Phosphorylation of smooth muscle myosin light chain by five different kinases

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Phosphorylation of the 20 kDa myosin light chain from smooth muscle by five different kinases was investigated. Three of the kinases (myosin light chain kinase, phosphorylase kinase, and cAMP-dependent protein kinase) phosphorylate serine residues, the fourth (casein-kinase-2) mainly threonine, and the fifth (glycogen synthase (casein) kinase-1) both serine and threonine. Isoelectric focusing analyses of ^{32}P-labelled chymotryptic peptides indicate that phosphorylase kinase and cAMP-dependent protein kinase phosphorylate the same site as myosin light chain kinase. However, both casein kinase-2 and glycogen synthase (casein) kinase-1 phosphorylate different sites.

Phosphorylation *Myosin light chain* *Calcium* *Cyclic AMP* *Isoelectric focusing* *Casein kinase*

1. INTRODUCTION

Phosphorylation of the 20 kDa light chains of smooth muscle myosin has been shown to regulate the actin-activated myosin ATPase activity [1]. These light chains are specifically phosphorylated by the Ca^{2+}, calmodulin-dependent light chain kinase [2]. However, kinases that are independent of Ca^{2+} and calmodulin have also been reported to phosphorylate the light chains [3–6]. It is not clear what relationship, if any, these kinases have to the Ca^{2+}, calmodulin-dependent enzyme. However, the latter enzyme becomes activated after mild proteolysis and loses its requirement for Ca^{2+} and calmodulin [7].

We have found that cyclic nucleotide- and Ca^{2+}-independent glycogen synthase (casein) kinase-1 (CK-1) also phosphorylates smooth muscle myosin light chain [8]. This prompted us to investigate the phosphorylation of myosin light chain by other glycogen synthase kinases. Here, we compare the phosphorylation of smooth muscle myosin light chain by CK-1, cAMP-independent casein kinase-2 (CK-2), phosphorylase kinase, cAMP-dependent protein kinase (A-kinase), and smooth muscle myosin light chain kinase.

2. EXPERIMENTAL

Myosin light chains and light chain kinase [2] were purified from turkey gizzard. The kinase was homogeneous whereas the light chain preparation was a mixture of the 20 kDa and 17 kDa light chains. The other kinases were from rabbit skeletal muscle. Both phosphorylase kinase [9] and CK-2 [12] were homogeneous preparations. A-kinase [10] and CK-1 [11] were highly purified according to the published methods.

Phosphorylation of the light chain by CK-1 or CK-2 was done at 30°C in a reaction mixture (0.05 ml) containing Tris–HCl buffer (pH 7.4), 25 mM; myosin light chain, 0.3 mg/ml; dithiothreitol, 2.5 mM; EGTA, 0.5 mM; KF, 5 mM; magnesium acetate, 9 mM; and [γ-^{32}P]ATP, 0.1 mM. The reaction mixtures also contain 5 μM cAMP for A-kinase, 0,1 mM $CaCl_2$ without EGTA for phosphorylase kinase, and 0.1 mM $CaCl_2$ and 0.5 μM calmodulin without EGTA for myosin light chain

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kinase. Reactions were initiated by the addition of 40 milliunits of A-kinase, CK-1, or CK-2; 1 μg/ml of myosin light chain kinase; or 5 μg/ml of phosphorylase kinase. Aliquots of the reaction mixtures were taken at timed intervals for the determination of ^{32}P incorporation as in [13]. One unit of A-kinase and CK-1 (or CK-2) is defined as the amount of enzyme catalyzing the incorporation of 1 nmol phosphate from [γ-^{32}P]ATP into 4 mg/ml of histone IIA and casein, respectively, per minute at 30°C.

^{32}P-Labelled light chain was hydrolyzed in 6 N HCl for 2 h at 110°C and phosphoamino acids analyzed by high-voltage electrophoresis at pH 3.5 [14]. Chymotryptic peptides were prepared and resolved by isoelectric focusing as in [15].

3. RESULTS

The 20 kDa myosin light chain from smooth muscle is phosphorylated by myosin light chain kinase [2], A-kinase [16], Ca^{2+}- and phospholipid-dependent kinase [17], and CK-1 [8]. In addition to these kinases, we found that CK-2 and phosphorylase kinase also phosphorylate the light chain (fig. 1A). Only the 20 kDa light chain is a substrate for all 5 kinases tested here (fig. 1B). No phosphorylation of the light chain was observed in the absence of added kinase. About 1 mol ^{32}P is incorporated into the light chain in reactions catalyzed by each of the kinases, with the exception of CK-1 which catalyzes a slightly higher extent of phosphorylation. Analysis of the amino acid residues in 20 kDa myosin light chain phosphorylated by these 5 kinases reveals that myosin light chain kinase, A-kinase, and phosphorylase kinase only phosphorylate serine (fig. 2). Myosin light chain kinase and A-kinase phosphorylated a serine residue in the myosin light chain [17]. CK-1 and CK-2 phosphorylate both

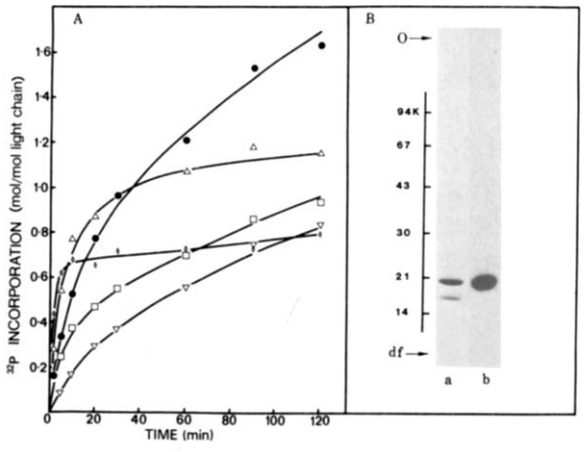

Fig. 1. Phosphorylation of myosin light chain by different kinases: (A) myosin light chains were phosphorylated under standard conditions by CK-1 (●), CK-2 (△), myosin light chain kinase (◇), phosphorylase kinase (□), and A-kinase (▽); (B) myosin light chains were resolved on a 7.5–20% gradient SDS–polyacrylamide slab gel after phosphorylation by CK-2; (a) Coomassie blue-stained gel; (b) autoradiogram of stained gel. Only the 20 kDa light chain was phosphorylated when the reaction was catalyzed by A-kinase, CK-1, phosphorylase kinase and myosin light chain kinase: (O) origin; (df) dye front.

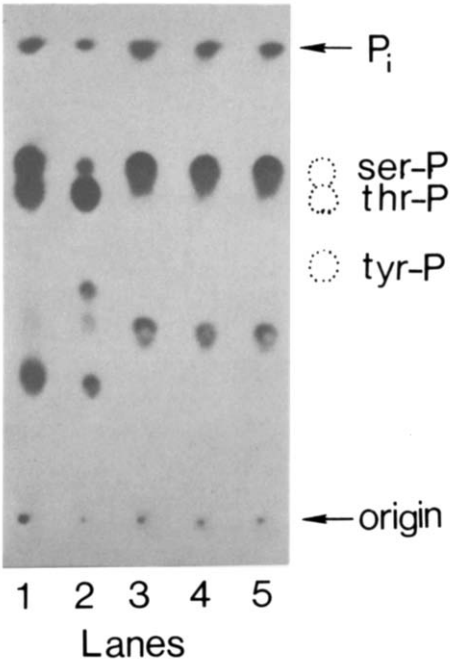

Fig. 2. Phosphoamino acid analysis of the light chain phosphorylated by different kinases. Myosin light chain was phosphorylated by CK-1 (lane 1), CK-2 (lane 2), A-kinase (lane 3), phosphorylase kinase (lane 4), and light chain kinase (lane 5) under standard conditions for 30 min. The phosphorylated light chain was then hydrolyzed in a 6 N HCl for 2 h at 110°C and analyzed by high-voltage electrophoresis at pH 3.5. Phosphorylated serine, threonine, and tyrosine were run as standards.

serine and threonine residues; however, the extents of phosphorylation of these two amino acids by these two kinases are different. CK-2 favors the phosphorylation of threonine (thr-P has 10-times as much radioactivity as ser-P) (fig. 2, lane 2) whereas CK-1 phosphorylates serine more favorably than threonine (the radioactivity in ser-P is 1.5-times more than thr-P) (fig. 2, lane 1). None of these kinases phosphorylates tyrosine.

A-kinase and myosin light chain kinase phosphorylate the same site on the light chain [16]. Addition of A-kinase or phosphorylase kinase after an initial phosphorylation of the light chain with myosin light chain kinase does not result in any additional phosphorylation. Further phosphorylation is observed, however, when CK-1 and CK-2 are separately added (fig. 3). These results suggest that phosphorylase kinase phosphorylates the same site as does A-kinase and the light chain kinase whereas CK-1 and CK-2

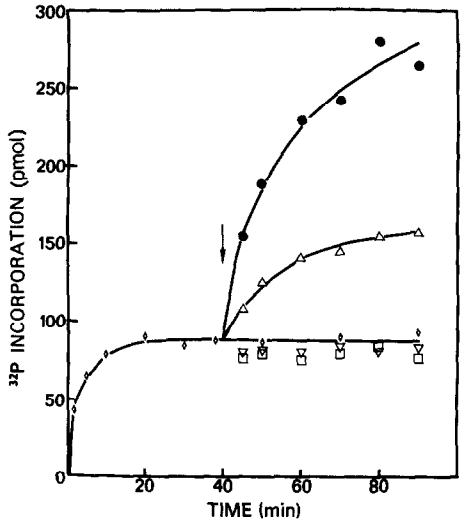

Fig. 3. Phosphorylation of myosin light chain by the different kinases after an initial phosphorylation with myosin light chain kinase. The light chain was initially phosphorylated by myosin light chain kinase (◇) under standard assay condition. After 40 min (↓) aliquots of the reaction mixture were removed and supplemented with: 0.5 mM EGTA and CK-1 (●); 0.5 mM EGTA and CK-2 (△); 0.5 mM EGTA, 5 μM cyclic AMP and A-kinase (▽); and phosphorylase kinase (□). The incubation was continued and samples were removed at different times for the determination of phosphate incorporation.

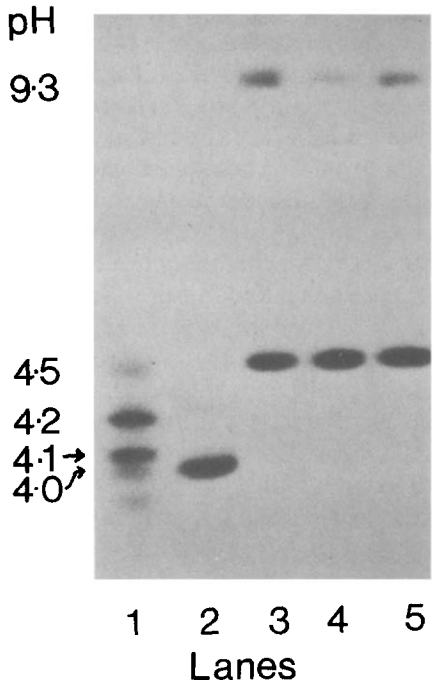

Fig. 4. Comparison of the ^{32}P-labeled chymotryptic peptides derived from light chain phosphorylated by different kinases. Light chain was phosphorylated by the various kinases for 30 min at 30°C under standard conditions and chymotryptic peptides were prepared. Isoelectric focusing of the chymotryptic peptides phosphorylated by CK-1 (lane 1), CK-2 (lane 2), A-kinase (lane 3), phosphorylase kinase (lane 4), and light chain kinase (lane 5) was done as in section 2.

phosphorylate different sites. Isoelectric focusing analysis indicates that the major chymotryptic peptides of the light chain phosphorylated by myosin light chain kinase, A-kinase, and phosphorylase kinase comigrate (fig. 4, lanes 3–5). These peptides are different from those phosphorylated by CK-1 (fig. 4, lane 1), and CK-2 (fig. 4, lane 2). The results indicate that CK-1 and CK-2 phosphorylate myosin light chain at sites different from that phosphorylated by A-kinase, myosin light chain kinase, and phosphorylase kinase.

4. DISCUSSION

These results indicate that smooth muscle myosin light chain contains many potential phosphorylation sites. Besides the site recognized

by myosin light chain kinase (as well as A-kinase and phosphorylase kinase), several other sites can be phosphorylated by CK-1 and CK-2. Skeletal muscle myosin P-light chain can be phosphorylated by a protease-activated kinase at a site different from that recognized by myosin light chain kinase [18]. Hence, it appears that myosin light chains from either smooth or skeletal muscle, when separated from the heavy chains, are potential substrates for many kinases.

The significance of the light chain phosphorylations catalyzed by CK-1, CK-2, phosphorylase kinase, and A-kinase is presently unknown. Unlike myosin light chain kinase, none of the other kinases phosphorylate intact myosin (unpublished). Since the light chain can be phosphorylated on either serine, threonine, or both amino acids, it may prove invaluable as a substrate to characterize serine and threonine protein phosphatases.

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