Vol. 102, No. 1, 1981 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
September 16, 1981 Pages 149-157

PHOSPHORYLATION OF SMOOTH MUSCLE ACTIN BY THE CATALYTIC SUBUNIT OF THE cAMP-DEPENDENT PROTEIN KINASE

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Received July 27,1981

SUMMARY

Partially purified smooth muscle (chicken gizzard) actomyosin contains two major substrates of cAMP-dependent protein kinase: a protein of M_r = 130,000, identified as the calmodulin-dependent myosin light chain kinase, and a protein of M_r = 42,000. This latter protein was shown by a variety of electrophoretic procedures to be actin. Purified smooth muscle actin also was phosphorylated by the catalytic subunit of cAMP-dependent protein kinase. The rate of phosphorylation of smooth muscle actin was significantly enhanced by depolymerization of actin. A maximum of 2.0 mol phosphate could be incorporated per mol G-actin. Skeletal muscle F-actin was not significantly phosphorylated by protein kinase; however, skeletal G-actin is a substrate for the protein kinase although its rate of phosphorylation was significantly slower than that of smooth muscle G-actin.

INTRODUCTION

It is generally recognized that relaxation of smooth muscle is controlled via a cAMP-dependent process (1,2). To date most of the attention has been centered on the influence of cAMP-induced phosphorylation on membrane associated processes (2-7). More recently, however, the possibility of regulation by cAMP at the level of the contractile proteins has become apparent (8). Adelstein et al. (9,10) showed that isolated turkey gizzard myosin light chain kinase can be phosphorylated by the catalytic subunit of cAMP-dependent protein kinase. This phosphorylation decreased the affinity of the myosin kinase for calmodulin, the Ca^{2+}-binding protein essential for the activity of the myosin kinase (11). It was suggested that the physiological effect of this phosphorylation would be a reduction in the activity of the

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Abbreviations: EGTA, ethylene glycol bis(β-aminoethyl ether)-N,N^1-tetraacetic acid; MLCK, myosin light chain kinase; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate.

0006-291X/81/170149-09$01.00/0

myosin kinase which would facilitate the subsequent relaxation of the muscle (9).

With a view to gaining further insight into the molecular events following an elevation in the level of cytosolic cAMP, we examined smooth muscle (chicken gizzard) actomyosin preparations for the presence of substrates of cyclic AMP-dependent protein kinase. In this communication we report that, in addition to myosin light chain kinase, actin is also a substrate of the protein kinase.

MATERIALS AND METHODS

[γ-^{32}P]ATP was purchased from New England Nuclear. The purified catalytic subunit of bovine heart cAMP-dependent protein kinase was from Sigma Chemical Co. (St. Louis, MO). The following proteins were prepared by methods described earlier: actomyosin (12), actin from smooth muscle thin filaments (13), skeletal muscle actin (14), smooth muscle tropomyosin (14,15) and chicken gizzard myosin light chain kinase (16). F-actins of both smooth and skeletal muscles were depolymerized as described by Kasai et al. (17).

Protein concentrations were determined by the biuret method (18) or according to Spector (19).

Polyacrylamide gradient slab gel electrophoresis in the presence of 0.1% SDS (SDS-gradient PAGE) was carried out at 30 mA employing the discontinuous buffer system of Laemmli (20). In order to separate smooth muscle actin and one of the tropomyosin components, which co-migrate on SDS-gradient PAGE, an identical slab gel was run with 8 M urea included in both the samples and the running gel. Coomassie blue-stained gels and autoradiograms were scanned at 550 nm using a Zeiss Spektralphotometer PM 6 attached to a Spectra-Physics SP 4050 printer/plotter, an SP 4020 data interface and an SP 4000 central processor. ^{32}P was detected in gel slices, after staining and destaining, by Cerenkov counting in plastic scintillation vials containing 15 mL deionized water.

RESULTS

Fig. 1 shows the effects of incubating a preparation of smooth muscle actomyosin with [γ-^{32}P]ATP in the presence and absence of the purified catalytic subunit of cAMP-dependent protein kinase. In the presence of protein kinase catalytic subunit, three phosphorylated proteins were observed of M_r = 130,000, 42,000 and 20,000. In the absence of the protein kinase, only the 20,000 dalton protein was phosphorylated. Based on its molecular weight and relative amount, this represented the regulatory light chain of myosin

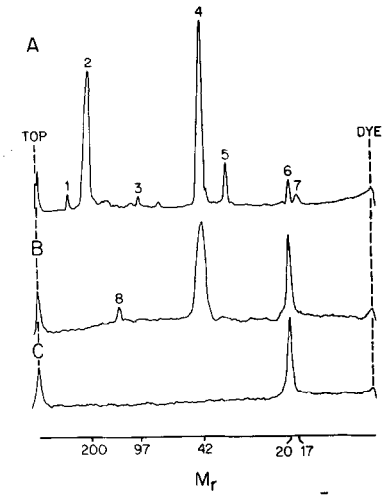

Fig. 1. Phosphorylation of actomyosin preparation by the catalytic subunit of cAMP-dependent protein kinase. Chicken gizzard actomyosin (1 mg/mL) was incubated with 25 mM Tris-HCl, pH 7.5, 4 mM $MgCl_2$, 1 mM EGTA, 1.4 mM [γ-^{32}P] ATP ($\sim$ 10,000 cpm/nmol), in the presence and absence of the purified catalytic subunit of cAMP-dependent protein kinase (E/S = 1:100), for 15 min at 25°C. Reactions were quenched by addition of an equal volume of boiling 0.5 M Tris-HCl, pH 6.8, 1% SDS, 30% glycerol, 1% 2-mercaptoethanol, 0.01% bromphenol blue (stop solution). Aliquots (50 µg protein) were subjected to SDS-gradient PAGE. A, densitometric scan at 550 nm of the Coomassie blue-stained gel. B and C, scans of autoradiograms of gels of actomyosin incubated with and without protein kinase, respectively. Major protein bands are identified by number as follows: 1 = filamin, 2 = myosin heavy chain, 3 = α-actinin, 4 = actin, 5 = tropomyosin, 6 = 20K light chain of myosin, 7 = 17K light chain of myosin, 8 = myosin light chain kinase. M_r = molecular weight x 10^{-3}.

that was phosphorylated by trace amounts of the Ca^{2+}-independent form of the myosin light chain kinase. In separate experiments, myosin light chain phosphorylation was enhanced greater than 10-fold in the presence of Ca^{2+} (data not shown). It was concluded, therefore, that the two higher M_r proteins were substrates of the cAMP-dependent protein kinase. The 130,000 dalton phosphoprotein can be identified as myosin light chain kinase since it co-migrated on SDS-polyacrylamide gradient gels with purified phosphorylated chicken gizzard myosin light chain kinase.

The 42,000 dalton protein was either actin, one of the tropomyosin components which in our electrophoretic system co-migrated with actin, or an unidentified protein. The possibility of phosphorylation of the tropomyosin subunit was discounted by electrophoresis in the presence of both SDS and

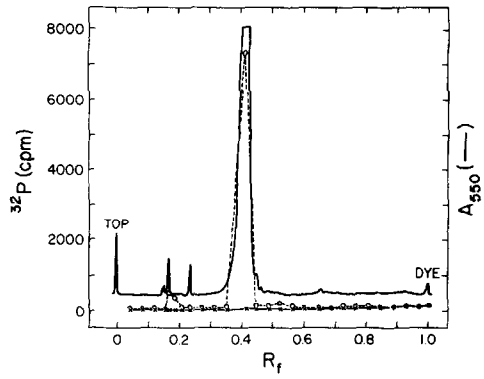

Fig. 2. Phosphorylation of purified smooth muscle actin by the catalytic subunit of cAMP-dependent protein kinase. Chicken gizzard actin (0.8 mg/mL) was incubated with 25 mM Tris-HCl, pH 7.5, 4 mM $MgCl_2$, 1 mM EGTA, 1.0 mM [γ-^{32}P] ATP ($\sim$ 10,000 cpm/nmol), in the presence and absence of the purified catalytic subunit of cAMP-dependent protein kinase (E/S = 1:100), for 4 h at 25°C. Aliquots were removed for determination of the stoichiometry of phosphate incorporation as described by Mrwa and Hartshorne (22). Remaining reaction mixtures were quenched by addition of an equal volume of boiling stop solution. Aliquots (10 µg protein) were subjected to SDS-gradient PAGE. Coomassie blue-stained gels were scanned at 550 nm (——) and then sliced for ^{32}P quantitation: samples treated with protein kinase catalytic subunit (O- - -O) and appropriate controls (X——X).

urea (21); ^{32}P counting of gel slices showed that the radioactive label remained with the actin band (R_f = 0.57), and the well-separated tropomyosin bands (R_f = 0.475 and 0.488) were not phosphorylated. These observations were confirmed by autoradiography. Furthermore, pure smooth muscle tropomyosin was not phosphorylated by the catalytic subunit of cAMP-dependent protein kinase (data not shown). One of the possibilities was therefore eliminated and the remaining choices for the labeled protein were either actin or an unidentified component.

In order to distinguish between these possibilities, smooth muscle actin was purified and examined as a possible substrate of cAMP-dependent protein kinase. A gel scan of the actin preparation showed it to be > 95% pure (Fig. 2). This preparation was phosphorylated (as described in the legend to Fig. 2) and 0.57 mol phosphate was incorporated per mol actin in the presence of protein kinase catalytic subunit, whereas no phosphorylation occurred in the absence of the catalytic subunit. The identity of the phos-

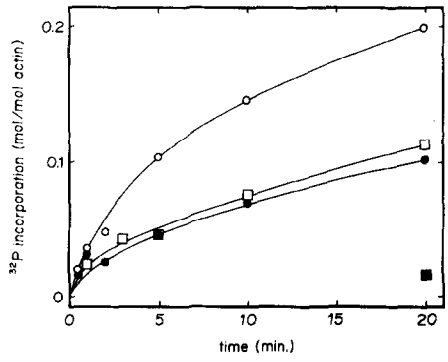

Fig. 3. Comparison of the rates of phosphorylation of smooth and skeletal muscle actins catalyzed by cAMP-dependent protein kinase. Smooth muscle G-(O) and F-(●) actins purified directly from thin filaments, and skeletal muscle G-(□) and F-(■) actins were incubated with the catalytic subunit of protein kinase under conditions described in the legend to Fig. 2, with the exception that the specific activity of [γ-^{32}P]ATP was ∿ 30,000 cpm/nmol. Phosphate incorporation was determined as described (22). Controls from which protein kinase catalytic subunit was omitted showed no ^{32}P incorporation.

phorylated protein was determined following SDS-gradient PAGE (see Materials and Methods) and the radioactivity profiles are shown in Fig. 2. Based on these data it is apparent that actin can be phosphorylated by the cAMP-dependent protein kinase. The identity of the phosphoprotein as actin was confirmed by isoelectric focusing, two dimensional electrophoresis and peptide mapping (data to be presented in a later publication).

Fig. 3 compares the initial rates of phosphorylation of smooth and skeletal muscle G- and F-actins catalyzed by the catalytic subunit of cAMP-dependent protein kinase. Smooth muscle F-actin was phosphorylated at a significant rate which was enhanced by depolymerization. On the other hand, no significant phosphate incorporation into skeletal F-actin occurred. Skeletal G-actin, however, was phosphorylated and is clearly a substrate of cAMP-dependent protein kinase. No phosphate incorporation was observed in the absence of the catalytic subunit of the cAMP-dependent protein kinase.

It was observed during these experiments that the catalytic subunit of the cAMP-dependent protein kinase was progressively inactivated under the

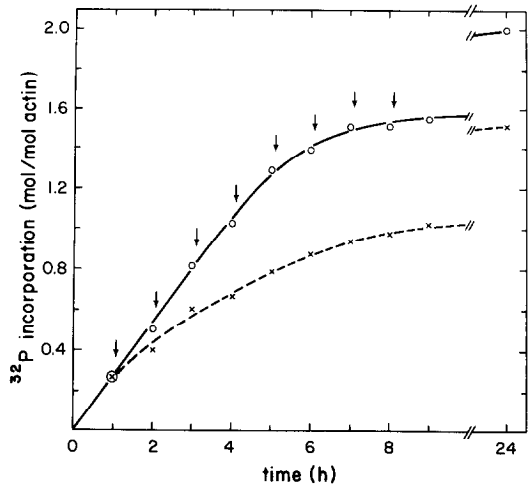

Fig. 4. The effect of periodic addition of protein kinase catalytic subunit on the phosphorylation of smooth muscle actin. Duplicate samples of chicken gizzard G-actin were phosphorylated as described in the legend to Fig. 3. Aliquots (0.25 mL) were withdrawn at selected time intervals for quantitation of protein-bound ^{32}P. Aliquots of the catalytic subunit of cAMP-dependent protein kinase were added to one actin sample (O——O) at regular intervals as indicated by the arrows; the ratio of E/S added each time was 1:100. No further addition of the catalytic subunit was made to the control actin sample (X- - -X).

chosen assay conditions. Therefore, in order to determine the maximum extent of phosphorylation of smooth muscle G-actin it was necessary to make repeated additions of the catalytic subunit and to follow the phosphorylation over a prolonged time period. These data are shown in Fig. 4. Up to 2 mol phosphate per mol actin could be incorporated under these conditions. (The time course of phosphorylation of G-actin with the catalytic subunit added only at the beginning of the experiment is also shown in Fig. 4.)

DISCUSSION

Investigations into regulation of smooth muscle contraction by cAMP at the level of contractile proteins to date has centered on phosphorylation of calmodulin-dependent myosin light chain kinase (9,10). Silver and DiSalvo (23) also demonstrated that myosin light chain phosphorylation in an actomyosin preparation from bovine aorta was diminished in the presence of cAMP and

cAMP-dependent protein kinase. This inhibition of myosin light chain phosphorylation was correlated with phosphorylation of a 100,000 dalton protein, tentatively identified as myosin light chain kinase, and with a decrease in the actomyosin Mg^{2+}-ATPase activity (23,24). It was pointed out, however, that actin or an unidentified co-migrating protein was also phosphorylated in the presence of cAMP and its dependent protein kinase (23). Our data suggest that this protein is actin.

The maximum extent of phosphorylation was determined to be 2 mol phosphate per mol G-actin. That this is specific for two sites in the actin molecule was suggested by isoelectric focusing and peptide maps (manuscript in preparation). Examination of the amino acid sequences of smooth (25) and skeletal (26) muscle actins, and in light of the known substrate requirements of the cAMP-dependent protein kinase (27), two likely phosphorylation sites were suggested, namely, serine 199 and serine 337. These serine residues have the neighboring sequences Arg-Gly-Tyr-Ser and Arg-Lys-Tyr-Ser, respectively.

Several previous reports have demonstrated the phosphorylation of actin-like proteins in various systems. Pratje and Heilmeyer (28) showed that skeletal muscle F-actin was phosphorylated by cAMP-dependent protein kinase but the level of incorporation was very low. This can be explained by our finding that G-actin is a preferred substrate for the cAMP-dependent protein kinase. Steinberg (29) observed that the nascent form of actin in S-49 mouse lymphoma cells was phosphorylated by cAMP-dependent protein kinase, although the level of incorporation was relatively low. It would be interesting to determine the difference, if any, between nascent actin and G-actin which as described above is phosphorylated by the cAMP-dependent protein kinase. Possibly the polymerization of the nascent form, or its combination with actin-binding proteins, renders it inaccessible to the cAMP-dependent protein kinase. Grazi et al. (30,31) have also shown that skeletal G- and F-actins interact with liver plasma membranes and become phosphorylated. However,

there is no indication that this was a cAMP-dependent process. The phosphorylation of the G-actin resulted in a loss of its ability to polymerize and to inhibit DNase I (31). Another membrane-associated phosphorylation of an actin-like protein was reported by Hofstein et al. (32), although once again the influence of cyclic nucleotides was not established.

Clearly the most interesting point to determine is whether or not the phosphorylation of actin plays a physiological role. From preliminary experiments we have observed that phosphorylation of G-actin prolongs the polymerization time and relieves the inhibition of DNase I, which is consistent with the findings of Grazi et al. (31). If one considers these results together with the fact that G-actin is a preferred substrate, then it is tempting to speculate that the physiological role of actin phosphorylation might be restricted to those cells (non-muscle) in which a pool of G-actin exists and may be involved in the regulation of actin polymerization.

ACKNOWLEDGMENTS

This work was supported by grants HL 23615 and HL 20984 from the National Institutes of Health. The authors are grateful to Ms. Maxine Celi for typing the manuscript.

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