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Phosphorylation of the myosin heavy chain

Its effect on actin-activated Mg^{2+}-stimulated ATPase in leukaemic myeloblasts

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Myosin purified from a murine myeloid leukaemia cell line (M1) that had been incubated with [^{32}P]orthophosphate incorporated ^{32}P into the heavy, but not the light, chain. When the heavy chain was dephosphorylated by bacterial alkaline phosphatase, myosin that had low actin-activated ATPase activity gained higher activity only in the presence of the light-chain kinase. In the absence of the light-chain kinase, however, the Mg^{2+}-stimulated ATPase activity of myosin was not activated by actin, regardless of phosphatase treatment. These results indicate that the activity of M1 myosin ATPase is regulated by phosphorylation of both the light and heavy chains. A scheme for this regulation by phosphorylation is presented and discussed.

An M1 cell line isolated from a myeloid leukaemia in an SL-strain mouse has been maintained *in vitro* (Ichikawa, 1969). The myosin purified from this cell line consists of one heavy chain (H) and three light chains (L_1, L_2, L_3) (Sagara *et al.*, 1982*a*). Activation of the myosin ATPase by actin required the presence of MLCK isolated from M1 cells that phosphorylated both the L_1 and L_2 light chains, but not the L_3- and H-chains (Sagara *et al.*, 1982*b*).

MLCK has been reported to be necessary for actin activation of myosin ATPase in smooth muscle (Sobieszek & Small, 1976; Gorecka *et al.*, 1976; Chacko *et al.*, 1977), macrophage (Trotter & Adelstein, 1979) and other non-muscle cells (Adelstein & Conti, 1975; Yerna *et al.*, 1979). Activities of the myosin from the cellular slime mould *Dictyostelium discoideum* (Kuczmarski & Spudich, 1980) and the myosin II from the protozoon *Acanthamoeba castellanii* (Collins & Korn, 1981), however, are depressed by phosphorylation of their heavy chains. In vertebrate cells, the heavy chains of macrophage (Trotter, 1982) and other non-muscle-cell myosins (Muhlrad & Oplatka, 1977; Hesketh *et al.*, 1978; Fecheimer & Cebra, 1982) have been shown to be phosphorylated, but the biological effect of the phosphorylation remains unknown.

We here report that preparations of M1-cell myosin show highly variable actin-activated ATPase activity even in the presence of MLCK. This variation probably depends on the extent of phosphorylation of the heavy chain.

Abbreviations used: MLCK, myosin light-chain kinase; Mg^{2+}-ATPase, Mg^{2+}-stimulated adenosine triphosphatase; SDS, sodium dodecyl sulphate; K^+-EDTA-ATPase, the ATPase activity in the presence of EDTA and K^+.

Materials and methods

Cell line and culture

M1 cells in suspension culture were harvested during two phases, the exponential and stationary phases. Cell density at the stationary phase was about 2×10^6 cells/ml. These cells were left another day without an addition of fresh medium, then harvested (Nagata *et al.*, 1980, 1982).

Preparation of M1-cell myosin

M1-cell myosin was prepared, with slight modification, as reported previously (Sagara *et al.*, 1982*a*). Cells were homogenized with 0.34M-sucrose containing 20mM-imidazole/HCl, 5mM-dithiothreitol, 2mM-ATP, 2mM-EGTA, 0.5mM-phenylmethanesulphonyl fluoride, 2μg of pepstatin A/ml, 2μg of leupeptin/ml and 1000 units of Trasylol/ml, pH 7.5. After centrifugation at 120000g for 90min, the supernatant was fractionated with $(NH_4)_2SO_4$. The precipitate obtained between 25 and 55%-satd. $(NH_4)_2SO_4$ was dialysed against 0.1M-KCl containing 10mM-imidazole/HCl, 0.5mM-dithiothreitol, pH 7.5, then dissolved in a solution containing 0.6M-KCl and 10mM-MgATP and centrifuged at 120000g for 30min. The supernatant was applied to a Sepharose 4B column, and eluted with 0.5M KCl solution containing 10mM-imidazole/HCl, 0.1mM-Mg-ATP and 0.5mM-dithiothreitol, pH 7.5.

Myosin fractions, detected with K^+-EDTA-

ATPase, were pooled and were dialysed against 0.1 M-KCl solution. After the addition of 1.0 M-$MgCl_2$ solution at a final concentration of 10 mM the myosin precipitate was collected and dissolved in a stock solution containing 0.3 M-KCl, 5% (w/v) sucrose, 10 mM imidazole/HCl, 1 mM-EDTA and 2 mM-dithiothreitol, pH 7.5.

These procedures yielded about 2 mg of myosin from 10 g wet weight of cells.

Phosphorylation of myosin in intact cells

Viable M1 cells (5×10^7 cells/ml) were incubated in 120 mM-NaCl/5 mM-KCl/1.7 mM-$CaCl_2$/1.2 mM-$MgCl_2$/10 mM-Tris/HCl/8 mM glucose containing 0.15 mCi of [^{32}P]orthophosphate/ml at 37°C for 60 min as described by Trotter (1982). Labelled cells were washed in 0.9% NaCl then combined with 4-fold the number of unlabelled cells, after which myosin was purified as described above. Myosin-bound phosphate was measured by the method of Stull & Buss (1977).

Treatment of myosin with phosphatase

Myosin solution (1–2 mg/ml) was incubated with bacterial alkaline phosphatase (0.02–0.05 mg/ml; Sigma type III-R or type III) in 0.4 M-KCl/2 mM-$MgCl_2$ / 2 mM - dithiothreitol / 20 mM - Tris / HCl (pH 7.5)/soya-bean trypsin inhibitor (0.05 mg/ml) at room temperature for 24 h. After incubation, myosin was separated from the phosphatase by Sepharose-4B chromatography.

Determination of ATPase activity

Assays for actin activation of myosin Mg^{2+}-ATPase activity were done at 35°C in 0.5 ml of 20 mM-imidazole/HCl (pH 7.0)/50–60 mM-KCl/5 mM-$MgCl_2$/1 mM-ATP, together with 100–200 μg of myosin/ml, 500 μg of actin/ml, and a sufficient amount of the MLCK preparation. Preparation of MLCK from M1 cells was described previously (Sagara *et al.*, 1982*b*). P_i was measured by the method of Pollard & Korn (1973).

Other methods

Rabbit skeletal-muscle actin was prepared by the method of Spudich & Watt (1971). SDS/poly-acrylamide-gel electrophoresis was performed as described by Laemmli (1970).

Results

The Mg^{2+}-ATPase activity of the myosin purified from M1 cells was activated by actin in the presence of MLCK, but there was considerable variation in activity from one preparation to another, individual activities varying from 10 to 130 nmol/min per mg. Without added MLCK, the Mg^{2+}-ATPase activity of the myosin was constantly low: myosin alone (~6 nmol/min per mg) and with actin (~8 nmol/min per mg). These variations in actin-activated ATPase activity were not due to different activities of the MLCK preparations used, because the same MLCK preparation gave different Mg^{2+}-ATPase activities with different preparations of myosin, as described above. Furthermore, the same preparation of myosin had almost the same value for activity with sufficient concentrations (1 to 10 μg/ml) of different preparations of MLCK.

The Mg^{2+}-ATPase activity of M1 myosin depends on the growth stage at which the cells were harvested. Myosin extracted from cells in the stationary phase always had low actin-activated ATPase activity, even in the presence of MLCK, whereas myosin from cells in the exponential phase had higher activities with rather large standard deviations (Table 1).

The actomyosin ATPase activity in the presence of MLCK rose when the 0.34 M-sucrose solution used for the extraction of myosin (see the Materials and methods section) was replaced by 30% (w/v) sucrose containing 40 mM-sodium pyrophosphate, 10 mM-Tris/HCl, 5 mM-dithiothreitol, pH 7.5, and several proteinase inhibitors, giving 60–120 nmol/min per mg even in the stationary phase. There was no significant difference in the final purities of the myosin preparations (~95%) whether the myosin was extracted in 0.34 M-sucrose or 30% (w/v) sucrose, as suggested by SDS/polyacrylamide gels stained with Coomassie Blue (Figs. 1*a* and 1*b*). The amounts of myosin-bound phosphate, however, were different, depending on the extraction solutions: 1.35 mol of P_i/mol of myosin for the myosin extracted in 0.34 M-sucrose, and 0.78 mol of P_i/mol of myosin for that extracted in 30% sucrose.

When M1 cells were incubated with [^{32}P]orthophosphate for 60 min at 37°C, after which myosin was isolated with 0.34 M-sucrose, [^{32}P]phosphate was incorporated only into the heavy chain of the myosin, not into the light chains (Fig. 2). About

Table 1. *Mg^{2+}-ATPase activity of myosin purified from M1 cells*

Cells at different growth stages were harvested and their myosins extracted with 0.34 M-sucrose solution as described in the Materials and methods section. Assays for the Mg^{2+}-ATPase of these myosins also are described in the Materials and methods section.

		Mg^{2+}-ATPase activity (nmol/min per mg)		
Stage of growth	Addition ...	None	Actin	Actin and MLCK
Exponential phase		6.2 ± 1.2	8.8 ± 1.9	58.6 ± 27.6
Stationary phase		6.3 ± 0.6	8.3 ± 1.4	17.3 ± 5.4

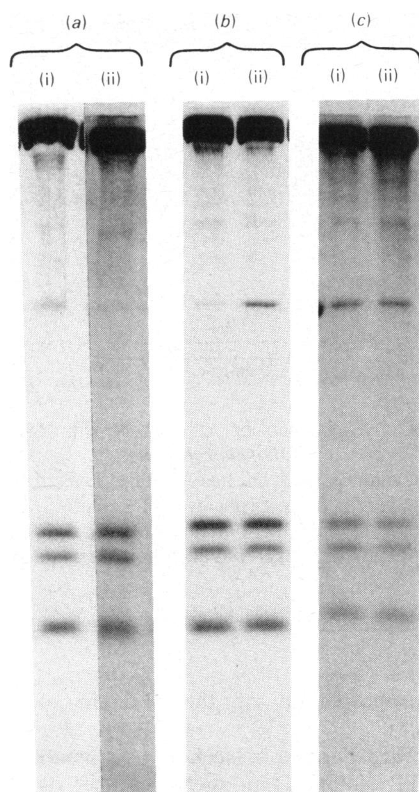


Fig. 1. *SDS/polyacrylamide-gel electrophoresis of M1-cell myosin*

Myosins from M1 cells at exponential phase (*a*) and stationary phase (*b,c*) were electrophoresed in 13% (w/v)-polyacrylamide gel and stained with Coomassie Blue. Myosins in slots (*a*)(i), (*b*)(i) and (*c*) were extracted in 0.34M-sucrose; myosins in slots (*a*)(ii) and (*b*)(ii) were extracted in 30% (w/v) (0.87M) sucrose. Slot (*c*) (i) shows myosin before alkaline phosphatase treatment, and slot (*c*)(ii) after the treatment.

90% of the radioactivity incorporated into the heavy chain was liberated during incubation with bacterial alkaline phosphatase.

In the absence of MLCK, no significant actin activation took place in the myosin ATPase regardless of phosphatase treatment. However, the myosin preparation that had low actin-activated ATPase activity acquired high activity in the presence of MLCK after initial treatment with phosphatase (Table 2). On SDS/polyacrylamide-gel electrophoresis the polypeptide-chain compositions of the myosins were not distinguishable before or after phosphatase treatment (Fig. 1*c*).

Discussion

Activation of M1-cell myosin ATPase by actin required the presence of MLCK, which phosphorylated the L_1- and L_2-chains but not the H-chain (Sagara *et al.*, 1982*b*). Activity varied considerably depending on the batch of myosin used, although there were no differences found in the SDS/polyacrylamide-gel electrophoresis of myosin preparations with different activities.

With the myosins from *Dictyostelium discoideum* (Kuczmarski & Spudich, 1980) and *Acanthamoeba castellanii* (Collins & Korn, 1981), phosphorylation of their H-chains depressed ATPase activity, and dephosphorylation restored it. *Acanthamoeba* myosin II extracted with 30% sucrose has been reported to show high activity compared with that obtained in other extractions. This high activity was always accompanied by less intensive phosphorylation of the H-chain (Collins & Korn, 1980). A 30% (0.87M)-sucrose solution (its mechanism is unknown) extracted myosin from M1 cells that was more highly activated by actin in the presence of MLCK than the myosin from the usual extraction with 0.34M-sucrose. It was also found that the myosin extracted in 30% sucrose contained a

Table 2. *Effects of the incubation of myosin with phosphatase on Mg^{2+}-ATPase activity*

Purified myosins (1–2mg/ml) were incubated with bacterial alkaline phosphatase (0.02–0.05mg/ml) for 24h at room temperature, then isolated by Sepharose 4B chromatography as described in the Materials and methods section. Myosins in Expts. 1–3 were prepared from M1 cells at stationary phase and that in Expt. 4 was from cells at exponential phase.

		Mg^{2+}-ATPase activity (nmol/min per mg)					
		Before incubation			After incubation		
Expt.	Addition ...	None	Actin	Actin and MLCK	None	Actin	Actin and MLCK
1		6.2	9.5	15.6	6.4	8.1	66.3
2		6.3	8.3	27.5	6.7	8.6	111.5
3		5.9	8.9	28.0	5.8	7.2	76.5
4		6.5	9.2	63.5	6.5	9.1	123.9

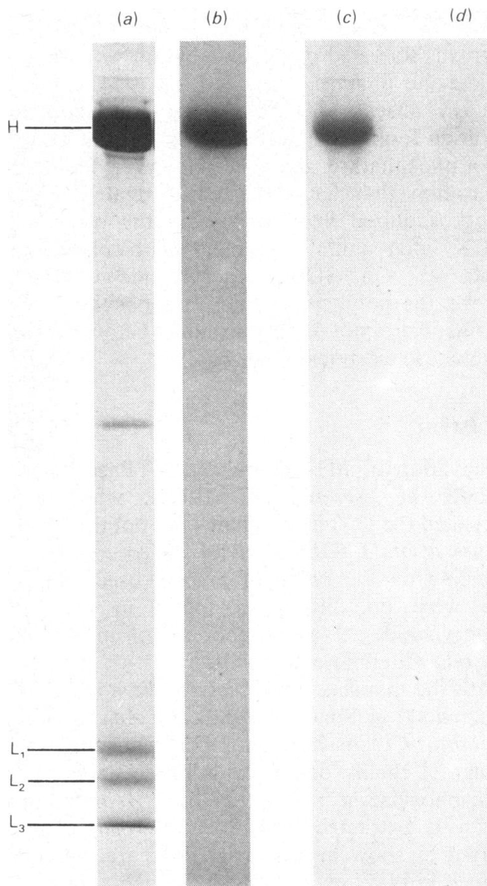
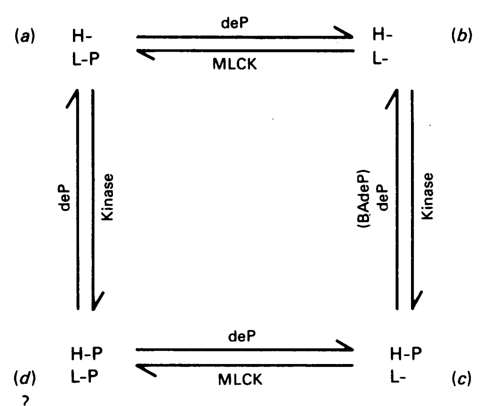


Fig. 2. *SDS/polyacrylamide-gel electrophoresis of M1-cell myosin incubated with* [^{32}*P*]*orthophosphate*

The myosin was purified from M1 cells that had been incubated with [^{32}P]orthophosphate as described in the Materials and methods section. Slot (*a*) shows the myosin preparation stained with Coomassie Brilliant Blue. The smallest light chain, L_3 (M_r 16000), was electrophoresed with Bromophenol Blue in this 10% (w/v) polyacrylamide gel. Slot (*b*), the fluorograph of slot (*a*), shows the ^{32}P incorporated into the heavy chain. The labelled myosins were incubated with (slot *d*) and without (slot *c*) bacterial alkaline phosphatase for 24 h at room temperature, then precipitated by adding 10% (w/v) trichloroacetic acid. Their precipitates were rinsed with acetone and diethyl ether, then dissolved in the sample buffer for SDS/polyacrylamide-gel electrophoresis, after which they were fluorographed.



Scheme 1. *Regulation of M1-cell myosin ATPase by phosphorylation*

Abbreviations used: H, heavy chain; L, light chains L_1 and L_2; P, incorporated phosphate; MLCK, M1-cell myosin light-chain kinase; deP, phosphatase; BAdeP, bacterial alkaline phosphatase.

smaller amount of phosphate than that extracted with 0.34 M-sucrose. Thus we assume that the variations found for the actin-activated myosin ATPase of M1 cells may be due to different extents of phosphorylation of the H-chain of myosin molecules.

Although purified M1-cell myosin incubated with [^{32}P]ATP solution in the presence of MLCK incorportated ^{32}P into its L_1- and L_2-chains, incubation of viable M1 cells with [^{32}P]orthophosphate phosphorylated the H-chain only. This phosphate incorporated into the H-chain was readily released on treatment with bacterial alkaline phosphatase. Further evidence supporting our assumption is that the myosin sample with low actin-activated ATPase showed higher actin activation after the myosin was treated with bacterial alkaline phosphatase. Unlike the myosin from *Acanthamoeba* and *Dictyostelium*, the activation of phosphatase-treated M1-cell myosin still required the presence of MLCK.

Our results suggest that the interaction of myosin ATPase with actin may be regulated by the phosphorylation of both the light chains (L_1 and L_2) and the heavy chain; with the light chains the action is stimulative, and with the heavy chain depressive. Therefore we have devised a scheme (Scheme 1) that shows four states of phosphorylation of M1 myosin.

Of these four states, only myosin A (in which only the light chain is phosphorylated) is capable of interacting with actin; this activates the ATPase. Myosin B and C represent inactive states. With myosin C, dephosphorylation of the H-chain is a prerequisite for activation by actin in the presence of MLCK. Otherwise, this myosin may be converted into inactive myosin D by MLCK. Myosin D, however, has yet to be identified in M1 cells. So the following possibility is not excluded: the phos-

phorylation of heavy chain prevents the phosphorylation of light chains and thus inhibits the activation of myosin ATPase by actin. According to our scheme, variations in the actin-activated ATPase of isolated myosin, even in the presence of MLCK, can be explained by the different contents of myosin B and C in individual myosin samples. This scheme also indicates that a heavy-chain kinase that differs from MLCK and endogenous phosphatase (about which nothing is known so far) are involved. Whether the differing activities of actin-activated myosin ATPase found at different growth stages of cells and with different extraction solutions is accounted for by differing contents of endogenous phosphatase in each sample requires further study.

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