Experimental Evidence for the Contractile Activities of Acanthamoeba Myosins IA and IB*

Hisao Fujisaki, Joseph P. Albanesi, and Edward D. Korn
From the Laboratory of Cell Biology, National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, Maryland 20205

The low-shear viscosity of 5–30 μM F-actin was greatly increased by the addition of 0.1–0.5 μM unphosphorylated Acanthamoeba myosins IA and IB. The increase in viscosity was about the same in 2 mM ADP as in the absence of free nucleotide, but was much less in 2 mM ATP. The single-headed monomolecular Acanthamoeba myosins were as effective as an equal molar concentration of two-headed muscle heavy meromyosin and much more effective than single-headed muscle myosin subfragment-1. These results suggest that Acanthamoeba myosins IA and IB can cross-link actin filaments as proposed in the accompanying paper (Albanesi, J. P., Fujisaki, H., and Korn, E. D. (1985) J. Biol. Chem. 260, 11174–11179) to explain the actin-dependent cooperative increase in actin-activated Mg$^{2+}$-ATPase activity as a function of the concentration of myosin I. Superprecipitation occurred when phosphorylated myosin IA or IB was mixed with F-actin. In addition to myosin I heavy chain phosphorylation, superprecipitation required Mg$^{2+}$ and ATP. ATP hydrolysis was linear during the time course of the superprecipitation, and inhibitors of ATP hydrolysis inhibited superprecipitation. A small, dense contracted gel was formed when the reaction was carried out in a cuvette, and a birefringent actomyosin thread resulted from superprecipitation in a microcapillary. The rate and extent of superprecipitation depended on the actin and myosin I concentrations with maximum superprecipitation occurring at an actin:myosin ratio of 7:1. These results provide strong evidence for the ability of Acanthamoeba myosins IA and IB to perform contractile and motile functions.

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The preparation of Acanthamoeba myosins IA and IB, myosin I heavy chain kinase, phosphorylated myosins IA and IB and rabbit skeletal muscle actin, the assays of ATPase activity, and electrophoretic analysis of proteins were as described in the accompanying paper (7). Just before use, the F-actin was pelleted, suspended in nucleotide-free buffer, and briefly sonicated to hydrolyze any remaining free or actin-bound ATP (11). Rabbit skeletal muscle myosin HMM$^*$ and S-1 were prepared as described by Weeds and Taylor (12). HMM and S-1 were further purified by chromatography on Sephadex G-200 (Pharmacia). Hexokinase (Type C-502) was obtained from Sigma.

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1. Presented at the annual meeting of the American Society of Cell Biology, Kansas City, MO, November 1984 (9), and the annual meeting of the Biophysical Society, Baltimore, MD, February 1985 (10).

2. The abbreviations used are: HMM, muscle myosin heavy meromyosin; S-1, muscle myosin subfragment-1; EGTA, ethylene glycol bis(β-aminoethyl ether)-N,N',N''-tetraacetic acid.
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**Fig. 1.** Effect of unphosphorylated *Acanthamoeba* myosin IB on the viscosity of F-actin. Viscosities were measured 60 min after mixing various concentrations of unphosphorylated myosin IB with fixed concentrations of F-actin in buffer containing 2 mM MgCl₂, 14 mM KCl, 0.1 mM CaCl₂, 6 mM imidazole, 0.3 mM dithiothreitol, 0.01% NaN₃, and 7% glycerol, pH 7.5, at 22 °C. The concentrations of F-actin are given in the figure. A, no added nucleotide; B, 2 mM ADP; C, 2 mM ATP.

**Fig. 2.** Effect of unphosphorylated *Acanthamoeba* myosin IA on the viscosity of F-actin. Viscosities were measured 60 min after mixing various concentrations of unphosphorylated myosin IA with 15 μM F-actin as described in Fig. 1. ○, no added nucleotide; △, 2 mM ADP; ■, 2 mM ATP.
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FIG. 3. Comparison of the effects of unphosphorylated Acanthamoeba myosin IB, HMM, and S-1 on the viscosity of F-actin. Viscosities were measured 5 min after the addition of various concentrations of unphosphorylated myosin IB ( ), HMM ( ), or S-1 ( ) to 15 μM F-actin as described for Fig. 1. A, no added nucleotide; B, 2 mM ADP; C, 2 mM ATP.

RESULTS

Interaction of Unphosphorylated Myosins IA and IB with F-actin—The increases in the low-shear viscosities of several fixed concentrations of F-actin as a function of the concentration of myosin IB are shown in Fig. 1. In the absence of free nucleotide (Fig. 1A), the viscosities of 15-30 μM F-actin increased sharply between 0 and 0.5 μM myosin IB, and a rigid gel formed at higher myosin concentrations. At 5 and 10 μM F-actin, peaks in the low-shear viscosity curves were obtained at 0.25 and 0.5 μM myosin IB, respectively, with decreases in viscosities at higher concentrations of myosin IB. Similar increases in low-shear viscosities of F-actin were observed in the presence of 2 mM ADP, but higher concentrations of myosin IB were required (Fig. 1B). The maximum viscosities obtained in the presence of 2 mM ATP (Fig. 1C) were very much lower (note the change in the scale), which is consistent with the known effect of ATP on the interaction of F-actin and myosins. Essentially identical results were obtained when myosin IA was added to 10 μM F-actin (Fig. 2), but the peak viscosities were higher with myosin IA and occurred at low concentrations of myosin. We interpret the increase in viscosity as indicating that myosins IA and IB cross-link actin filaments into networks and the decrease in viscosity at higher myosin:actin ratios as indicative of the formation of more oriented bundles of cross-linked filaments.

The ability of unphosphorylated myosin IB to increase the viscosity of F-actin is compared to that of HMM and S-1 in Fig. 3. In the absence of free nucleotide, and in the presence of 2 mM ADP or 2 mM ATP, unphosphorylated myosin IB was as effective as an equimolar concentration of HMM, and both of these proteins were much more effective than an equal concentration of S-1. In these experiments, the viscosities were measured after 5 min because acto-HMM and acto-S-1 would have hydrolyzed a considerable fraction of the ATP during the 60-min incubation period used in the experiments described in Figs. 1 and 2.

Interaction of Phosphorylated Myosins IA and IB with F-actin—Phosphorylated myosins IA and IB behaved similarly to the unphosphorylated myosins in the absence of free nucleotide or in the presence of 2 mM ADP. But consistent viscosity measurements could not be obtained when phosphorylated myosins IA and IB were added to F-actin in the presence of ATP because of the formation of a contracted thread. The thread was very difficult to see under bright light, more readily detected under dark field, and easily visualized under crossed polarizers (Fig. 4). The thread consisted of highly ordered filaments because it changed in color from blue to red when the polarizers were rotated by 90°. The thread did not form when unphosphorylated myosin I was used.

The time course of the superprecipitation of actomyosin I was readily followed and photographed when it was allowed to occur in a spectrophotometer cuvette (Fig 5). The initially clear solution (0 min) became turbid and sufficiently gelled to trap air bubbles by 4 min; contraction began at about 10 min and was almost complete by 22 min. When air bubbles were present, the contracted gel floated to the top of the cuvette (Fig. 5), but when the bubbles were carefully removed
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Fig. 4. Formation of a birefringent actomyosin I thread. Phosphorylated (left) or unphosphorylated (right) Acanthamoeba myosin IB (0.7 μM) and F-actin (5 μM) were mixed in the buffer described for Fig. 1 containing 2 mM ATP, and the solutions were sucked into microcapillaries. Photographs were taken after 20 min at 22 °C with the capillaries placed between crossed polarizers. The actomyosin I thread was red against a purple background. When the capillaries were rotated 90°, the thread was blue. The inner diameter of the microcapillaries is 1.2 mm.

Fig. 5. Photographic record of the time course of superprecipitation. Phosphorylated Acanthamoeba myosin IA (0.75 μM) was mixed with F-actin (2 μM) in the buffer described for Fig. 1 containing 2 mM ATP and incubated in a 10 × 3-mm cuvette at 25 °C. Photographs of the 10-mm face were taken at 2-min intervals between 0 and 32 min. Only the results at 4, 10, 14, and 22 min are shown.

from the solutions a smaller, denser gel formed and sank.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of equal aliquots of the original mixture, the contracted gel, and the remaining solution (Fig. 6) demonstrated that essentially all of the actin and myosin were in the contracted gel. Fig. 6 also demonstrates the degree of purity of the myosin I used in these experiments and the complete absence of Acan-
thamoeba myosin II (heavy chains of 185,000 Da). The myosin IB was of equal purity (3). The minor components seen in the electrophoretic gel between the positions of the myosin IA heavy chain and actin were from the kinase preparation. The kinase had neither cross-linking nor superprecipitation activity in the absence of myosin I.

If the contraction of actomyosin I gels illustrated in Figs. 4 and 5 were true superprecipitation, it should depend on ATP hydrolysis and, therefore, require Mg2+, ATP, and that the myosin I heavy chain be phosphorylated. To test the requirements for superprecipitation, more quantitative assays were carried out by measuring the increase in turbidity at 660 nm. The increase in absorbance always correlated with the formation of a gel that subsequently contracted with a consequent decrease in absorbance as the contracted gel moved out of the light path.

Superprecipitation did not occur when unphosphorylated myosin IB was added to F-actin but then occurred rapidly after the subsequent addition of myosin I heavy chain kinase (Fig. 7). Solutions prepared with 0.5 mM EDTA did not superprecipitate until excess MgCl2 (2.5 mM) was added (Fig. 8); addition of 2.5 mM CaCl2 had no effect (data not shown). To demonstrate a requirement for ATP, glucose and hexokinase were first added to the myosin I to remove the ATP
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**FIG. 7. Requirement for heavy chain phosphorylation of Acanthamoeba myosin I for superprecipitation.** At zero time, 0.5 μM unphosphorylated myosin IB was mixed with 2 μM F-actin in the buffer described for Fig. 1 containing 2 mM ATP. Superprecipitation was monitored by the absorbance at 660 nm during incubation at 25 °C. At 22 min (arrow), myosin I heavy chain kinase (about 5 nM) was added, and superprecipitation began immediately.

**FIG. 8. Requirement for Mg²⁺ for superprecipitation.** At zero time, 0.5 μM phosphorylated myosin IA was mixed with 2 μM F-actin in the buffer described for Fig. 1 except for the addition of 0.5 mM EDTA and the omission of the 2 mM MgCl₂. The solution was incubated at 25 °C, and the absorbance monitored at 660 nm. At 13 min (arrow), 2.5 mM MgCl₂ was added, and superprecipitation began immediately.

**FIG. 9. Requirement for ATP for superprecipitation.** A, to remove the ATP from the solution after phosphorylation of myosin IA with heavy chain kinase, 1 μM phosphorylated myosin IA in 200 μM ATP was incubated with hexokinase (25 units/ml) and 400 μM glucose for 5 min at 25 °C. Then, at zero time, 2 μM F-actin was added to a final concentration of 0.3 μM phosphorylated myosin IA in the buffer described for Fig. 1 without ATP. Suppreprecipitation was monitored by the absorbance at 660 nm. At 28 min (arrow), 2 mM ATP was added, and superprecipitation began immediately. B, control experiments in which neither hexokinase nor glucose was added (-----), only hexokinase was added (-----), or only glucose was added (-----).

remaining from the phosphorylation of the myosin heavy chain. When F-actin was added, superprecipitation did not occur until the readdition of ATP (Fig. 9A). Neither glucose nor hexokinase alone had any effect on superprecipitation (Fig. 9B). The time courses of increase in $A_{660}$ and ATP hydrolysis are compared in Fig. 10. It can be seen that ATP hydrolysis occurred at a constant rate during the period of superprecipitation. In this experiment, the solution was stirred continuously to prevent formation of a solid gel so that aliquots could be removed to assay ATP hydrolysis. N-Ethylmaleimide (0.25 mM) and sodium vanadate (0.8 mM) inhibited both ATP hydrolysis and superprecipitation (data not shown), and EGTA had no effect on either.

Finally, the extent of maximum superprecipitation was measured as a function of the F-actin concentration with 0.3 and 0.6 μM phosphorylated myosin IA. The times required to reach the maximum increase in turbidity were longer at the lowest and highest ratios of actin:myosin than at intermediate ratios; the maximum increase in turbidity occurred at a molar ratio of actin:myosin of 7:1 at both myosin concentrations (Fig. 11).

**DISCUSSION**

The inability of Acanthamoeba myosins IA and IB to form filaments has made it difficult to understand how these single-headed, monomeric molecules could function with F-actin in contractile or motile processes. In the accompanying paper (7), we proposed that the myosin I monomer might have two actin-binding sites (or undergo actin-dependent dimerization) in order to explain the actin-dependent, myosin-cooperative increase in actin-activated Mg²⁺-ATPase activity. It is a requirement of this model that myosins IA and IB be capable of cross-linking actin filaments. The increase in low-shear...
viscosity of F-actin solutions upon the addition of unphosphorylated or phosphorylated myosins IA and IB provides experimental support for this hypothesis. It is interesting that the single-headed myosins IA and IB increase the viscosity of F-actin as much as an equimolar concentration of two-headed HMM and that the Acanthamoeba myosins are much more effective than single-headed S-1.

Supereprecipitation has been regarded as a model of actomyosin contractile activity since the early work of Szent-Györgyi (18). Myosin filaments have been thought to be essential for such activity; HMM (19) and S-1 (20) can cross-link but do not superprecipitate F-actin confirms our earlier conclusions (3) that heavy chain phosphorylation is required for a step in the actomyosin ATPase catalytic cycle but not for binding of myosin to F-actin. Supereprecipitation appears to be optimal at an actin:myosin ratio of 7:1 which, possibly only coincidentally, corresponds to one myosin for every half-pitch of the actin helix. Ando and Scales (20) showed that S-1 could cross-link actin filaments into bundles with the same optimal actin:myosin ratio. This bundling appeared not to reflect supereprecipitation, but it does suggest that S-1, like the Acanthamoeba myosins IA and IB, contains two actin-binding sites (20).

The catalytic and active sites of Acanthamoeba myosins IA and IB have been mapped (21), but we still know too little about the chemistry of the heavy chains of the myosin I isoenzymes to suggest the structural basis for the two actin-binding sites (or for the alternative possibility of actin-induced formation of dimers or higher oligomers). Similarly, there is yet no basis for speculation on the nature of the ATP-dependent conformational change in the heavy chains that would lead to supereprecipitation. Nonetheless, it does seem clear from the data in this paper, and from other evidence (8), that Acanthamoeba myosins IA and IB can support actin-dependent contractile and motile activity.

REFERENCES
Superprecipitation of F-actin by Acanthamoeba Myosin I