

# Expression of *Dictyostelium* Myosin Tail Segments in *Escherichia coli*: Domains Required for Assembly and Phosphorylation

Theresa J. O'Halloran, Shoshana Ravid, and James A. Spudich

Department of Cell Biology, Stanford University School of Medicine, Stanford, California 94305

**Abstract.** The assembly of myosins into filaments is a property common to all conventional myosins. The ability of myosins to form filaments is conferred by the tail of the large asymmetric molecule. We are studying cloned portions of the *Dictyostelium* myosin gene expressed in *Escherichia coli* to investigate functional properties of defined segments of the myosin tail. We have focused on five segments derived from the 68-kD carboxyl-terminus of the myosin tail. These have been expressed and purified to homogeneity from *E. coli*, and thus the boundaries of each segment within the myosin gene and protein sequence are known. We identified an internal 34-kD segment of the tail, N-LMM-34, which is required and sufficient for assembly. This 287-amino acid domain represents the smallest tail segment purified from any myosin that is capable of forming highly ordered paracrystals characteristic of myosin. Because the assembly of *Dictyostelium* myosin can be regulated by phosphorylation

of the heavy chain, we have studied the in vitro phosphorylation of the expressed tail segments. We have determined which segments are phosphorylated to a high level by a *Dictyostelium* myosin heavy chain kinase purified from developed cells. While LMM-68, the 68-kD carboxyl terminus of *Dictyostelium* myosin, or LMM-58, which lacks the 10-kD carboxyl terminus of LMM-68, are phosphorylated to the same extent as purified myosin, subdomains of these segments do not serve as efficient substrates for the kinase. Thus LMM-58 is one minimal substrate for efficient phosphorylation by the myosin heavy chain kinase purified from developed cells. Taken together these results identify two functional domains in *Dictyostelium* myosin: a 34-kD assembly domain bounded by amino acids 1533–1819 within the myosin sequence and a larger 58-kD phosphorylation domain bounded by amino acids 1533–2034 within the myosin sequence.

**D**ICTYOSTELIUM DISCOIDEUM is an ameboid microorganism capable of many kinds of movement, ranging from transport of intracellular organelles to translocation of the entire cell. Like other eukaryotes, *Dictyostelium* has an organized cytoskeleton containing, in part, motors thought to drive cellular motility. One cytoskeletal component is the protein myosin, which exists in at least two forms in *Dictyostelium*. A conventional form, designated myosin in this paper, contains a 240-kD heavy chain protein and light chains of 16 and 18 kD (Clarke and Spudich, 1974). An unconventional form of myosin, known as myosin I, contains a heavy chain of 116 kD (Côté et al., 1985). The conventional myosin in *Dictyostelium* shares structural homology with myosins purified from muscle tissues. At the amino terminus of the molecule, two polypeptide heavy chains each fold into globular head domains, each of which binds two light chains. The heavy chains emerge from the heads and associate with each other in an alpha-helical coiled-coil, forming an elongated tail domain.

T. J. O'Halloran's present address is the Department of Cell Biology and Neuroscience, University of Texas Southwestern Medical Center, Dallas, TX 75235.

Recent studies of *Dictyostelium* mutants highlight the importance of myosin filaments in a nonmuscle cell. *Hmm* cells have wild-type myosin replaced with a myosin unable to polymerize because it lacks the carboxyl terminus of the tail (De Lozanne and Spudich, 1987). Mutant *Dictyostelium* cells have also been created that are completely devoid of wild-type myosin (Manstein et al., 1989). Both mutants are unable to complete the developmental cycle and are also unable to undergo cytokinesis. The identical phenotypes of the two mutants demonstrates that the enzymatic head domain is not sufficient for myosin function in vivo; a tail capable of polymerization into filaments is also required.

Rod-shaped structures with the dimensions of purified myosin filaments have been visualized by immunofluorescence microscopy in *Dictyostelium* cells (Yumura and Fukui, 1985). These studies demonstrate the dynamic nature of myosin filaments. Myosin assembles in the posterior end of a cell undergoing chemotaxis and, at other times, in the cleavage furrow of a dividing cell. Such changes in myosin localization are correlated with phosphorylation of the heavy chain in vivo (Berlot et al., 1985; 1987; Nachmias et al., 1989). In vitro studies have suggested that these phosphory-

lations may regulate myosin assembly (Kuczmariski and Spudich, 1980; Côté and McCrea, 1987; Ravid and Spudich, 1989).

To understand the contributions from specific regions of the myosin molecule to myosin assembly and phosphorylation, we have focused on the carboxyl terminus of the molecule, the myosin tail. Here we report the functional properties of defined segments of the *Dictyostelium* myosin tail expressed and purified from *Escherichia coli*. This approach allows us to study purified tail segments with defined amino acid boundaries. Because the proteins are produced in *E. coli*, they have not been phosphorylated by endogenous *Dictyostelium* kinases, a condition that might alter their assembly and ability to be further phosphorylated. These studies identify an internal 34-kD domain with solubility and assembly characteristics of myosin filaments, and longer 58- and 68-kD segments that serve as substrates equivalent to myosin for a myosin heavy chain kinase.

## Materials and Methods

### Construction of Expression Vectors

The expression plasmid encoding LMM-58 has been described (De Lozanne et al., 1987). DNA fragments encoding other segments of the tail of *Dictyostelium* myosin were subcloned from pBgl 4.5, a plasmid containing the 3' end of the *Dictyostelium* myosin heavy chain gene (De Lozanne et al., 1988). For the construct expressing LMM-68, the 2.0-kb Eco RI-Bgl II fragment encoding the 68-kD carboxyl terminus of the *Dictyostelium* myosin gene (1.8-kb coding sequence and 0.2-kb 3' flanking sequence) was subcloned into the Eco RI-Bam HI sites of the plasmid pIN-I-A2, gift of Dr. M. Inouye (University of Medicine and Dentistry of New Jersey at Rutgers) (Masui et al., 1983). The resulting expression plasmid encoded the 584 amino acids from the carboxyl-terminal end of the *Dictyostelium* myosin gene (residues 1533–2116 of the protein sequence; Warrick et al., 1986) with 4 additional amino acids derived from vector sequence added to the amino terminus.

The expression vector for N-LMM-34 was constructed by subcloning the 0.9-kb Eco RI-Xho II fragment from pBgl 4.5 into the Eco RI-Bam HI sites of the plasmid pIN-I-A2. To bring a stop codon from the vector into the reading frame of the myosin fragment, the plasmid was subsequently opened with Bam HI, filled in with Klenow fragment of polymerase I, and religated. The resulting expression plasmid encoded a 287-amino acid 34-kD myosin segment (residues 1533–1819 of the myosin protein sequence). Seven additional amino acids derived from vector sequence were added to the myosin segment; four amino acids to the amino terminus and three amino acids to the carboxyl terminus.

The expression vector for N-LMM-37 was constructed by subcloning the 0.95-kb Eco RI-Cla I fragment from pBgl 4.5 into the Eco RI and Bam HI sites of pIN-I-A2. The Cla I site of the insert and the Bam HI site of the expression vector were previously blunted by filling in with the Klenow fragment of DNA polymerase I. This vector encoded a 316-amino acid 37-kD myosin segment (from 1533–1848 residues of the myosin sequence) with four additional amino acids derived from vector sequence added to the amino terminus and three amino acids added to the carboxyl terminus.

The expression vector for C-LMM-34 was constructed by subcloning the 1.1-kb Xho II-Bgl II fragment (0.9-kb coding sequence and 0.2-kb noncoding sequence) into the Bam HI site of pIN-I-A2. To correct the reading frame for this construct, the Eco RI site was opened, filled in with the Klenow fragment of DNA polymerase I, and religated before subcloning the appropriate myosin gene fragment. This resulted in an expression plasmid encoding a 298-amino acid 34-kD myosin segment (from 1819 to 2116 residues of the myosin protein sequence) and 10 additional amino acids derived from vector sequence added to the amino terminus of the myosin sequence. An alternate expression vector for C-LMM-34 without noncoding sequence was constructed from a 0.9-kb Xho II-Hind III fragment from the 3' end of pBgl 4.5. This fragment was cloned into the Eco RI-Hind III sites of pIN-I-A3. The Xho II site of the 0.9-kb fragment and the Eco RI site of the plasmid were previously blunted by filling in with the Klenow fragment of DNA polymerase I. To create a stop codon in the reading frame, the Hind III site

was opened, filled in with the Klenow fragment of DNA polymerase I, and religated. This created a Nhe I site that was opened, filled in, and religated. This vector encoded a 297-amino acid 34-kD myosin tail segment (residues 1819–2115) with five amino acids added to the amino terminus and two amino acids added to the carboxyl terminus from vector sequence. In addition to the 34-kD myosin tail segment, this vector also expressed a second 30-kD myosin fragment. This 30 kD fragment could have arisen from proteolysis of the 34-kD myosin segment or from a second translation start within the 0.9-kb sequence. Results from assembly and phosphorylation experiments were indistinguishable for C-LMM-34 expressed from either construct.

Expression plasmids were transformed into *E. coli* LE392 or DH5 $\alpha$  made competent with CaCl<sub>2</sub>. Constructs were verified after cloning either with diagnostic restriction digests or were sequenced directly. Recombinant DNA procedures were performed by standard procedures (Maniatis et al., 1982).

### Purification of the Expressed Proteins

An overnight culture of bacteria containing the appropriate expression plasmid was diluted 1:100 into fresh media. Cells were grown to an OD<sub>600</sub> of 0.8 in fermenters containing 12 or 200 liters of LB media with 50  $\mu$ g/ml of ampicillin. The harvested cells were weighed, resuspended in 5 vol of lysis buffer (50 mM Tris, pH 7.5, 10 mM EDTA, 48 mM sodium pyrophosphate, 30% sucrose, 0.2 mM PMSF, 0.7  $\mu$ g/ml pepstatin A, 0.7  $\mu$ g/ml leupeptin) per gram of cell pellet and lysozyme was added to 1 mg/ml. After 10 min at 0°C the lysate was frozen in dry ice and thawed at 22°C to aid cell lysis. All subsequent steps were performed at 4°C. The lysate was sonicated using a sonifier, (Heat Systems-Ultrasonics, Inc., Farmingdale, NY), with 30-s bursts until no longer viscous and then sedimented at 100,000 g for 45 min. The supernatant was placed in a boiling water bath in 50-ml aliquots, stirred for 8–10 min until most proteins denatured, and then centrifuged at 27,000 g for 30 min. The supernatant was dialyzed overnight into DEAE buffer (10 mM Tris, pH 7.5, 25 mM NaCl, 1 mM EDTA, 1 mM DTT) and applied to a 100-ml column of DEAE-Sepharose Fast Flow (Pharmacia Fine Chemicals, Uppsala, Sweden), equilibrated in the same buffer. The column was eluted with a linear gradient from DEAE buffer to DEAE buffer containing an additional 500 mM NaCl (500 ml total volume). Fractions of 3.5 ml were collected and fractions enriched in the expressed protein (as assessed by immunoblots stained with anti-*Dictyostelium*-myosin serum) were pooled. Purified LMM-68, LMM-58, and N-LMM-34 were collected as a pellet after dialysis against assembly buffer (10 mM Tris, pH 7.5, 2 mM MgCl<sub>2</sub>, 50 mM NaCl) and centrifugation at 100,000 g for 45 min. The proteins were resuspended in storage buffer (20 mM Tris, pH 7.5, 600 mM NaCl, 5 mM EDTA, 0.02% sodium azide). C-LMM-34 did not precipitate in assembly buffer and was purified by gel filtration chromatography. DEAE-sepharose fractions of C-LMM-34 were concentrated to a volume of 0.5–1.5 ml with a Centriprep spin column (Amicon Corp., Danvers, MA) and loaded in 0.5-ml aliquots onto a prepacked Superose-6 column (100 mm  $\times$  30 mm; Pharmacia Fine Chemicals) equilibrated in assembly buffer. Under these conditions, C-LMM-34 eluted before contaminating proteins. 0.5-ml fractions were collected, and fractions of purified C-LMM-34 (as assessed by immunoblots of the fractions stained with anti-*Dictyostelium*-myosin serum) were pooled for subsequent study.

Because myosin tail fragments seemed to adsorb easily to sticky surfaces, column fractions were collected in plastic tubes. In addition, dialysis membranes and Centriprep columns were blocked with 1% BSA in TBS (50 mM Tris, pH 7.5, 150 mM NaCl) and washed several times with 1 M NaCl washes before use. Protein concentrations were determined by densitometry of Coomassie-stained gels using rabbit muscle LMM as a standard. The concentration of rabbit muscle LMM was determined by absorption at 280 nm assuming an extinction coefficient of 0.3.

### Electron Microscopy

Rotary shadowing of expressed tail segments in 70% glycerol, 10 mM Tris, pH 7.5, 150 mM NaCl was as described (Flicker et al., 1985). For negative stain microscopy, samples were applied to carbon-coated formvar grids for 30 s followed by 1% aqueous uranyl acetate applied for 30 s. Grids of rotary-shadowed samples and negatively stained samples were examined with an electron microscope (201; Philips Electronic Instruments, Inc., Mahwah, NJ). The magnification was calibrated using negatively stained tropomyosin paracrystals that have a repeating periodicity of 395 Å (Flicker et al., 1985). Optical diffraction patterns were obtained according to De Rosier and Klug (1972).

## Electrophoresis and Immunoblotting

SDS-polyacrylamide gels were used for electrophoresis of proteins, and stained with Coomassie brilliant blue (Laemmli, 1970). Gels were scanned with a scanning laser densitometer (Ultrascan XL Laser Densitometer; LKB Instruments Inc., Bromma, Sweden). The areas of peaks were evaluated using the Gelscan XL software program that integrated the area under a Gaussian curve fitted to each peak. Immunoblot analysis was performed according to the method of Towbin et al. (1979) using polyclonal anti-*Dictyostelium* myosin serum diluted 1:1,000 as the primary antibody (De Lozanne et al., 1985), and affinity-purified goat-anti rabbit IgG horseradish peroxidase conjugate (1:2,000) as the secondary antibody (Bio-Rad Laboratories, Richmond, CA). Antibody-conjugates were visualized with 4-chloro-1-naphthol following the manufacturer's protocol (Bio-Rad Laboratories).

## Phosphorylation of *Dictyostelium* Myosin and Expressed Segments

A myosin heavy chain kinase from developed *Dictyostelium* cells was purified as described (Ravid and Spudich, 1989). Myosin purified from *Dictyostelium* (Griffith et al., 1987) or expressed myosin segments (0.5–1 mg/ml) were incubated at 22°C with the myosin heavy chain kinase (0.01 mg/ml) in a reaction mixture containing 10 mM Hepes pH 7.5, 6 mM MgCl<sub>2</sub>, 0.2 mM [ $\gamma$ -<sup>32</sup>P]ATP (500 cpm/pmole) and 1 mM DTT. The reaction was initiated by addition of ATP and stopped by addition of 10% trichloroacetic acid. After a 15 min incubation with 10% TCA at 0°C, the reaction mixture was pelleted in a microfuge (Eppendorf instruments made by Brinkmann Instruments, Inc., Westbury, NY), washed once with 10% TCA and then resuspended in 20  $\mu$ l SDS sample buffer and electrophoresed on an SDS-polyacrylamide gel. After staining with Coomassie brilliant blue, the gels were dried and exposed (XAR-5 film; Eastman Kodak Co., Rochester, NY) with an intensifying screen (Dupont Co., Wilmington, DE) at -80°C. To determine incorporation of <sup>32</sup>P, the protein bands were quantitated by scanning densitometry and then excised from the gel and counted in a scintillation counter (LS 7500; Beckman Instruments, Inc., Palo Alto, CA).

## Results

### Expression of Myosin Tail Segments and Purification from *E. coli*

We have studied five different segments of the tail of *Dictyostelium* myosin expressed in and purified from *E. coli*. The expressed proteins were completely soluble upon cell lysis indicating that neither extensive aggregation of the expressed proteins nor partition into bacterial inclusion bodies had occurred. The tail segments were expressed with varying efficiencies: whereas the level of N-LMM-34 was equivalent to major proteins of the bacterial lysate (Fig. 1, lane 3), LMM-68, N-LMM-37, and C-LMM-34 were relatively minor proteins (Fig. 1, lanes 1, 5, and 7). Although the molecular masses predicted from the sequences of expressed LMM-68, N-LMM-34, N-LMM-37, and C-LMM-34 are 69, 34, 38, and 37 kD, respectively, the purified proteins have apparent molecular masses on SDS-polyacrylamide gels of 78, 40, 45, and 44 kD, respectively. This aberrant migration on gels may reflect the highly charged nature of the myosin tail sequence (Warrick et al., 1986).

We used a purification scheme based on heat denaturation and removal of proteins sensitive to heat. Immunoblots of the supernatants revealed that the vast majority of each of the five segments remained soluble after heat treatment. Subsequent DEAE chromatography resulted in further purification and removal of nucleic acids. We used these partially purified preparations for the initial characterization of the expressed proteins. We further purified the myosin tail segments to

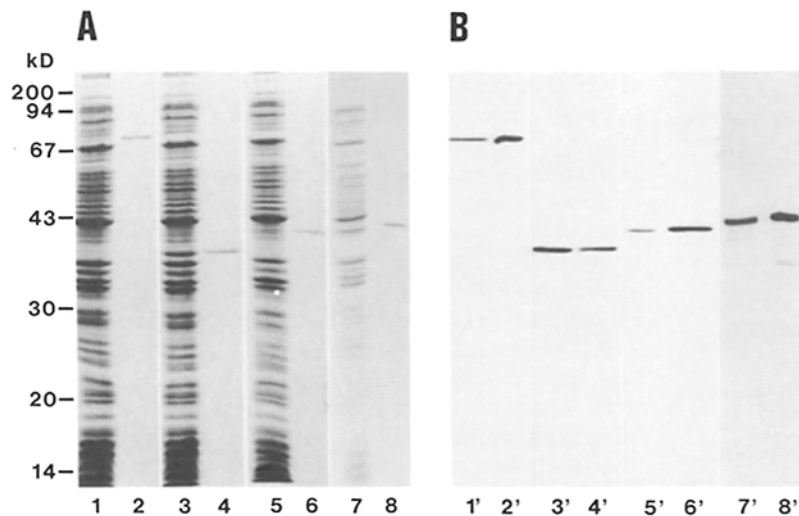
homogeneity to ensure that contaminating proteins had not altered their properties. This last purification step was achieved by gel filtration for C-LMM-34 or by dialysis into assembly buffer and sedimentation for all other constructs. We found no difference in the behavior of the expressed proteins whether partially purified or purified to homogeneity (data not shown). The heat treatment did not affect the properties of the expressed tail segments; the functional properties of LMM-58 or LMM-68 were identical purified by this method or whether purified by selective solubilities in different buffers (De Lozanne et al., 1987). Immunoblots stained with a polyclonal antiserum against *Dictyostelium* myosin (Fig. 1 B) revealed that the expressed proteins in the bacterial lysates comigrate with the final purified proteins, indicating that proteolysis did not occur during purification. The yield of expressed proteins from 50 g of bacterial cells was 0.7 mg for LMM-68, 20 mg for N-LMM-34, 5 mg for C-LMM-37, and 1 mg for C-LMM-34.

### The Expressed Tail Segments Have Properties Predicted for Native Myosin Tail Segments

As shown in Fig. 2, the tail segments purified from *E. coli* were flexible rods, like the tail of *Dictyostelium* myosin, with lengths (Table I) close to the predicted lengths for alpha-helical coiled-coils calculated from their sequences (McLachlan, 1984). In relation to the ~185-nm *Dictyostelium* myosin tail, N-LMM-34 and C-LMM-34 were calculated to occupy positions 98–141 and 141–185 nm, respectively, from the head-tail junction (Table I). Consistent with these assignments, immunoblots showed reaction of the anti-*Dictyostelium* myosin monoclonals My 5 and My 4 with N-LMM-34, N-LMM-37, and LMM-68, but not with C-LMM-34 (Table I). These antibodies have been previously mapped to 120 and 135 nm from the head-tail junction of *Dictyostelium* myosin (Peltz et al., 1985; Flicker et al., 1985).

### Assembly Properties of Expressed Proteins

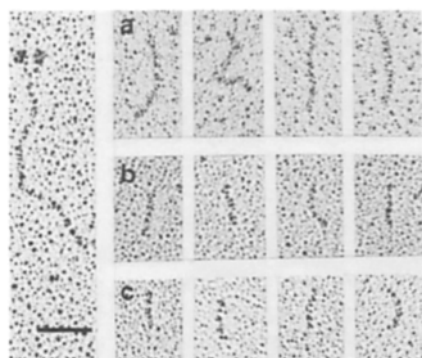
Like other myosins, *Dictyostelium* myosin forms filaments in low ionic strength buffers and solubilizes in higher salt concentrations. *Dictyostelium* myosin has the unusual property of being soluble in very low salt buffers which lack magnesium (Kuczmarzski et al., 1987). Similar to *Dictyostelium* myosin, we found LMM-68, LMM-58, N-LMM-37, and N-LMM-34 to be insoluble in buffer containing 50 mM NaCl and soluble in high salt buffers containing 500 mM NaCl. C-LMM-34 remained soluble in both buffers. The solubility of the largest construct, LMM-68, and its amino-terminal and carboxyl-terminal halves was compared directly with that of purified *Dictyostelium* myosin in a range of salt concentrations. The solubility dependence on ionic strength of *Dictyostelium* myosin, LMM-68 and N-LMM-34 was very similar (Fig. 3). All were insoluble in low salt, and solubilized with increasing salt concentrations. Interestingly, whereas *Dictyostelium* myosin requires the absence of magnesium for solubility in very low ionic strength buffers, LMM-68 and N-LMM-34 were insoluble regardless of the presence of magnesium. In contrast, the carboxyl-terminal half, C-LMM-34, remained soluble in all salt concentrations. Neither the inclusion of 2–10 mM MgCl<sub>2</sub> nor lowering the pH to 6.8 changed the solubility of C-LMM-34, although *Dictyostelium* myosin, LMM-68 and N-LMM-34



**Figure 1.** SDS-polyacrylamide gel and immunoblot of purified myosin tail segments. (A) A 12.5% SDS-polyacrylamide gel stained with Coomassie brilliant blue of lysates of *E. coli* transformed with appropriate expression vectors and of *Dictyostelium* myosin tail segments purified from the *E. coli* lysates. (B) An immunoblot of a duplicate gel stained with a polyclonal anti-*Dictyostelium* myosin serum. Lanes 1 and 1', lysate of *E. coli* expressing LMM-68; lanes 2 and 2', purified LMM-68; lanes 3 and 3', lysate of *E. coli* expressing N-LMM-34; lanes 4 and 4', purified N-LMM-34; lanes 5 and 5', lysate of *E. coli* expressing C-LMM-34; lanes 6 and 6', purified C-LMM-34; lanes 7 and 7', lysate of *E. coli* expressing N-LMM-37; lanes 8 and 8', purified N-LMM-37.

continued to precipitate in these conditions at low salt concentrations (data not shown). To determine whether C-LMM-34 would precipitate if dialyzed into low salt in the presence of an assembly competent segment, samples of C-LMM-34 were mixed with either LMM-68 or N-LMM-34 and dialyzed into low salt. C-LMM-34 remained soluble while LMM-68 and N-LMM-34 continued to precipitate (data not shown).

All assembly competent segments formed highly ordered paracrystals with an observed transverse periodicity of 14 nm. Shown are examples of the largest segment, LMM-68 and the shortest segment, N-LMM-34 (Fig. 4). A novel repeat of 4.6 nm was observed in favorable areas. Optical diffraction patterns of electron micrographs of the paracrystals confirmed the presence of the 14- and 4.6-nm repeats. The diffraction patterns also revealed an additional reflection corresponding to 3.5 nm in some areas of LMM-68; this measurement is reminiscent of a 3.8-nm reflection observed in optical diffraction patterns of thick filaments of skeletal muscle stained with uranyl acetate (Hanson et al., 1971). Paracrystals were not observed in samples in high salt buffers. Paracrystals were never seen with samples of C-LMM-34 in any buffer.



**Figure 2.** The expressed proteins have morphologies expected for *Dictyostelium* myosin tail segments. Rotary shadowed molecules are shown. On the left is an electron micrograph of *Dictyostelium* myosin. Individual molecules of LMM-68 (row a); N-LMM-34 (row b); and C-LMM-34 (row c) are shown on the right. Bar, 50 nm.

### Phosphorylation of the Expressed Tail Segments

To examine the ability of the expressed myosin tail segments to be phosphorylated, the expressed proteins were incubated with  $\gamma$ - $^{32}$ P-labeled ATP and a myosin heavy chain kinase purified from developed cells (Ravid and Spudich, 1989). Phosphorylation levels of *Dictyostelium* myosin and the expressed tail segments were monitored as a function of time of incubation of equal molar amounts of substrate. LMM-68 and LMM-58 were phosphorylated to the same extent and with similar kinetics as purified *Dictyostelium* myosin throughout the course of the assay. In contrast, even with extended incubation, the degree of phosphorylation of either N-LMM-34, N-LMM-37, or C-LMM-34 was <15% of either LMM-68 or *Dictyostelium* myosin (Fig. 5).

### Discussion

Comparison of the properties of the tail segments with purified *Dictyostelium* myosin, summarized in Fig. 6, identifies functional domains within the tail. A 34-kD segment, N-LMM-34, bounded by amino acids 1533–1819 can assemble into structures with assembly and structural features characteristic of myosin filaments. With regard to phosphorylation kinetics, the larger domain, LMM-58, which contains amino acids 1533–2034 of myosin, is the smallest domain that can serve as a substrate equivalent to myosin for a *Dictyostelium* myosin heavy chain kinase. Several subdomains of LMM-58 are not phosphorylated efficiently.

The solubility of *Dictyostelium* myosin tail fragments in low salt buffer has been addressed in previous studies that studied proteolytic myosin fragments. Peltz et al. (1981) found a 102-kD chymotryptic peptide of *Dictyostelium* myosin to be insoluble in low salt buffers. In an analysis of myosin fragments produced with chymotrypsin and mapped with defined monoclonal antibodies, Pagh et al. (1984) identified an 85-kD insoluble fragment and concluded that a region comprising 50–80% of the tail was important for insolubility in low salt buffer. However these studies analyzed mixtures of several proteolytic fragments, and thus the contribution of interactions between different segments of the myosin tail to produce insolubility could not be addressed. In contrast with

Table I. Physical Properties of *Dictyostelium* Myosin and Expressed Tail Segments

Protein	Amino acid boundaries	Polyclonal anti-myosin reactivity	Monoclonal My4 reactivity	Monoclonal My5 reactivity	Length* (nm $\pm$ SD)
Myosin	1-2116	+	+	+	180 <sup>‡</sup> , 189 <sup>§</sup>
LMM-68	1533-2116	+	+	+	78 $\pm$ 15
LMM-58	1533-2034	+	+	+	74 $\pm$ 7 <sup>  </sup>
N-LMM-34	1533-1819	+	+	+	39 $\pm$ 4
N-LMM-37	1533-1848	+	+	+	N.D.
C-LMM-34	1819-2116	+	-	-	37 $\pm$ 6

\* Estimated by rotary shadowing;  $n = 25$  in all cases.

<sup>‡</sup> Flicker et al., 1985.

<sup>§</sup> Clavier et al., 1982.

<sup>||</sup> De Lozanne et al., 1987.

previous studies, we have purified to homogeneity myosin segments with defined amino acid boundaries. We can therefore extend previous conclusions to state that N-LMM-34 (comprising the region  $\sim 53\%$ – $76\%$  from the head-tail junction) is not only important, it is sufficient for insolubility in low salt buffer. Indeed, despite a large difference in mass, the solubility of the smaller 34-kD N-LMM-34 is strikingly similar to that of the larger 240 kD heavy chain of the native myosin molecule in a range of salt concentrations.

We have also examined the structure of the material formed in low salt. The insoluble aggregates that form in low ionic strength buffer differ in many respects from myosin filaments that are bipolar structures with a defined length and

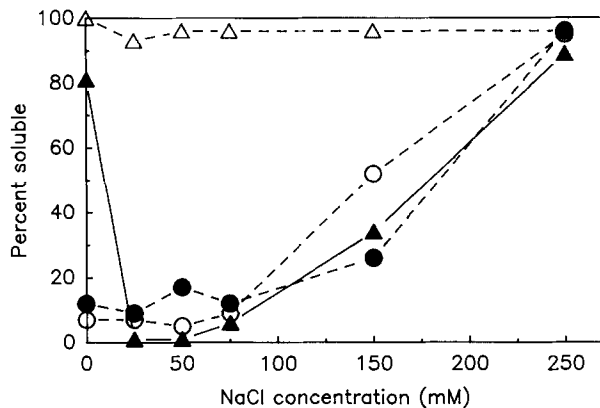
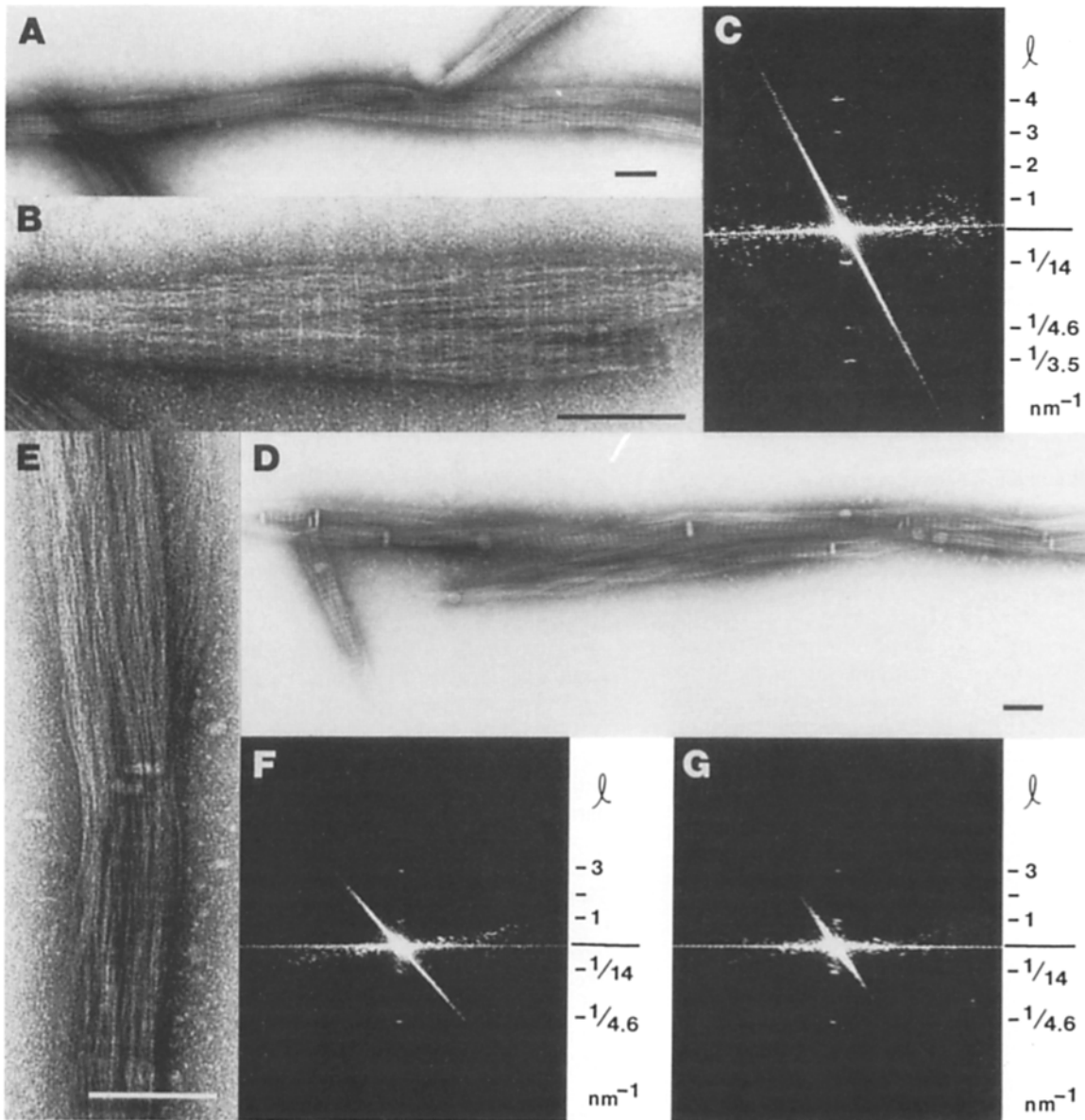


Figure 3. Solubility of three *Dictyostelium* myosin tail segments in a range of salt concentrations. Equivalent molar concentrations (3–5  $\mu$ M) of *Dictyostelium* myosin and expressed *Dictyostelium* myosin tail segments were dialyzed into 10 mM imidazole, 1 mM DTT, 0.1 mM EDTA (pH 7.5), and appropriate NaCl concentrations. The samples were centrifuged at 100,000 g for 15 min and equivalent amounts of supernatants and pellets were subjected to SDS-PAGE. Proteins in the gel were stained with Coomassie brilliant blue and their relative amounts were determined by scanning densitometry. The solubility of *Dictyostelium* myosin and the largest tail segment, LMM-68 and its amino- and carboxyl-terminal halves are shown. The percentage of each protein that remained in the supernatant is shown as a function of salt concentration. (closed triangles) purified *Dictyostelium* myosin; (open circles) LMM-68; (closed circles) N-LMM-34; and (open triangles) C-LMM-34. Note that, except at zero salt concentration, myosin, LMM-68 and N-LMM-34 exhibited similar solubility in a range of salt concentrations. In contrast, C-LMM-34 remained soluble in all salt concentrations.

width. The paracrystals formed from the expressed myosin segments are both longer and wider than the bipolar filaments formed by intact *Dictyostelium* myosin (Stewart and Spudich, 1979). However the paracrystals are similar to myosin filaments in that they contain a 14-nm repeat. A repeat or parallel stagger of 14 nm is a hallmark of myosin filaments from both muscle and nonmuscle sources, including filaments of *Dictyostelium* myosin (Huxley and Faruqi, 1983; Pagh and Gerisch, 1986). The 14-nm repeat present in paracrystals of muscle myosin tail segments is thought to arise from the parallel interaction of individual molecules, each staggered from its neighbor by 14 nm (Bennett, 1981; Quinlan and Stewart, 1987). The 14-nm stagger observed in paracrystals of N-LMM-34 suggests that the amino acid sequence contained therein may dictate the assembly and parallel spacing of myosin molecules within a thick filament. This is supported by a recent study in which areas of interactions between *Dictyostelium* myosin assembled into parallel dimers was measured by electron microscopy. The area most likely to be in contact between two myosin molecules (75–125 nm from the head-tail junction) is similar to the area of the tail demarcated by N-LMM-34 (Pasternak et al., 1989). N-LMM-34 is the smallest domain yet studied of a myosin tail segment from any species or tissue that can form paracrystals and indeed may be a minimal domain capable of assembly into paracrystals sampled by a 14-nm repeat. The 287 amino acids contained in N-LMM-34 approaches the theoretical limit of the 198-amino acid repeat of myosin rods necessary for a 14-nm parallel stagger between complementary groups of positive and negative charge in the myosin tail (McLachlan, 1984). Our attempts to express a smaller subdomain of N-LMM-34 resulted in a protein that was not stable in *E. coli* extracts.

Comparison of phosphopeptides in two-dimensional maps of myosin or bacterially expressed LMM-58 phosphorylated by *Dictyostelium* myosin heavy chain kinases derived from either developed cells or vegetative cells shows near identity (De Lozanne et al., 1987; Wagle et al., 1988). These experiments indicate that the phosphorylation sites for one or more myosin heavy chain kinases are localized within LMM-58. Here we have examined a time course of phosphorylation of *Dictyostelium* myosin or myosin tail segments not as a means to identify phosphorylated residues, but rather as a guide to identify parameters of the tail important for efficient phosphorylation. The kinase we used has been purified to homogeneity from developed cells and characterized extensively.

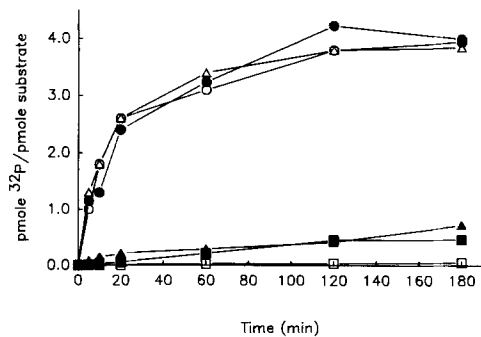


**Figure 4.** Paracrystals of LMM-68 and N-LMM-34 stained with uranyl acetate. (A) A typical filamentous paracrystal observed in samples of LMM-68. A higher magnification (B) displays transverse periodicities of 14 and 4.6 nm. (C) Optical diffraction pattern of B. The optical diffraction pattern confirms the periodicities; 14- and 4.6-nm reflections are seen. An additional 3.5-nm reflection is also revealed. (D) A typical paracrystal observed in samples of N-LMM-34. A view at higher magnification (E) displays an area where transverse periodicities of 4.6 nm (top) and 14 nm (bottom) are evident. Optical diffraction patterns corresponding to the top region (F) and bottom region (G) of the paracrystal shown in E confirm the periodicities. Ordinates of the diffraction patterns correspond to the long axes of the paracrystals. The layer lines ( $l$ ) are indicated on the top portion of the diffraction patterns and their corresponding distances in nanometers is below. Bar, 100 nm.

It appears to be distinct from previously described *Dictyostelium* kinases (Ravid and Spudich, 1989). We show that the extent and kinetics of phosphorylation by this purified heavy chain kinase are virtually indistinguishable between myosin, LMM-68 or LMM-58. Thus, neither the region of the tail adjacent to the myosin heads (analogous to the S-2 region of skeletal muscle myosin) nor the 10-kD carboxyl terminus of the tail are necessary for phosphorylation by the kinase.

Phosphorylation sites on the myosin heavy chain have been roughly mapped to a domain 32 kD from the tip of the tail (Pagh et al., 1984). Wagle et al. (1988) have suggested that

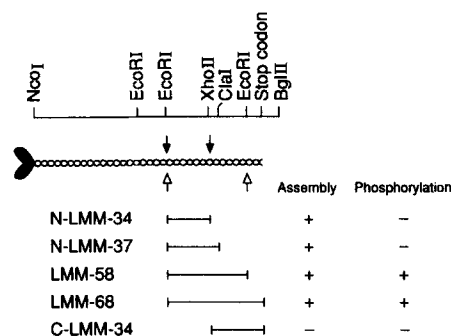
amino acid 1823, one in a cluster of four threonines might be a phosphorylation site for a kinase from vegetative cells. Vaillancourt et al. (1988) have directly identified two phosphorylated threonines at positions 1833 and 2029. Since all phosphorylation sites of LMM-58 and LMM-68 are present in either N-LMM-34 or C-LMM-34, our results suggest that additional parameters are necessary for efficient phosphorylation. Notably, all substrates for the kinase assemble into either filaments or paracrystals in low salt buffers in which the kinase is active. Thus, the low efficiency of phosphorylation could be either because of the absence of phosphorylation



**Figure 5.** Time course of phosphorylation of expressed *Dictyostelium* myosin tail segments. After incubation for various time intervals with a myosin heavy chain kinase purified from developed cells, *Dictyostelium* myosin, LMM-68, N-LMM-37, N-LMM-34, and C-LMM-34 were subjected to SDS-PAGE and stained with Coomassie brilliant blue. The gels were scanned to determine protein concentrations. To quantitate incorporation of  $^{32}\text{P}$ , the protein bands were excised from the gels and counted in a scintillation counter. The phosphorylation of each protein throughout a time course is shown. Note that both the extent and kinetics of phosphorylation of *Dictyostelium* myosin (open circles), LMM-68 (open triangles), and LMM-58 (closed circles) are very similar. However, at maximal phosphorylation, N-LMM-37 (closed triangles), N-LMM-34 (open squares), and C-LMM-34 (closed squares) are phosphorylated only to levels <15% of the extent of *Dictyostelium* myosin.

sites on N-LMM-34 or the inability of C-LMM-34 to form paracrystals. We designed N-LMM-37 with the purpose of including threonine residues 1823 and 1833, potential candidates for phosphorylation, in a small assembly competent segment. Yet N-LMM-37 did not serve as an efficient substrate suggesting that one minimal domain for efficient phosphorylation is limited to the boundaries of LMM-58, amino acids 1533–2034.

The recent discovery of homologous recombination in



**Figure 6.** Schematic representation of functional domains within the tail of *Dictyostelium* myosin. The top line shows a map of restriction sites of the tail portion of the *Dictyostelium* myosin heavy chain gene (Warrick et al., 1986). The drawing depicts the native form of the myosin molecule with the limits of the assembly domain, N-LMM-34 (solid arrows), and the limits of the phosphorylation domain, LMM-58 (open arrows), indicated. Below the molecule are the five myosin tail segments studied; lines represent the correspondence of each segment to the gene and the myosin molecule. The functional properties of each segment are summarized on the right.

*Dictyostelium* makes possible the creation of *Dictyostelium* mutants containing specifically altered myosin proteins (De Lozanne and Spudich, 1987). Thus, it is now possible to create mutants with altered myosin domains and to assess the contribution of these alterations to the dynamics of myosin assembly and phosphorylation within living cells. The creation of myosin molecules with alterations in N-LMM-34 may reveal the contribution of this domain to the assembly of myosin filaments within *Dictyostelium* cells. Similarly, myosin mutants can now be constructed that lack C-LMM-34, a region we show here to be important for myosin phosphorylation in vitro. Ultimately, we hope to understand how functional domains such as these contribute to myosin function within living cells.

We would like to thank Arturo De Lozanne for advice and helpful discussions throughout the course of this work.

T. J. O'Halloran was the recipient of National Institutes of Health (NIH) postdoctoral grant GM 11607; S. Ravid was the recipient of a Weizmann Postdoctoral Fellowship. This study was supported by NIH grant GM-30387 to J. A. Spudich.

Received for publication 10 July 1989 and in revised form 19 September 1989.

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