PHOSPHORYLATION OF FIBROBLAST MYOSIN

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1. Introduction

The fact that myosin might be phosphorylated has been known for more than a decade [1]. However, only recently have special kinases been described, in striated muscle [2] and in platelets [3], which catalyze the phosphorylation of one of the light-chains of myosin. The myosin light-chain kinase isolated from platelets was found to be capable of transferring the terminal-phosphate of γ-labeled $AT^{32}P$ to the 20 000 dalton light-chain of platelet myosin and to the same light-chain in smooth-muscle, fibroblast and rhabdomyosarcoma myosin but it does not affect striated muscle myosin [4]. It was suggested that this phenomenon may have a physiological significance as the actin-activated ATPase activity of platelet myosin increases after phosphorylation [5]. Considering the possibility that the phosphorylation of myosin may play a role in the regulation of cell mobility it was interesting to look for endogenous myosin phosphorylating activity in other non-muscle cells such as fibroblasts.

So far there is only one indication (in the case of cardiac myosin [6,7]) that the heavy-chain of myosin, in addition to 20 000 and 18 500 dalton light-chains, might be phosphorylated. In the following we will show that both heavy and light chains of fibroblast myosin can be phosphorylated by incubating fibroblast extracts with ATP.

2. Experimental procedure

Mouse 3T3 cells (obtained from Miss T. Koch, Weizmann Institute of Science) were grown on Falcon plastic dishes in Dulbecco's modified Eagle medium supplemented with 10% calf serum, 100 U/ml penicillin and 100 μg/ml streptomycin.

Platelet myosin light-chain kinase was prepared from human platelets by the method of Daniel and Adelstein [3].

Isolation of fibroblast actomyosin was carried out with some modification according to Ostlund and Pastan [8]. All manipulations were accomplished at 0–4°C. After reaching confluency, the cells were washed twice with phosphate buffered saline (0.15 M NaCl, 135 mM potassium phosphate and 1 mM DTT, pH 7.0) and twice with 0.15 M NaCl and 1 mM DTT. They were then scraped from the surface of the container by means of a bent glass-pipette, suspended in 0.15 M NaCl and 1 mM DTT and centrifuged at 8000 rev/min for 10 min. The cell-pellet obtained was frozen and kept at −20°C. Samples were thawed and extracted with four vol. 0.6 M KCl, 2 mM DTT, 20 mM Tris–acetate, pH 7.4 and 3% butanol for 1 h with intermittent manual stirring. The resulting cell-lysate was centrifuged at 12 000 rev/min for 45 min and dialyzed against 100 vol. 30 mM KCl, 1 mM EDTA and 10 mM imidazole, pH 7.0, for 2–8 h. The actomyosin fraction which was precipitated as a con-

sequence of the lowering of the ionic strength was collected by centrifugation at 10 000 rev/min for 10 min. The actomyosin pellet was dissolved in one-fifth of the original volume of 0.6 M KCl, 1 mM DTT, 1 mM EDTA and 20 mM Tris–acetate, pH 7.4.

Either the supernatant of the cell-lysate or the dissolved actomyosin after dialysis were phosphorylated, essentially by the method of Daniel and Adelstein [3]. [γ-^{32}P]ATP (purchased from The Nuclear Research Center, Negev, Israel) was premixed with cold ATP to give a final radioactivity of 20 μCi/ml. The incubation mixture contained: 0.6 M KCl, 1 mM DTT, 20 mM Tris–acetate, pH 7.4, 3 mM $MgCl_2$ and 31 μM ATP. The reaction (at 37°C for 15 min) was stopped by the addition of 5 mM EDTA which inhibits both kinase and phosphatase activities. Actomyosin was precipitated from the phosphorylated lysate and dissolved as described above.

The phosphorylated actomyosin preparations were applied to a Sepharose-4B column (0.9 X 40 cm) which was equilibrated with the same medium and chromatography was accomplished at a rate of about 3 ml/h.

SDS–Polyacrylamide (5.5%) gel electrophoresis was performed following the method of Fairbanks [9]. The gels were stained with Coomassie Brilliant Blue and scanned by the Gilford Gel Scanner at 540 nm. After scanning, the gels were cut to 4 mm slices with a razor-blade and their radioactivity determined in a toluene–PPO cocktail using a Packard Tricarb liquid scintillation spectrometer.

K^+-Activated ATPase activity, expressed as nmol P_i/min/ml eluate, was measured at 25°C in a 4 ml solution containing 100 μg protein, 0.6 M KCl, 1 mM ATP, 20 mM Tris–acetate, pH 8.0 and 5 mM EDTA (the latter was present in order to chelate Mg^{2+} [10]). The P_i released was measured by the modified Fiske-Subbarow procedure of Bárány et al. [11].

3. Results

The elution profiles of the actomyosin obtained from the phosphorylated lysate, with respect to protein content, radioactivity and K^+-activated ATPase activity are shown in fig.1. The protein peak in the void volume exhibited K^+-activated ATPase activity which is characteristic of myosin ATPase. Part of the

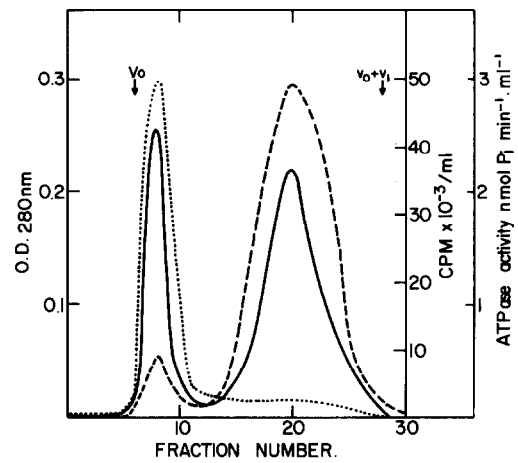

Fig.1. Chromatography on Sepharose-4B of actomyosin obtained from phosphorylated fibroblast lysate. Actomyosin solution (0.3 ml, 0.5 mg/ml) was applied to the column. Fractions (1.5 ml) were collected and analyzed for protein content (optical density at 280 nm (———)); radioactivity (cpm X 10^{-3} (- - - -)) and K^+-activated ATPase activity, nmol Pi/min/ml (. . . . .). Void volume and total volume of the column are indicated at v_o and $v_o + v_i$ respectively.

^{32}P-radioactivity was associated with this peak suggesting that phosphorylation took place. The free [γ-^{32}P]ATP and ^{32}P, were eluted in a second peak which also contained small molecular weight proteins. Analysis by SDS–polyacrylamide gel electrophoresis (fig.2) of the first peak showed that it consisted mainly of myosin and actin; however, the presence of other minor constituents can also be seen, one of them having a molecular weight higher than that of the heavy-chain of myosin. These minor constituents do not contain radioactivity above the background level. Of the total radioactivity, 50% was found to be attached to the heavy-chain and 40% to the 20 000 dalton light-chain of myosin, indicating that both subunits have been phosphorylated.

The distribution of protein, radioactivity and ATPase activity was essentially the same as in fig.1 when phosphorylation was accomplished at a later stage, i.e. when [^{32}P]ATP was applied to the actomyosin obtained after precipitation at low ionic strength and dissolution. However, SDS–gel electrophoresis (fig.3) revealed that now 80% of the radioactivity was attached to the 20 000 dalton light-chain of myosin while the phosphorylation of the myosin heavy-chain was negligible.

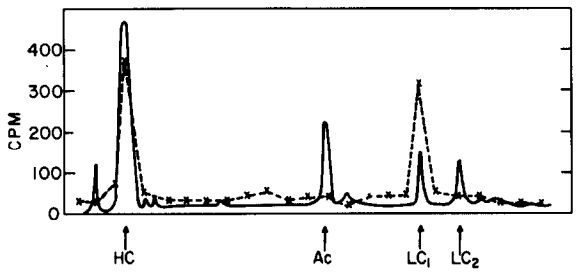

Fig.2. Densitometer and radioactivity tracing of SDS–polyacrylamide gel electrophorogram of the collected fractions of the first, unabsorbed, peak of the Sepharose-4B gel chromatography (shown in fig.1). The protein was concentrated by dialysis against 40% polyethylene glycol (20 000 dalton) and 50 μg/protein applied to the polyacrylamide column. Symbols: HC, myosin heavy-chain (200 000 dalton); A_C, actin (43 000 dalton); LC_1, myosin light-chains 20 000 dalton; LC_2, myosin light-chains 15 000 dalton. The positions of these polypeptide chains were verified by using authentic markers: chicken-gizzard myosin [14] and chicken-breast actin [15]. Densitometer (———) and radioactivity (X——X).

We attempted to phosphorylate myosin which might be located on the outside surface of the plasma membrane of fibroblasts. To achieve this myosin light-chain kinase isolated from platelets and [γ-^{32}P]ATP were added to intact 3T3 cells which grew on the surface of Falcon plastic dishes. The cells were harvested and their actomyosin isolated as described in Experimental procedure. No radioactivity associated with myosin could be detected. This experiment was repeated using intact cells in suspension. In this case cells were harvested by the addition of either 0.25% trypsin or 0.2% EDTA (both in phosphate buffered saline). Phosphorylation of myosin could not be observed in either of these experiments

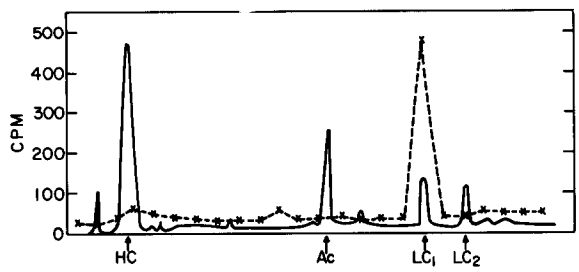

Fig.3. Densitometer and radioactivity tracing of SDS–polyacrylamide gel electrophorogram of the collected fractions of the first peak of the Sepharose-4B chromatography of actomyosin which was phosphorylated just before chromatography. For details and symbols, see figs.1 and 2.

4. Discussion

Both the heavy- and the 20 000 dalton light-chains of fibroblast myosin could be phosphorylated by endogenous kinases if [γ-^{32}P]ATP was added to the supernatant of cell-lysates. However, only the 20 000 dalton light-chain was labeled if the phosphorylation reaction was carried out after precipitating the actomyosin from the lysate. It thus appears that two different myosin kinases are present in fibroblasts: one is responsible for the phosphorylation of the heavy-chain while the other can phosphorylate the light-chain. The light-chain kinase apparently co-precipitates with myosin when the ionic strength is lowered (and also fractionates with myosin during ammonium sulphate fractionation [5]) while the heavy-chain kinase is probably less strongly bound and could have been removed from the fraction containing myosin by lowering the ionic strength. Such an interpretation may account for the fact that phosphorylation of the heavy chain of cardiac myosin [6,7] could so far be achieved only in in vivo experiments while in more or less purified in vitro systems the phosphorylation of light-chains only could be observed [2,3].

The extent of phosphorylation of myosin was calculated from the specific activity of [γ-^{32}P]ATP and was found to be 0.3–0.5 mol/mol light- or heavy-chain. This value could not be increased by the addition of exogenous platelet light-chain kinase. One may assume that the reason for this rather low extent of phosphorylation is the presence in fibroblasts of an active myosin phosphatase. A finding which supports this assumption is that in the absence of EDTA the amount of phosphate bound to myosin quickly decreases with time so that if chromatography was performed 24 h after phosphorylation only traces of radioactivity were associated with the myosin fraction.

We could not detect any phosphorylated myosin when platelet kinase and [γ-^{32}P]ATP were added to 3T3 mouse fibroblasts cells. On the other hand, after our experiments have been completed, it was reported by Olden et al. [12] that labeling of the surface components of L-929 mouse fibroblasts with ^{125}I by the lactoperoxidase-catalyzed iodination procedure revealed the presence of myosin. A more careful re-examination of our preliminary experiments [13] on the inhibition of normal and SV40 transformed fibroblast (mouse 3T3) aggregation by rabbit skeletal-

muscle F-actin showed that the effect could be partially reversed by ATP or pyrophosphate (unpublished results) suggesting the binding of the added F-actin to surface myosin as well as to some other receptor. It could be that while surface myosin was accessible to iodination, its light-chains were not within reach of exogenous blood platelet light-chain kinase.

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