Expression in *Escherichia coli* of a Functional *Dictyostelium* Myosin Tail Fragment

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Abstract. The amino acid sequence of the myosin tail determines the specific manner in which myosin molecules are packed into the myosin filament, but the details of the molecular interactions are not known. Expression of genetically engineered myosin tail fragments would enable a study of the sequences important for myosin filament formation and its regulation. We report here the expression in *Escherichia coli* of a 1.5-kb fragment of the *Dictyostelium* myosin heavy chain gene coding for a 58-kD fragment of the myosin tail. The expressed protein (DdLMM-58) was purified to homogeneity from the soluble fraction of *E. coli* extracts. The expressed protein was found to be functional by the following criteria: (a) it appears in the electron microscope as a 74-nm-long rod, the predicted length for an α-helical coiled coil of 500 amino acids; (b) it assembles into filamentous structures that show the typical axial periodicity of 14 nm found in muscle myosin native filaments; (c) its assembly into filaments shows the same ionic strength dependence as *Dictyostelium* myosin; (d) it serves as a substrate for the Dictyostelium myosin heavy chain kinase which phosphorylates myosin in response to chemoattractant signaling; (e) in its phosphorylated form it has the same phoshoamino acids and similar phosphopeptide maps to those of phosphorylated *Dictyostelium* myosin heavy chain; (f) it competes with myosin for the heavy chain kinase. Thus, all the information required for filament formation and phosphorylation is contained within this expressed protein.

Myosin, a motive force-producing protein found in eukaryotic cells, is thought to be involved in many cellular functions including chemotaxis, cell division, and cytoplasmic streaming (Warrick and Spudich, 1987). Myosin purified from most cell types consists of two heavy chains (∼200 kD) and two pairs of light chains (∼20 kD). The amino-terminal half of each heavy chain folds together with a pair of light chains into a globular head and the carboxyl-terminal halves twist around each other to form an α-helical coiled-coil tail. Myosin assembles into thick filaments both in muscle and nonmuscle cells.

In striated muscle cells, myosin thick filaments are stable structures that are arranged in a semicrystalline array with actin filaments. The individual myosin molecules in a thick filament are packed with a stagger of 14 nm (Huxley et al., 1983). This packing is dictated by the nature of the sequence of the myosin tail (McLachlan, 1984). All myosin tail sequences analyzed display three kinds of repeating features (Warrick and Spudich, 1987). A seven-residue repeat in which the first and fourth amino acids have small hydrophobic side chains is involved in the formation of an α-helical coiled coil. A 28-residue repeat in which positive and negative amino acids have an alternating pattern is thought to be necessary for the electrostatic interaction of myosin tails within the myosin filament. Finally a 196-residue repeat has been revealed by Fourier analysis and is thought to consist of modulations of the charge distribution set by the 28-residue repeat (McLachlan and Karn, 1983). It has been suggested that the 196-residue repeat determines the stagger of 14 nm which myosin molecules have in thick filaments. Despite all these analyses there is no precise model of how the tail sequence determines the structure of the thick filaments.

In nonmuscle cells the myosin filaments are thought to be transient structures that are assembled and disassembled in different parts of the cell in response to different signals. Therefore myosin filament assembly in these cells must be a finely regulated process. Nonmuscle cells seem to employ at least two mechanisms to regulate filament assembly. Assembly can be regulated by phosphorylation of the light chain, as for thymus myosin (Smith et al., 1983), or by phosphorylation of the heavy chain, as for myeloblasts (Sagara et al., 1983), *Acanthamoeba* (Collins and Korn, 1981) and *Dictyostelium* myosin (Kuczmaszki and Spudich, 1980). In *Dictyostelium* the myosin heavy chain and 18-kD light chain are rapidly phosphorylated in vivo in response to the chemoattractant cAMP. The myosin phosphorylation kinetics are the same as those observed for cAMP-induced cell shape changes (Berlot et al., 1985). This chemotactic response leads to the aggregation of thousands of cells and their development into a fruiting body (Tomchik and Devreotes, 1981).

The mechanism of myosin assembly into thick filaments...
and its regulation could be studied in detail if one were able to produce homogeneous preparations of different fragments from the myosin tail and analyze their assembly properties. Previously, protease cleavage has been used as a tool to produce myosin fragments. In this way it was shown that the filament-forming capacity is located in the carboxyl-terminal region of the α-helical coiled-coil tail called light meromyosin (LMM) (Lowey et al., 1969). However, this approach is limited to the proteolytic sites that occur naturally in the molecule. In fact, preparation of an LMM-like fragment from nonmuscle myosins has not been reported. A major limitation has been the inability to obtain homogeneous populations of proteolytic subfragments of LMM, in order to further localize the critical sites involved in assembly. In addition, muscle myosin preparations contain the products of several genes expressed in the same tissues (Gauthier and Lowey, 1977), thus making it impossible to obtain truly homogeneous preparations of proteolytic tail fragments. Expression in *Escherichia coli* of different myosin heavy chain gene fragments would not have these limitations. In addition, site-directed mutagenesis of the expressed proteins could be applied to examine the relationships between sequence and function of the myosin tail.

To understand further the structure–function relationships for *Dictyostelium* myosin, we isolated and sequenced the entire *Dictyostelium* myosin heavy chain gene (*mhcA* gene) (De Lozanne et al., 1987; Warrick et al., 1986). Comparison between the *Dictyostelium* and muscle myosin tail sequences revealed that the 7-, 28-, and 196-amino acid repeats described above are also present in *Dictyostelium* myosin. Gene disruption experiments have been utilized to create *Dictyostelium* cells in which the native myosin molecule has been replaced by a myosin fragment that lacks the LMM portion (De Lozanne and Spudich, 1987). These cells have a block in cytokinesis and in development indicating an essential role of the LMM portion for these cell functions.

We report here the expression in *E. coli* of a fragment of the *Dictyostelium* *mhcA* gene. The expressed protein, DdLMM-58, contains only a portion of the LMM region of the tail, has been purified to homogeneity, is capable of assembly, and is recognized by the *Dictyostelium* myosin heavy chain kinase which phosphorylates myosin in response to chemotactic signaling.

**Materials and Methods**

**DdLMM-58 Expression and Purification**

The 1.5-kb EcoRI fragment from the tail portion of the *Dictyostelium* *mhcA* gene (Fig. 1) was subcloned into the EcoRI site of the plasmid pIN-1-A2 (Masui et al., 1983). This fragment containing a portion of the LMM region was introduced into *E. coli* LE392. Cells were grown in 6 liters of LB media to an OD~600 of 1.0, harvested, and washed in 100 mM KCl, 10 mM Tris (pH 7.5) and centrifuged at 100,000 g for 60 min. The supernatant, which contained the DdLMM-58 protein and nucleic acids, was dialyzed into DEAE column buffer (10 mM Tris (pH 7.5), 1 mM EDTA, 200 mM KCl). A 20 ml DEAE column was equilibrated with column buffer and then the dialysate was loaded onto the column. Under these conditions the protein flowed through the column and the nucleic acids remained bound. The flow-through was passed through the column twice to assure complete removal of the nucleic acids. The flow-through was then dialyzed into low salt buffer and centrifuged as before. The pellet was resuspended in 1 ml of high-salt buffer and centrifuged again as before. The supernatant was then used in all experiments. The concentration of DdLMM-58 was determined by densitometry of Coomassie-stained gels using rabbit muscle LMM as a standard (OD~580 at 280 nm = 3.0).

**Electron Microscopy**

Rotary shadowing of DdLMM-58 molecules in 70% glycerol and 0.3 M ammonium acetate was as described (Flicker et al., 1985). For negative-stain microscopy, samples were applied to carbon-coated formvar grids for 30 s followed by negative staining with 1% aqueous uranyl acetate. Grids of rotary-shadowed samples and negatively stained samples were examined with a Philips 201 electron microscope at ×30,000. The magnification was calibrated using negatively stained troponymosin paracrystals which have a repeat of 395 Å (Flicker et al., 1985).

**Phosphorylation Assay**

DdLMM-58 (1–20 µg) was added to 200 µl of ice-cold lysis buffer (0.2% Triton X-100, 2 mM MgCl₂, 7.5 mM Tris (pH 7.5)) to which 100-µl aliquots of developed cell suspension (2 × 10⁷ cells/ml) were then added. Triton-insoluble fractions were then prepared and phosphorylated as described (Berlot et al., 1985). Subsequent to phosphorylation, myosin and DdLMM-58 were immunoprecipitated with a polyclonal myosin antibody and subjected to SDS-polyacrylamide gel electrophoresis as described (Berlot et al., 1985). Phosphorylation rates were determined by measuring the amount of 3²P incorporated into known amounts of myosin or DdLMM-58 during linear 2-min reactions on ice. These values were compared with the specific radioactivity of the ATP in the reaction mix. The amounts of phosphorylation were determined by measuring Cerenkov radiation of the myosin or DdLMM-58 bands cut out of the polyacrylamide gels, and the amounts of myosin or DdLMM-58 in the reactions were determined by densitometry using a standard curve of known amounts of myosin or DdLMM-58 loaded onto the same gel.

**Phosphoamino Acid Analysis**

Phosphoamino acid generation and analysis were performed as described (Berlot et al., 1987).

**Phosphopeptide Mapping**

Phosphopeptide mapping was performed as described (Berlot et al., 1987) except that DdLMM-58 (4.5 µg) and myosin heavy chain (0.66 µg) were digested with both trypsin and chymotrypsin (50 µg of each added three times at 12-h intervals).

**Results**

**Expression in *E. coli* of a Myosin Fragment**

A 1.5-kb EcoRI fragment of the *Dictyostelium* *mhcA* gene (Fig. 1) was selected for expression in *E. coli*. This fragment
codes for 58 kD from the tail portion of *Dictyostelium* myosin from residues 1533–2034 of the protein sequence (Warrick et al., 1986). The fragment was cloned into the expression vector pIN-I-A2 (Masui et al., 1983) and the construction was named pDdLMM-58. This vector uses the strong lipoprotein promoter to express a fusion protein of the inserted sequence with 4 amino acids at the amino terminus and 40 amino acids at the carboxyl terminus. The first 4 amino acids derive from the polylinker sequence and the last 40 amino acids derive from the lipoprotein sequence. The fusion protein was named DdLMM-58 and its expected size is 64 kD.

The plasmid pDdLMM-58 was introduced into *E. coli* LE392 cells. The transformed cells expressed DdLMM-58 in a constitutive manner, and its expression did not affect the growth rate of the bacteria. The level of DdLMM-58 expression in late-log cell cultures was determined to be ~0.1 mg of protein per gram of wet cell pellet (data not shown).

**Figure 2.** Purification of the *E. coli* expressed DdLMM-58. (A) Coomassie Blue-stained polyacrylamide gel of DdLMM-58 at different stages of purification (see Materials and Methods): lane 1, crude cell lysate; lane 2, high-speed supernatant of the lysate; lane 3, precipitate formed during dialysis; lane 4, soluble fraction after ethanol precipitation; lane 5, DEAE flow-through; lane 6, supernatant of the dialysate after DEAE chromatography; lane 7, pellet of the dialysate after DEAE chromatography. (B) Western blot stained with a polyclonal antibody directed against *Dictyostelium* myosin: lanes same as in A. *M*<sub>r</sub> standards indicated in kilodaltons, k.

**Purification of DdLMM-58**

A purification protocol for DdLMM-58 was designed based on the properties of self-assembly and resistance of an α-helical coiled coil to ethanol denaturation (see Materials and Methods). DdLMM-58 was found to be in the soluble fraction of the *E. coli* extract (Fig. 2 A, lane 2). This fraction was diazylized into a buffer that permits assembly of DdLMM-58 into filamentous structures which were collected by sedimentation (Fig. 2 A, lane 3). The major purification step consisted in the ethanol denaturation of the *E. coli* proteins. In the presence of ethanol and high salt all proteins were precipitated, including DdLMM-58, however, only DdLMM-58 and a few contaminants were resolubilized from that precipitate (Fig. 2 A, lane 4). This fraction also contained large amounts of nucleic acids which were removed by DEAE chromatography. The final product was 98% pure as estimated by scanning gel densitometry (Fig. 2 A, lane 7).

**Assembly of DdLMM-58**

The assembly of *Dictyostelium* myosin into filamentous structures is dependent upon ionic strength (Kuczmarski and Spudich, 1980). To ascertain if the *E. coli* expressed DdLMM-58 fragment has the same properties, it was diazylized into buffers with different salt concentrations. The samples were centrifuged to separate the soluble and precipitated fractions and their relative amounts were determined by densitometry.

**Figure 3.** Assembly of DdLMM-58. Purified DdLMM-58 at 0.2 mg/ml in 10 mM Tris, pH 7.5, 1 mM EDTA, 0.6 M KCl was diazylized into 10 mM Tris, pH 7.5, 0.1 mM EDTA, and various amounts of KCl. The samples were centrifuged at 100,000 g for 30 min and equivalent amounts of supernatants and pellets were subjected to electrophoresis on a 7.5% polyacrylamide gel. Proteins in the gel were stained with Coomassie Blue and their relative amounts were determined by scanning densitometry. (●) DdLMM-58; (▲) phosphatased myosin (taken from Kuczmarski and Spudich, 1980).
electron microscopy of DdLMM-58. Collection of six representative DdLMM-58 molecules compared with a molecule of Dictyostelium myosin shadowed at the same time. Bar, 50 nm.

Electron Microscopy of DdLMM-58

The purified DdLMM-58 molecules were visualized by rotary shadow electron microscopy under conditions in which they do not assemble into macromolecular structures. As can be seen in Fig. 4, DdLMM-58 has a rod shape with an average length of 74 nm (±7 nm, n = 51). Assuming an α-helical rise of 1.485 Å per residue (McLachlan, 1984), the predicted length for a coiled coil 501 amino acids long (the size of DdLMM-58) would be 74 nm.

In low ionic strength, muscle myosin LMM forms large paracrystals or tactoids that can be visualized by negative-stain electron microscopy. These paracrystals show a marked transverse periodicity of 14 nm that originates from the packing of the LMM molecules in the paracrystal (Chowrashi and Pepe, 1977) (Fig. 5 A). This repeat is identical to that of muscle myosin molecules in native thick filaments (Huxley et al., 1983). Expressed DdLMM-58 filamentous structures stained with uranyl acetate and visualized in the electron microscope also displayed a transverse periodicity of ~14 nm (Fig. 5 B).

DdLMM-58 Is Phosphorylated by a Dictyostelium Myosin Heavy Chain Kinase

Previous experiments demonstrated that cAMP stimulation of intact Dictyostelium amebas results in transient increases in the amounts of phosphorylation of the myosin heavy chain and 18-kD light chain in vivo and increases in the corresponding phosphorylation rates as measured in vitro (Berlot et al., 1985). The heavy chain kinase responsible for the heavy chain phosphorylation increase was found to be in a Triton X-100-insoluble fraction of ameba extracts (Berlot et al., 1987).

The E. coli expressed DdLMM-58 was used in an in vitro phosphorylation assay to determine if it is a substrate for the Triton-insoluble kinase. Developed amebas were lysed into a Triton reaction mixture containing DdLMM-58 and the Triton-insoluble fraction was labeled with [γ-32P]ATP (see Figure 5.

Figure 4. Rotary-shadowing electron microscopy of DdLMM-58. Collection of six representative DdLMM-58 molecules compared with a molecule of Dictyostelium myosin shadowed at the same time. Bar, 50 nm.

Figure 5. Negatively stained paracrystals of DdLMM-58 and rabbit LMM. Rabbit muscle chymotryptic LMM was prepared as described (Margossian and Lowey, 1982). (A) Chymotryptic rabbit LMM and (B) DdLMM-58 paracrystals formed by dialysis into 10 mM Tris, pH 7.5, 5 mM MgCl2, 50 mM KCl were adsorbed to carbon-coated grids and stained with uranyl acetate. A tropomyosin paracrystal was used as a standard for the measurement of the axial repeats. Bar, 100 nm.
Figure 6. DdLMM-58 is phosphorylated by a Triton-insoluble kinase. Myosin (lanes 1 and 2) and DdLMM-58 (lane 2) were phosphorylated in vitro and then immunoprecipitated with a polyclonal antibody to myosin (see Materials and Methods). (A) Coomassie-stained SDS-polyacrylamide gel (10%). (B) Autoradiograph of A.

Materials and Methods). The endogenous myosin and the added DdLMM-58 fragment were then immunoprecipitated from the assay mixture using a polyclonal anti-myosin IgG and subjected to electrophoresis on SDS-polyacrylamide gels. Fig. 6 shows the Coomassie-stained gel (A) and autoradiograph (B) of the proteins immunoprecipitated from the phosphorylation mixture. The control mixture (lane 1) shows that the only 32P-labeled immunoprecipitated Dictyostelium protein comigrated with the myosin heavy chain. When DdLMM-58 was present in the assay (lane 2) it was also phosphorylated and immunoprecipitated.

Phosphoamino Acid and Phosphopeptide Analysis of DdLMM-58

DdLMM-58 was phosphorylated on both threonine and serine by the insoluble kinase (Fig. 7). As was previously shown for the myosin heavy chain phosphorylated in vitro, there was relatively more phosphothreonine than phosphoserine (Berlot et al., 1987). Phosphothreonine accounted for 95% of the phosphorylated residues of DdLMM-58. In a phosphoamino acid map of myosin heavy chain which was phosphorylated and analyzed in parallel with DdLMM-58 (data not shown), phosphothreonine accounted for 98% of the phosphorylated residues.

We compared the specificity of the sites phosphorylated on myosin heavy chain and DdLMM-58 by analyzing the phosphopeptides produced after digestion with an excess of trypsin and chymotrypsin. The peptide maps from both proteins shared seven spots (Fig. 8, A and B) although the relative intensities of some spots varied. In the peptide map of myosin (Fig. 8 A) spots 1, 2, 3, 4, 5, and 6 predominated. In the map of DdLMM-58 (Fig. 8 B) the most intense spots were 2, 5, 6, and 7. The intensities of spots 1, 3, and 4 were reduced and that of 7 was increased compared with the phosphopeptide map of the myosin heavy chain. There were also a few minor spots in the DdLMM-58 map that were not seen in the myosin map. A phosphopeptide map prepared from a mixture of the digests in Fig. 8, A and B (Fig. 8 C) confirmed that spots 1-7 in the myosin heavy chain map comigrate with spots 1-7 in the DdLMM-58 map.

The presence of multiple spots in the above peptide maps does not necessarily indicate that there are multiple sites of phosphorylation. It is possible that the peptides overlap or represent different oxidation states of the same peptide. In this regard, the maximum amount of phosphorylation obtained on both myosin heavy chain and DdLMM-58 was <1 mol of phosphate/mol of protein. The level of phosphorylation was determined (Berlot et al., 1985) by measuring the incorporation of [32P]phosphate into myosin and calculating the moles of phosphate incorporated using the known specific radioactivity of the [32P]ATP. In these experiments myosin incorporated 0.6 mol of phosphate/mol of heavy chain whereas 0.015 mol of phosphate/mol of DdLMM-58 was incorporated.

DdLMM-58 Competes with Myosin Heavy Chain for the Insoluble Kinase

Because the specificity of the insoluble kinase(s) phosphorylating DdLMM-58 and myosin heavy chain appeared to be...
very similar, we investigated whether DdLMM-58 could inhibit phosphorylation of myosin heavy chain by serving as an alternate substrate for the myosin heavy chain kinase. We found that DdLMM-58 reduced the myosin heavy chain phosphorylation rate (0.3 mol phosphate/mol of heavy chain per min) to a minimum of 33 % of the uninhibited rate (Fig. 9). The amount of phosphate incorporated into DdLMM-58 was directly proportional to the amount of DdLMM-58 incubated in the assay at all concentrations tested and was equal to 0.008 mol phosphate/mol DdLMM-58 per min. Half of the maximal inhibition was attained at a molar ratio of DdLMM-58 to myosin heavy chain of ~1/5. At this molar ratio rabbit skeletal muscle LMM, which was not phosphorylated in the assay, inhibited Dictyostelium myosin heavy chain phosphorylation by only 7% (data not shown).

Discussion

Previous studies on the mechanism of assembly of myosin into thick filaments have analyzed the sedimentation behavior of proteolytic fragments from rabbit muscle myosin (Nyitray et al., 1983) and Dictyostelium myosin (Pagh et al., 1984). The conclusion from both studies is that there is a small region in the LMM portion that is responsible for assembly into a sedimentable form. However, these studies were done with a mixed population of proteolytic fragments and did not determine whether the sedimentable material is assembled into organized structures. In this report we describe the expression in E. coli of a homogeneous subfragment of the LMM portion of the Dictyostelium myosin tail. Analysis of its appearance in the electron microscope, its assembly properties, and its pattern of phosphorylation by a Dictyostelium myosin heavy chain kinase demonstrates that the expressed protein is functional and contains all the sequence information necessary for filament formation and phosphorylation.

The DdLMM-58 filamentous structures show an axial repeat close to the 14-nm repeat typical of muscle myosin filaments and previously seen in Dictyostelium myosin filaments (Stewart and Spudich, 1979; Pagh and Gerisch, 1986). This implies that Dictyostelium myosin assemblies into filaments with the same basic structure as muscle myosins. Our results show that the structural information needed for the correct assembly into filaments with the 14-nm repeat is contained within this 58-kD fragment of the myosin tail. We have also shown that the assembly of this fragment has the same ionic strength dependence as the entire Dictyostelium myosin molecule, indicating that the interactions between the fragment molecules must be as strong as the ones within the myosin filament. We have not proven that the DdLMM-58 fragment assembles into an α-helical coiled-coil structure but it is likely that...
this is the case because the fragment appears in the electron microscope as a rod of the predicted length. Furthermore, this conformation is undoubtedly required for the assembly of the fragment into organized paracrystals and also for its recognition as a substrate by the myosin heavy chain kinase.

We have shown by analysis of phosphorylated amino acids and phosphopeptides that the DdLMM-58 fragment is phosphorylated in a specific manner by the myosin heavy chain kinase. The phosphopeptide maps obtained after digestion of DdLMM-58 and myosin heavy chain with an excess of trypsin and chymotrypsin are similar, but not identical. The differences between the two phosphopeptide maps could be due to the peptides that arise from the termini of DdLMM-58. These peptides would be expected to differ in size from the corresponding fragments produced from myosin heavy chain, which would be bounded on one side by a cleavage site present in DdLMM-58 and on the other side by a cleavage site outside of DdLMM-58. In addition, the presence of lipoprotein-derived amino acids in DdLMM-58 could alter the cleavage sites.

The ability of DdLMM-58 to inhibit phosphorylation of myosin heavy chain by the Triton-insoluble kinase demonstrates that the same kinase phosphorylates both proteins. One possible explanation for why the heavy chain phosphorylation rate is not completely inhibited by DdLMM-58 is that there is more than one heavy chain kinase and DdLMM-58 is only phosphorylated by one or a subset of them. Alternatively, it is possible that the myosin heavy chain contains other phosphorylation site(s) in addition to those shared by DdLMM-58. A third possibility is that the Triton-insoluble heavy chain kinase and myosin may be associated in vivo in a specific cytoskeletal structure which presents an accessibility barrier to added DdLMM-58. In favor of such an association is the finding that dilution of the heavy chain phosphorylation reaction does not affect the phosphorylation rate (Berlot et al., 1985). Also of note is the observation that cAMP stimulation of Dictyostelium amebas causes increases in the amount of Triton-insoluble myosin that correlate with increases in the myosin heavy chain phosphorylation rate (Berlot et al., 1987). A specific association of myosin heavy chain and its kinase may also explain why the rate of phosphorylation of myosin heavy chain is greater than that of DdLMM-58. A more complete characterization of myosin heavy chain and DdLMM-58 phosphorylation awaits purification of the myosin heavy chain kinase.

It is now possible to introduce mutations in the sequence of the expressed protein and to study their effects on assembly and phosphorylation. This system can also be used to express different fragments of the Dictyostelium myosin heavy chain tail and to study the effects of phosphorylation on their assembly properties. The study of these fragments should reveal important information about the mechanism of filament formation and its regulation by phosphorylation.

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