the two peptides isolated from a tryptic digest of MlcD. Dark boxes indicate residues that are identical in all four proteins. Light shading is used to highlight residues at the X, Y, Z, -X and -Z positions of the four predicted EF-hand loops (EF1–EF4). In the canonical EF-hand, these residues are involved in chelating the Ca²⁺ ion.

cocktail (Sigma). Following centrifugation at 15000 *g* for 15 min, the supernatant was mixed with anti-FLAG epitope M2 antibody beads (Sigma) for 1 h at 4 °C. Beads were collected by centrifugation and washed five times with lysis buffer. Immunoprecipitated proteins were eluted from the beads with 2× SDS sample buffer, resolved by SDS/PAGE and subjected to immunoblot analysis using an anti-FLAG epitope M5 antibody (Sigma), an anti-*Dictyostelium* calmodulin antibody (a gift from Dr Thierry Soldati, Imperial College of Science Technology and Medicine, London, U.K.) and rabbit polyclonal antibodies against MyoB, MyoD [28] and MyoC (a gift from Dr John Hammer, III, National Institutes of Health, Bethesda, MD, U.S.A.) [12]. Immunoblots were visualized using a horseradish-peroxidase-conjugated secondary antibody and ECL[®] (enhanced chemiluminescence; Amersham Biosciences).

Overlay analysis

MlcD and bacterially expressed human calmodulin were biotinylated using *N*-hydroxysuccinimidobiotin (Sigma) according to the manufacturer's protocol. The behaviour of the biotinylated proteins was identical with that of the unmodified proteins, as judged by measurements of the Ca²⁺-induced change in intrinsic fluorescence for MlcD and the Ca²⁺-induced mobility shift on SDS/PAGE for calmodulin. Overlay assays were performed by resolving GST–IQ motif proteins by SDS/PAGE, followed by transfer to ImmobilonP (Millipore). Membranes were blocked for 2 h with TBS containing 1% (w/v) BSA and 0.1% (v/v) Tween 20 and were then probed with 1.2 μM biotinylated calmodulin or MlcD for 1 h at room temperature. After three 15 min washes with blocking buffer, biotinylated proteins were detected using horseradish-peroxidase-conjugated avidin (Sigma) and ECL[®].

RESULTS

Sequence analysis of the *Dictyostelium* MyoD light chain

Dictyostelium MyoD is a long-tailed class I myosin consisting of a heavy chain with a molecular mass of 125 kDa [18]. SDS/PAGE

analysis of a number of preparations of purified MyoD consistently showed that a protein with a molecular mass of approx. 16 kDa co-purified with the 125 kDa heavy chain (Figure 1A). The stoichiometry of the 16 kDa protein to the MyoD heavy chain was in the range 1.7–2.4:1 for four separate preparations of MyoD. In accordance with this stoichiometry, two putative IQ motifs can be recognized in the neck region of the MyoD heavy chain (see below).

Following SDS/PAGE, the 16 kDa protein was transferred on to nitrocellulose and was digested with trypsin. The resulting peptides were resolved by chromatography over a reverse-phase HPLC column and subjected to automated Edman sequence analysis, yielding the sequences XLGQNPSQSEINEILR and QAVGSGDGAINYEPF. Exact matches to both tryptic peptide sequences were identified in the translation of two cDNA clones obtained by the *Dictyostelium discoideum* cDNA project in Japan (GenBank[®] accession numbers C24640 and AU075930). Both cDNAs, which derive from the slug stage of development, code for a previously undescribed protein, which we have termed myosin light chain D (MlcD). The cDNA encoding MlcD was amplified by reverse transcriptase (RT)-PCR from RNA extracted from growth-phase *Dictyostelium* and by PCR from genomic DNA. Analysis of these products confirmed the sequence of MlcD and showed that the *mlcD* gene is interrupted by a single 133 bp intron (Figure 1B). Conceptual translation of the nucleotide sequence shows that MlcD is 147 residues in length with a molecular mass of 16483 Da, consistent with the size of the light chain associated with MyoD as estimated by SDS/PAGE (Figure 1C). MlcD is quite an acidic protein, with a pI of 4.38 and a net negative charge at pH 7.0 of –11.

Phylogenetic analysis of MlcD

When BLAST searches using the amino-acid sequence of MlcD were carried out against known *Dictyostelium* proteins, MlcD was found to be most closely related to calmodulin (44% sequence identity). As with calmodulin, four helix–loop–helix EF-hand Ca²⁺-binding motifs can be recognized in the MlcD sequence (Figure 1C). A phylogenetic tree generated from sequence alignments of *Dictyostelium* small (< 25 kDa) four EF-hand proteins

Figure 2 Phylogenetic analysis of MlcD

Comparison of the amino-acid sequence of MlcD with other *Dictyostelium* small four EF-hand proteins and *Acanthamoeba* MICLC. Members of the CTER subfamily are indicated. Alignment and bootstrapping analysis was carried out using the ClustalX software package and dendrograms were constructed using Treeview. Circles indicate nodes that have bootstrap values of > 750 , whereas squares indicate nodes that have bootstrap values of between 500 and 750 (out of 1000). Nodes with bootstrap values < 500 are not marked. Abbreviations and accession numbers are CBP1 (calcium binding protein 1; P42529); CBP2 (AAC47039); CBP3 (AAD17692); CBP4a (CAC04145); CBP4b (CAC04146); CBP5 (BAB63905); CBP6 (BAB63906); CBP7 (BAB63907); CBP8 (BAB63909); CBP9 (BAB63908); calcineurin B (AAL96710); calfuminin (BAA06266); calmodulin (AAA33172); CalB (calmodulin-like protein; AAB60882); myosin II RLC (P13833); myosin II ELC (A28127); NCS (neuronal Ca^{2+} -sensor homologue; AAL86951); NH (neurocalcin homologue; AAL96715); *Acanthamoeba* MICLC (AAB17871).

shows that MlcD is also closely related to CalB, a calmodulin-related protein, and the myosin II essential light chain (ELC) and regulatory light chain (RLC) (Figure 2). The high degree of sequence similarity between MlcD and calmodulin, which functions as the light chain for several types of myosin I, and the myosin II ELC and RLC, is entirely consistent with the functional identification of MlcD as a MyoD light chain.

When compared with proteins from other organisms, MlcD exhibited a high degree of sequence identity with many different types of calmodulin, a result that reflects the highly conserved nature of the calmodulin sequence. Excluding calmodulin, MlcD was found to share the greatest degree of sequence identity (43%) with the *Acanthamoeba* myosin IC light chain (MICLC) (Figure 2). Interestingly, phylogenetic analysis indicates that the heavy chains of MyoD and *Acanthamoeba* myosin IC are more closely related to each other than they are to any of the other *Dictyostelium* or *Acanthamoeba* myosin I heavy chains [34]. The close relationship exhibited by both the heavy and light chains of MyoD and *Acanthamoeba* myosin IC suggests that the two proteins are functional homologues.

Expression of FLAG-tagged MlcD in *Dictyostelium*

Overexpression of a FLAG-tagged MlcD in *Dictyostelium* had no noticeable effect on cell growth rates in suspension or on Petri

Figure 3 FLAG-MlcD expressed in *Dictyostelium* associates with MyoD, but not with MyoB or MyoC

(A) An extract of *Dictyostelium* cells expressing FLAG-MlcD was chromatographed over a phosphocellulose column. Column fractions were monitored for protein concentration (●), K^+ /EDTA-ATPase activity (Δ) and conductivity (broken line). The K^+ /EDTA-ATPase activity indicates the elution positions of MyoB, MyoC and MyoD [28], which were pooled as indicated. (B) Immunoblot analysis of the flow-through (FT) and the MyoB, MyoC and MyoD pools from the phosphocellulose column using anti-FLAG M5 antibody. (C) Lysates of *Dictyostelium* cells expressing FLAG-MlcD were immunoprecipitated using anti-FLAG M2-Sepharose. Samples of the cell lysate and the material precipitated by the anti-FLAG resin (FLAG IP) were immunoblotted using antibodies specific for the FLAG-epitope tag and the MyoB, MyoC and MyoD heavy chains. (D) Lysates of *Dictyostelium* cells expressing FLAG-MlcD were incubated with an excess of anti-FLAG M2-Sepharose in order to bind virtually all of the FLAG-MlcD. Samples of the cell lysate, the immunodepleted supernatant remaining after incubation with anti-FLAG resin (FLAG ID) and the material bound to the anti-FLAG resin (FLAG IP) were immunoblotted with antibodies specific for the FLAG epitope and MyoD heavy chain.

plates, on the organization of the actin cytoskeleton or on the ability of cells to complete the developmental cycle (results not shown). The ability of FLAG-MlcD to associate with myosin I was examined by chromatographing lysates from cells expressing FLAG-MlcD over a phosphocellulose column. This column has previously been demonstrated to resolve all three of the long-tailed *Dictyostelium* myosin I isoforms (MyoB, MyoC and MyoD) (Figure 3A) [28]. The highly acidic MlcD would not be expected to bind to this column unless it was associated with another protein. Indeed, immunoblot analysis showed that a significant amount of FLAG-MlcD was present in the flow-through from the phosphocellulose column, suggesting that FLAG-MlcD may be able to exist on its own as a soluble and stable protein in the *Dictyostelium* cytosol (Figure 3B). The fractions containing

MyoB, MyoC and MyoD were detected and pooled based on assays of their K^+ /EDTA-ATPase activity (Figure 3A). Immunoblot analysis showed that MlcD co-purified with the MyoD pool, but not with MyoB or MyoC (Figure 3B). The small amount of MlcD present in the MyoB pool was due to contamination with MyoD and was eliminated when MyoB was further separated from MyoD by chromatography over a MonoQ column (results not shown).

The interaction of MlcD with the long-tailed myosin I isoenzymes was examined further by immunoprecipitation experiments. Lysates from cells expressing FLAG-MlcD were immunoprecipitated with anti-FLAG M2 resin and probed with antibodies to MyoB, MyoC and MyoD. MyoD, but not MyoB or MyoC, co-immunoprecipitated with the FLAG-MlcD (Figure 3C). Addition of an excess of anti-FLAG M2 resin resulted in the depletion of more than 90% of the total MyoD from cell lysates, showing that the overexpressed FLAG-MlcD can be very efficiently incorporated into MyoD (Figure 3D).

To test whether or not MyoB, MyoC or MyoD was associated with calmodulin, the three myosin I isoforms were immunoprecipitated from cell extracts and were probed with a polyclonal antibody against *Dictyostelium* calmodulin. Calmodulin was not detected in any of the immunoprecipitated fractions (results not shown). Surprisingly, when purified MyoB was subjected to SDS/PAGE, no light chain could be detected either by Coomassie Blue staining or by silver staining (results not shown). This result suggests that the MyoB light chain may not be tightly associated, and is lost during the purification procedure. MyoC could not be purified to sufficient homogeneity to identify the presence of an associated light chain.

Ca^{2+} -binding properties of MlcD

The classical EF-hand is characterized by a sequence of 12 amino acids in which residues at positions 1, 3, 5, 7, 9 and 12 (usually designated X, Y, Z, -Y, -X and -Z) co-ordinate Ca^{2+} [35,36]. Aspartic, asparagine or serine residues are commonly present in the X, Y, Z and -X positions, where they provide side-chain oxygen ligands to chelate Ca^{2+} , whereas a glutamic residue almost always occupies the -Z position and contributes both side-chain oxygens to co-ordinate calcium. Although the N-terminal EF-hand of MlcD (EF1) conforms to the consensus Ca^{2+} -binding sequence, the other three EF-hands (EF2–EF4) have deletions or non-conservative substitutions that might render them non-functional: EF2 has glycine at the X position and lacks a glutamic residue that could fill the -Z position, EF3 has lysine, glycine and aspartic residues at the Z, -X and -Z positions respectively, and EF4 has valine and proline residues at the X and -Z positions respectively (Figure 1C).

The metal-ion-binding properties of bacterially expressed MlcD, which has two vector-derived amino acids (glycine and serine) at the N-terminus that are not present in MlcD, were analysed by ESI MS (positive-ion mode). In the absence of metal ions, recombinant MlcD displayed a molecular mass of 16664 Da, which is in close agreement with the mean isotopic mass calculated from the sequence (16663 Da) (Figure 4). Upon addition of $10\ \mu\text{M}$ Ca^{2+} , a major peak appeared at $M + 40$ and a minor peak appeared at $M + 80$, corresponding to MlcD with one or two bound Ca^{2+} . Under similar conditions, four Ca^{2+} could be detected bound to calmodulin (results not shown), in agreement with a previous study [37]. The ability of MlcD to bind La^{3+} , a tight-binding Ca^{2+} analogue with a mass of 139 Da, was also examined. In the presence of $2.5\ \mu\text{M}$ La^{3+} , a major peak was obtained that corresponded to MlcD bound to a single La^{3+} (Figure 4). A second much less intense peak sometimes appeared under these

Figure 4 ESI MS of MlcD

Deconvoluted spectra are shown for MlcD in (A) the absence of metal ions, (B) with $10\ \mu\text{M}$ $CaCl_2$ or (C) with $5\ \mu\text{M}$ $LaCl_3$. The expected molecular mass for apo-MlcD is 16663 Da, Ca^{2+} is 40 Da and La^{3+} is 139 Da.

conditions that corresponded to MlcD with two bound La^{3+} . The results obtained with MlcD are consistent with the presence of one higher-affinity metal-binding site (presumably contributed by EF1) and one lower-affinity site.

The single tryptophan residue present in MlcD (Trp³⁹) is located in the linker region between EF1 and EF2 (Figure 1C). The fluorescence intensity of Trp³⁹ served as a useful probe for monitoring structural transitions induced by metal ions binding to EF1. Addition of either Ca^{2+} or Mg^{2+} decreased the fluorescence intensity of Trp³⁹ by close to 30% (Figure 5A). This corresponded to a decrease in the quantum yield from 0.18 to 0.12 when emission spectra were standardized to a value of 0.13 for free tryptophan in the same buffer [38]. The change in fluorescence of Trp³⁹ could be fully reversed upon chelation of Ca^{2+} or Mg^{2+} by addition of EDTA. In the presence of $1\ \text{mM}$ Mg^{2+} , addition of Ca^{2+} produced only a small additional decrease in fluorescence intensity, suggesting that both metal ions induce a comparable change in the environment of Trp³⁹ (Figure 5A). Titrations performed with Ca^{2+} and Mg^{2+} yielded changes in the fluorescence intensity of MlcD that were consistent with the presence of a single metal-ion-binding

Figure 5 Fluorescence emission spectra of MlcD

(A) The fluorescence emission spectrum of MlcD (10 μ M) was measured in the absence of metal ions (—) or in the presence of 1 mM Ca^{2+} (---), 1 mM Mg^{2+} (.....), or 1 mM Ca^{2+} and Mg^{2+} (— · — · —) dotted and dashed lines). Following the addition of 1 mM Ca^{2+} or Mg^{2+} , the addition of 5 mM EDTA yielded a fluorescence emission spectrum identical with that of MlcD in the absence of metal ions (—). (B) The fluorescence emission of MlcD (10 μ M) at 345 nm was monitored as the concentrations of Ca^{2+} (●) and Mg^{2+} (○) were varied. The calculated fitted curves were obtained as described in the Experimental section. The maximal change in fluorescence intensity as determined from the calculated fitted curves was set to 100 %.

site (Figure 5B). Apparent dissociation constants (K_d') of 53 ± 1 μ M for Ca^{2+} and 425 ± 25 μ M for Mg^{2+} were obtained.

Studies were carried out to determine if the conformational change induced by Ca^{2+} altered the properties of MlcD. Unlike calmodulin, which increases its mobility on SDS/PAGE in the presence of Ca^{2+} , no change in the electrophoretic mobility of MlcD was observed in the presence of Ca^{2+} (Figure 6A). The binding of Ca^{2+} exposes hydrophobic residues on the surface of calmodulin, which results in Ca^{2+} -dependent binding to hydrophobic target sites on proteins and to resins such as phenyl-Sepharose [39]. In contrast, MlcD was unable to bind phenyl-Sepharose in the presence of Ca^{2+} , suggesting that Ca^{2+} does not promote the exposure of a hydrophobic group on the surface of MlcD (Figure 6B). The binding of Ca^{2+} can also reduce the affinity of calmodulin for myosin IQ motifs, leading to dissociation of calmodulin from the myosin heavy chain [7]. Pull-down experiments using actin filaments showed that MlcD did not dissociate from the MyoD heavy chain in the presence of millimolar levels of Ca^{2+} (Figure 6C). In addition, the actin-activated MgATPase activity of MyoD was unaffected by the presence or absence of millimolar Ca^{2+} .

Interaction of MlcD and calmodulin with MyoD IQ motifs

The generalized consensus sequence for IQ motifs is (I/L/V)QXXRXXXX(R/K) [40]. Two closely spaced IQ motifs

Figure 6 Effects of Ca^{2+} on the properties of MlcD

(A) Calmodulin and MlcD were subjected to SDS/PAGE in the presence of either 10 mM Ca^{2+} or 10 mM EGTA. Calmodulin, but not MlcD, displayed a Ca^{2+} -dependent shift in mobility. (B) Calmodulin and MlcD were applied to a column of phenyl-Sepharose in the presence of 1 mM Ca^{2+} and were then eluted with buffer containing 1 mM EGTA. Calmodulin, but not MlcD, bound to the phenyl-Sepharose column in a Ca^{2+} -dependent manner. (C) MyoD was incubated with actin in the presence of either 1 mM EGTA or 1 mM Ca^{2+} . Following high-speed centrifugation (100 000 g for 20 min), the resulting supernatant and actin pellet fractions were analysed by SDS/PAGE. Coomassie Blue staining showed that MlcD did not dissociate from MyoD in the presence of Ca^{2+} .

can be recognized in the MyoD neck region, but differ from the consensus IQ motif sequence by having residues other than arginine and lysine at positions 6 and 11 (Figure 7A). Inspection of the IQ motif sequences shows that the first motif (IQa) is considerably more hydrophobic than the second motif (IQb). Indeed, IQa and IQab (which spans both IQ motifs) were insoluble when expressed in bacteria as GST-fusion proteins, whereas GST-IQb was soluble. GST-IQa and GST-IQab were also highly degraded when expressed in bacteria. Addition of a His₆ tag to the C-terminus of the fusion proteins significantly decreased proteolysis and also provided a means to verify that the IQ motif was present (Figure 7B). In overlay experiments, IQa, IQb and IQab bound both MlcD and calmodulin in a Ca^{2+} -independent manner (Figure 7B).

Figure 7 Binding of MlcD and calmodulin to the MyoD IQ motifs

(A) The sequence of the MyoD neck region contains two recognizable IQ motifs (IQa and IQb). (B) GST-fusion proteins containing one or both of the MyoD IQ motifs (GST-IQa, GST-IQb and GST-IQab) were subjected to SDS/PAGE and either stained with Coomassie Blue (CB) or transferred on to ImmobilonP and probed with an antibody that recognizes the His₆ tag (6xHis) present at the C-terminus of the fusion protein. Overlay assays were carried out with biotinylated MlcD (MlcD) or with biotinylated calmodulin (calmodulin). Biotinylated proteins were detected using horseradish-peroxidase-conjugated avidin and ECL[®].

DISCUSSION

In the present study, we show that MyoD, a long-tailed *Dictyostelium* myosin I, is associated with two copies of a 16.5 kDa light chain. Sequence analysis showed that the MyoD light chain is a novel calmodulin-related protein that we have termed MlcD. FLAG-MlcD expressed in *Dictyostelium* was efficiently incorporated into MyoD, confirming that MlcD functions as a MyoD light chain. Co-immunoprecipitation studies and SDS/PAGE analysis provided evidence that only MlcD, and not calmodulin, is associated with MyoD. Any calmodulin present on MyoD would easily have been detected, both because calmodulin is larger than MlcD and because it exhibits a mobility shift in the presence of calcium. The exclusive association of MlcD with MyoD is in contrast with the ability of both calmodulin and MlcD to bind to the isolated MyoD IQ motifs in an overlay assay (Figure 7B). However, because the overlay assays are not quantitative, this result does not rule out the possibility that the MyoD IQ motifs exhibit a higher affinity for MlcD than for calmodulin. Alternatively, it is possible that sequences outside the minimal IQ motif may be involved in the specific recognition of MlcD. Further

studies on the MyoD neck region will be required to identify the features that confer specificity for MlcD.

Although specialized light chains are invariably found as subunits of conventional myosin (myosin II), only a handful of unconventional myosins are known to utilize light chains other than calmodulin. These include human myosin X [41] and *Toxoplasma gondii* myosin A (a class XIV myosin) [42], which are associated with calmodulin-like proteins and some class V myosins that bind both myosin II light chains and calmodulin [43,44]. Before the present study, *Acanthamoeba* myosin IC was the only myosin I known to associate with a specialized light chain, a calmodulin-related protein termed MICLC [45].

MlcD shares a high degree of sequence identity with calmodulin and, like calmodulin, has four EF-hand motifs. ESI MS showed, however, that MlcD retains only a single primary metal-binding site. Examination of the MlcD sequence suggests that the metal-binding site corresponds to the N-terminal EF-hand, EF1, since EF2 suffers from extensive deletions, and EF3 and EF4 contain non-conservative substitutions at some of the critical positions required to co-ordinate Ca²⁺. A second, lower-affinity metal-binding site was detected in MlcD, indicating that that one of the other EF-hands (EF2–EF4) may be able to bind metal ions weakly. Alternatively, examination of calmodulin by ESI MS has identified a total of nine Ca²⁺-binding sites, comprising the four high affinity EF-hand binding sites and five auxiliary, low-affinity binding sites [46]. Thus it is possible that the lower-affinity site detected in MlcD represents a poorly defined auxiliary site.

MlcD underwent a conformational change upon binding of Ca²⁺ or Mg²⁺ that could be detected as a change in the fluorescence intensity of Trp³⁹, the only tryptophan residue present in MlcD. Ca²⁺ and Mg²⁺ induced comparable conformational changes in MlcD, and the presence of Mg²⁺ prevented the Ca²⁺-induced conformational change, suggesting that both metal ions compete for a similar site. The affinity of MlcD for Ca²⁺ (K_d of approx. 50 μ M) is much too low for MlcD to act as a physiological Ca²⁺ sensor in *Dictyostelium*, where intracellular free Ca²⁺ concentrations range from 40 nM in unstimulated cells to a peak value of 160 nM in aggregation-competent cells stimulated by cAMP [47]. Moreover, at the millimolar Mg²⁺ concentrations present in the cytosol, the metal binding site of MlcD (K_d for Mg²⁺ of approx. 450 μ M) would be almost fully occupied by Mg²⁺. The metal-binding properties of MlcD share a number of points of similarity with the Ca²⁺-insensitive RLCs associated with vertebrate skeletal- and smooth-muscle myosin. The muscle myosin RLCs comprise four EF-hands, but only the N-terminal EF-hand (EF1) is functional [48,49]. Both Ca²⁺ and Mg²⁺ bind to the RLCs, but the affinities for the two metal ions are such that the site is occupied by Mg²⁺ under physiological conditions [50,51]. Ca²⁺ and Mg²⁺ both induce a conformational change in the skeletal muscle myosin RLC that reduces its Stokes radius and may increase its affinity for the heavy chain [51,52]. At present, there is no evidence that the conformational change induced in MlcD by Ca²⁺ and Mg²⁺ is required for MlcD to bind the MyoD heavy chain. Actin-precipitation studies showed that MlcD remained attached to MyoD in the presence and absence of bivalent cations.

All known myosin light chains belong to the calmodulin, troponin C, myosin II ELC and RLC (CTER) subfamily of four EF-hand proteins. CTER subfamily members are congruent proteins that diverged from a single precursor of full length without subsequent domain shuffling [53]. A search of data compiled by the *Dictyostelium* cDNA project in Japan (<http://www.csm.biol.tsukuba.ac.jp/cDNAproject.html>) and the near-complete *Dictyostelium* genome project (<http://db.dictybase.org>) reveals 19 small (< 25 kDa) four EF-hand proteins, only five of which (MlcD, calmodulin, CalB, and the myosin II RLC and ELC) group

with the CTER subfamily (Figure 2). Unless several additional members of the *Dictyostelium* CTER subfamily remain to be discovered, each of the eleven *Dictyostelium* unconventional myosins cannot be associated with a specialized light chain. Based on this analysis, it might be predicted that calmodulin functions as a generic light chain for most *Dictyostelium* unconventional myosins, as it does for unconventional myosins in other organisms. It should be noted, though, that neither MyoB or MyoC were found to co-immunoprecipitate with calmodulin. Whether this result simply reflects a weak association between MyoB and MyoC and the calmodulin light chain or whether both myosins have specialized light chains requires further investigation. The prospect that MlcD functions as a light chain for other unconventional myosins in addition to MyoD must also be considered. A significant amount of FLAG-MlcD was not bound to MyoD, as judged by its elution in the flow-through from the phosphocellulose column (Figure 3B), suggesting either that it exists on its own as a soluble protein or that it associates with other, as yet unidentified, proteins.

The motile activities of several mammalian myosin I isoenzymes associated with calmodulin are modulated by physiological levels of Ca^{2+} [24–27]. In some instances, elevated levels of Ca^{2+} cause calmodulin to dissociate from the heavy chain, resulting in the complete loss of motor activity [24–26]. MyoD, through its association with MlcD, may be specialized to operate under conditions where Ca^{2+} levels are elevated and where myosins I with calmodulin light chains may be inactivated. Gene disruption studies have provided convincing evidence that MyoB, MyoC and MyoD have distinct roles in processes such as pseudopod formation, phagocytosis, motility and chemotactic streaming [13,14,16,18]. The cellular locations of the long-tailed myosin I isoenzymes also differ. In stationary-stage cells, MyoB and MyoD concentrate at the leading edge of lamellipodia and at sites of cell–cell contact, but in starved, aggregated cells, MyoB remains at these sites while MyoD relocates to the cytoplasm [54]. Furthermore, MyoB, but not MyoC or MyoD, is recruited to the plasma membrane of highly motile, streaming cells [55]. The results presented here suggest that some of the functional differences between MyoB, MyoC and MyoD are likely to depend not only on differences between the heavy chains, but also on the nature of the associated light chain.

MlcD exhibits a high degree of sequence similarity to MICLC, the light chain bound to *Acanthamoeba* myosin IC (Figure 2) [45]. Although the Ca^{2+} -binding properties of MICLC have not been characterized, examination of the MICLC sequence indicates that, similar to MlcD, EF-hands 2–4 may be non-functional (Figure 1B). Early studies showed that the actin-activated MgATPase activity of *Acanthamoeba* myosins IA and IB are not Ca^{2+} -sensitive [56], but the possibility that Ca^{2+} might regulate myosin I has not been examined in detail [45]. The heavy chains of MyoD and myosin IC are also closely related, supporting the view that these two myosins are functional counterparts that evolved from a common ancestor [34]. Based on the similarity of both the heavy and light chains, it might be predicted that MyoD and *Acanthamoeba* myosin IC would share a conserved function in amoeboid motility, but this does not seem to be the case. Myosin IC is the only one of the three known *Acanthamoeba* myosin I isoforms to concentrate around the contractile vacuole complex [57,58]. Antibodies that inhibit the activity of myosin IC interfere with the expulsion of water by the contractile vacuole and lead to cell lysis when the cells are exposed to a hypotonic stress [59]. In contrast, MyoD is not present on the contractile vacuole and MyoD-null cells are not hypersensitive to hypotonic media [16]. Additional studies are required to reconcile the discrepancy between the conserved structures of MyoD

and *Acanthamoeba* myosin IC and their apparently divergent functions.

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REFERENCES

- Soldati, T., Geissler, H. and Schwarz, E. C. (1999) How many is enough? Exploring the myosin repertoire in the model eukaryote *Dictyostelium discoideum*. *Cell. Biochem. Biophys.* **30**, 389–411
- De La Roche, M. A. and Côté, G. P. (2001) Regulation of *Dictyostelium* myosin I and II. *Biochim. Biophys. Acta* **1525**, 245–261
- Trybus, K. M. (1994) Role of myosin light chains. *J. Muscle. Res. Cell Motil.* **15**, 587–594
- Uyeda, T. Q. P., Abramson, P. D. and Spudich, J. A. (1996) The neck region of the myosin motor domain acts as a lever arm to generate movement. *Proc. Natl. Acad. Sci. U.S.A.* **93**, 4459–4464
- Tafari, S. R., Rushforth, A. M., Kuczmarski, E. R. and Chisholm, R. L. (1989) *Dictyostelium discoideum* myosin: isolation and characterization of cDNAs encoding the regulatory light chain. *Mol. Cell. Biol.* **9**, 3073–3080
- Chisholm, R. L., Rushforth, R. S., Pollenz, R. S., Kuczmarski, E. R. and Tafari, S. R. (1988) *Dictyostelium discoideum* myosin: isolation and characterization of cDNAs encoding the essential light chain. *Mol. Cell. Biol.* **8**, 794–810
- Barylko, B., Binns, D. D. and Albanesi, J. P. (2000) Regulation of the enzymatic and motor activities of myosin I. *Biochim. Biophys. Acta* **1496**, 23–35
- Osherov, N. and May, G. S. (2000) *In vivo* function of class I myosins. *Cell Motil. Cytoskeleton* **47**, 163–173
- Schwarz, E. C., Neuhaus, E. M., Kistler, C., Henkel, A. W. and Soldati, T. (2000) *Dictyostelium* myosin IK is involved in the maintenance of cortical tension and affects motility and phagocytosis. *J. Cell Sci.* **113**, 621–633
- Zot, H. G., Doberstein, S. K. and Pollard, T. D. (1992) Myosin I moves actin filaments on a phospholipid substrate: implications for membrane targeting. *J. Cell Biol.* **116**, 367–376
- Fujisaki, H., Albanesi, J. P. and Korn, E. D. (1985) Experimental evidence for the contractile activities of *Acanthamoeba* myosin IA and IB. *J. Biol. Chem.* **260**, 11183–11189
- Jung, G., Rimmert, K., Wu, X., Volosky, J. M. and Hammer, III, J. A. (2001) The *Dictyostelium* CARMIL protein links capping protein and the Arp2/3 complex to type I myosins through their SH3 domains. *J. Cell Biol.* **153**, 1479–1498
- Jung, G. and Hammer, III, J. A. (1990) Generation and characterization of *Dictyostelium* cells deficient in a myosin I heavy chain isoforms. *J. Cell Biol.* **110**, 1955–1964
- Wessels, D., Murray, J., Jung, G., Hammer, III, J. A. and Soll, D. R. (1991) Myosin IB null mutants of *Dictyostelium* exhibit abnormalities in motility. *Cell Motil. Cytoskeleton* **20**, 301–315
- Titus, M. A., Wessels, D., Spudich, J. A. and Soll, D. (1993) The unconventional myosin encoded by the *myoA* gene plays a role in *Dictyostelium* motility. *Mol. Biol. Cell* **4**, 233–246
- Jung, G., Wu, X. F. and Hammer, III, J. A. (1996) *Dictyostelium* mutants lacking multiple classic myosin I isoforms reveal combinations of shared and distinct functions. *J. Cell Biol.* **133**, 305–323
- Novak, K. D., Peterson, M. D., Reedy, M. C. and Titus, M. A. (1995) *Dictyostelium* myosin I double mutants exhibit conditional defects in pinocytosis. *J. Cell Biol.* **131**, 1205–1221
- Jung, G., Fukui, Y., Martin, B. and Hammer, III, J. A. (1993) Sequence, expression pattern, intracellular localization and targeted disruption of the *Dictyostelium* myosin ID heavy chain isoforms. *J. Biol. Chem.* **268**, 14981–14990
- Bement, W. M. and Mooseker, M. S. (1995) TEDS rule: a molecular rationale for differential regulation of myosins by phosphorylation of the heavy chain head. *Cell Motil. Cytoskeleton* **31**, 87–92
- Brzeska, H. and Korn, E. D. (1996) Regulation of class I and class II myosins by heavy chain phosphorylation. *J. Biol. Chem.* **271**, 16983–16986
- Wu, C., Lee, S.-F., Furmaniak-Kazmierczak, E., Côté, G. P., Thomas, D. Y. and Leberer, E. (1996) Activation of myosin-I by members of the Ste20p protein kinase family. *J. Biol. Chem.* **271**, 31787–31790
- Lee, S.-F., Egelhoff, T. T., Mahasneh, A. and Côté, G. P. (1996) Cloning and characterization of a *Dictyostelium* myosin I heavy chain kinase activated by Cdc42 and Rac. *J. Biol. Chem.* **271**, 27044–27048
- Brzeska, H., Young, R., Knaus, U. and Korn, E. D. (1999) Myosin I heavy chain kinase: cloning of the full-length gene and acidic lipid-dependent activation by Rac and cdc42. *Proc. Natl. Acad. Sci. U.S.A.* **96**, 394–399

- 24 Williams, R. and Coluccio, L. M. (1994) Novel 130-kDa rat liver myosin-1 will translocate actin filaments. *Cell Motil. Cytoskeleton* **27**, 41–48
- 25 Wolenski, J. S., Hayden, S. M., Forscher, P. and Mooseker, M. S. (1993) Calcium-calmodulin and regulation of brush border myosin-I MgATPase and mechanotransduction. *J. Cell Biol.* **122**, 613–621
- 26 Zhu, T., Sata, M. and Ikebe, M. (1996) Functional expression of mammalian myosin I β : analysis of its motor activity. *Biochemistry* **35**, 513–522
- 27 Stoffer, H. E. and Bahler, M. (1998) The ATPase activity of Myr3, a rat myosin I, is allosterically inhibited by its own tail domain and by Ca²⁺ binding to its light chain calmodulin. *J. Biol. Chem.* **273**, 14605–14611
- 28 Lee, S.-F. and Côté, G. P. (1993) Isolation and characterization of three *Dictyostelium* myosin-I isozymes. *J. Biol. Chem.* **268**, 20923–20929
- 29 Aebersold, R. (1989) Internal amino acid sequence analysis of proteins after *in situ* protease digestion on nitrocellulose. In *A Practical Guide to Protein and Peptide Purification for Microsequencing* (Matsudaira, P., ed.), pp. 71–88, Academic Press, San Diego
- 30 Nellen, W., Datta, S., Raymond, C., Sivertsen, A., Mann, S., Crowley, T. and Firtel, R. A. (1987) Molecular biology in *Dictyostelium*: tools and applications. *Methods Cell Biol.* **28**, 67–100
- 31 Pearlstone, J. R., Chandra, M., Sorenson, M. M. and Smillie, L. B. (2000) Biological function and site II Ca²⁺-induced opening of the regulatory domain of skeletal troponin C are impaired by invariant site I or II Glu mutations. *J. Biol. Chem.* **275**, 35106–35115
- 32 Lee, S.-F. and Côté, G. P. (1995) Purification and characterization of a *Dictyostelium* protein kinase required for the actin-activated MgATPase activity of *Dictyostelium* myosin I. *J. Biol. Chem.* **270**, 11776–11782
- 33 Levi, S., Polyakov, M. and Egelhoff, T. T. (2000) Green fluorescent protein and epitope tag fusion vectors for *Dictyostelium discoideum*. *Plasmid* **44**, 231–238
- 34 Lee, W. L., Ostap, E. M., Zot, H. G. and Pollard, T. D. (1999) Organization and ligand binding properties of the tail of *Acanthamoeba myosin*-IA. Identification of an actin-binding site in the basic (tail homology-1) domain. *J. Biol. Chem.* **274**, 35159–35171
- 35 Nelson, M. R. and Chazin, W. J. (1998) Structures of EF-hand Ca²⁺-binding proteins: diversity in the organization, packing and response to Ca²⁺ binding. *Biometals* **11**, 297–318
- 36 Marsden, B. J., Shaw, G. S. and Sykes, B. D. (1990) Calcium binding proteins: elucidating the contributions to calcium affinity from an analysis of species variants and peptide fragments. *Biochem. Cell Biol.* **68**, 587–601
- 37 Doherty-Kirby, A. L. and Lajoie, G. A. (2002) Investigation of calcium-binding proteins using electrospray ionization mass spectrometry. *Methods Mol. Biol.* **173**, 161–174
- 38 Pearlstone, J. R., Borgford, T., Chandra, M., Oikawa, K., Kay, C. M., Herzberg, O., Moul, J., Herklotz, A., Reinach, F. C. and Smillie, L. B. (1992) Construction and characterization of a spectral probe mutant of troponin C: application to analyses of mutants with increased Ca²⁺ affinity. *Biochemistry* **31**, 6545–6553
- 39 Chin, D. and Means, A. R. (2000) Calmodulin: a prototypical calcium sensor. *Trends Cell Biol.* **10**, 322–328
- 40 Bahler, M. and Rhoads, A. (2002) Calmodulin signaling via the IQ motif. *FEBS Lett.* **513**, 107–113
- 41 Rogers, M. S. and Strehler, E. E. (2001) The tumor-sensitive calmodulin-like protein is a specific light chain of human unconventional myosin X. *J. Biol. Chem.* **276**, 12182–12189
- 42 Herm-Gotz, A., Weiss, S., Stratmann, R., Fujita-Becker, S., Ruff, C., Meyhofer, E., Soldati, T., Manstein, D. J., Geeves, M. A. and Soldati, D. (2002) *Toxoplasma gondii* myosin A and its light chain: a fast, single-headed, plus-end-directed motor. *EMBO J.* **21**, 2149–2158
- 43 Stevens, R. C. and Davis, T. N. (1998) Mlc1p is a light chain for the unconventional myosin Myo2p in *Saccharomyces cerevisiae*. *J. Cell Biol.* **142**, 711–722
- 44 Espindola, F. S., Suter, D. M., Partata, L. B., Cao, T., Wolenski, J. S., Cheney, R. E., King, S. M. and Mooseker, M. S. (2000) The light chain composition of chicken brain myosin-Va: calmodulin, myosin-II essential light chains, and 8-kDa dynein light chain/PIN. *Cell Motil. Cytoskeleton* **47**, 269–281
- 45 Wang, Z. Y., Sakai, J., Matsudaira, P. T., Baines, I. C., Sellers, J. R., Hammer, III, J. A. and Korn, E. D. (1997) Amino acid sequence of the light chain of *Acanthamoeba* myosin IC. *J. Muscle Res. Cell Motil.* **18**, 395–398
- 46 Lafitte, D., Capony, J. P., Grassy, G., Haiech, J. and Calas, B. (1995) Analysis of the ion binding sites of calmodulin by electrospray ionization mass spectrometry. *Biochemistry* **34**, 13825–13832
- 47 Yumura, S., Furuya, K. and Takeuchi, I. (1996) Intracellular free calcium responses during chemotaxis of *Dictyostelium* cells. *J. Cell Sci.* **109**, 2673–2678
- 48 Bagshaw, C. R. and Kendrick-Jones, J. (1980) Identification of the divalent metal ion binding domain of myosin regulatory light chains using spin-labelling techniques. *J. Mol. Biol.* **140**, 411–433
- 49 Houdusse, A. and Cohen, C. (1996) Structure of the regulatory domain of scallop myosin at 2 Å resolution: implications for regulation. *Structure* **4**, 21–32
- 50 Blumenschein, T. M. and Reinach, F. C. (2000) Analysis of affinity and specificity in an EF-hand site using double mutant cycles. *Biochemistry* **39**, 3603–3610
- 51 Alexis, M. N. and Gratzel, W. B. (1978) Interaction of skeletal myosin light chains with calcium ions. *Biochemistry* **17**, 2319–2325
- 52 Bagshaw, C. R. and Kendrick-Jones, J. (1979) Characterization of homologous divalent metal ion binding sites of vertebrate and molluscan myosins using electron paramagnetic resonance spectroscopy. *J. Mol. Biol.* **130**, 317–336
- 53 Nakayama, S., Moncrief, N. D. and Kretsinger, R. H. (1992) Evolution of EF-hand calcium-modulated proteins. II. Domains of several subfamilies have diverse evolutionary histories. *J. Mol. Evol.* **34**, 416–448
- 54 Morita, Y. S., Jung, G., Hammer, III, J. A. and Fukui, Y. (1996) Localization of *Dictyostelium* myoB and myoD to filopodia and cell-cell contact sites using isoform-specific antibodies. *Eur. J. Cell Biol.* **71**, 371–379
- 55 Senda, S., Lee, S. F., Côté, G. P. and Titus, M. A. (2001) Recruitment of a specific amoeboid myosin I isoform to the plasma membrane in chemotactic *Dictyostelium* cells. *J. Biol. Chem.* **276**, 2898–2904
- 56 Maruta, H., Gadas, H., Collins, J. H. and Korn, E. D. (1979) Multiple forms of *Acanthamoeba* myosin I. *J. Biol. Chem.* **254**, 3624–3630
- 57 Baines, I. C., Brzeska, H. and Korn, E. D. (1992) Differential localization of *Acanthamoeba* myosin I isoforms. *J. Cell Biol.* **119**, 1193–1203
- 58 Kong, H. H. and Pollard, T. D. (2002) Intracellular localization and dynamics of myosin-II and myosin-IC in live *Acanthamoeba* by transient transfection of EGFP fusion proteins. *J. Cell Sci.* **115**, 4993–5002
- 59 Doberstein, S. K., Baines, I. C., Wiegand, G., Korn, E. D. and Pollard, T. D. (1993) Inhibition of contractile vacuole function *in vivo* by antibodies against myosin I. *Nature (London)* **365**, 841–843

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