Phosphorylation and Activation of Smooth Muscle Myosin by *Acanthamoeba* Myosin I Heavy Chain Kinase*

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*Acanthamoeba* myosin I heavy chain kinase activates the actin-activated Mg\(^{2+}\)-ATPase activity of the *Acanthamoeba* myosin I isoenzymes, myosins IA and IB, by phosphorylating a single site within the myosin heavy chain. In this paper, we report that myosin I heavy chain kinase phosphorylates isolated turkey gizzard smooth muscle myosin light chains, gizzard smooth muscle heavy meromyosin, and intact gizzard smooth muscle myosin, all in the absence of Ca\(^{2+}\) and with specific activities close to those measured for purified Ca\(^{2+}\)/calmodulin-dependent gizzard smooth muscle myosin light chain kinase. Myosin I heavy chain kinase incorporates a maximum of 2 mol of phosphate/mol of heavy meromyosin, both by itself and together with smooth muscle myosin light chain kinase (the light chain kinase alone incorporates 1.6 mol of phosphate/mol of heavy meromyosin). Both kinases phosphorylate intact smooth muscle myosin to a maximum of 2 mol of phosphate/mol of myosin. Myosin I heavy chain kinase fully activates the actin-activated Mg\(^{2+}\)-ATPase of both myosin and heavy meromyosin. Two-dimensional tryptic peptide maps of isolated light chains phosphorylated by myosin I kinase show the same phosphopeptide as for light chains phosphorylated by the light chain kinase. These results support the conclusion that myosin I heavy chain kinase phosphorylates gizzard smooth muscle myosin I isoenzyme at the same site within the heavy chain kinase. The results suggest that the amino acid sequence around the phosphorylation site within the heavy chain of *Acanthamoeba* myosin I isoenzymes may be similar to the primary sequence around the phosphorylation site within the smooth muscle myosin light chain.

Myosin I heavy chain kinase has recently been purified to homogeneity from the soil amoeba *Acanthamoeba castellanii* (1). Myosin I heavy chain kinase is an approximately globular protein containing one polypeptide of *M* \(_r\) = 107,000. The kinase phosphorylates the heavy chain, but not the light chain, of the single-headed *Acanthamoeba* myosin I isoenzymes, myosins IA and IB (1–3). Myosin I heavy chain kinase phosphorylates both myosin I isoenzymes at one site within the heavy chain, which has been shown to be a serine residue in the case of myosin IB (1). Maximal phosphorylation of myosin IA and IB results in a 20-fold increase in their actin-activated Mg\(^{2+}\)-ATPase activities over the unphosphorylated enzymes (3, 4). The purified kinase has a high specific activity toward myosin IA and IB, about 4 \(\mu\)mol of phosphate incorporated/min-mg (30 °C) at concentrations of myosin I that are well below saturating levels (1). The kinase requires Mg\(^{2+}\) but is not dependent on Ca\(^{2+}\), Ca\(^{2+}\)/calmodulin, or cAMP for activity (1).

Investigation of myosin I heavy chain kinase substrate specificity revealed that the kinase also phosphorylates isolated turkey gizzard smooth muscle myosin light chains at a high rate (1, 5). In this paper, we extend this observation, showing that myosin I heavy chain kinase phosphorylates intact gizzard smooth muscle myosin and heavy meromyosin at high rates, that myosin I heavy chain kinase fully activates the actin-activated Mg\(^{2+}\)-ATPase activity of smooth muscle myosin and heavy meromyosin, and that this heavy chain kinase phosphorylates the same site within the 20,000-Da myosin light chain as does purified Ca\(^{2+}\)/calmodulin-dependent gizzard smooth muscle myosin light chain kinase.

**EXPERIMENTAL PROCEDURES**

**Proteins**—*Acanthamoeba* myosin I heavy chain kinase was purified to greater than 95% homogeneity, as judged by SDS-PAGE, by the procedure of Hammer et al. (1). *Acanthamoeba* myosin IB was purified as described by Maruta et al. (6), as modified by Albanesi et al. (4). Rabbit skeletal muscle F-actin, purified according to Eisenherg and Kiely (7), was a gift of Dr. Lois Greene (National Institutes of Health).

Turkey gizzard smooth muscle myosin and heavy meromyosin were prepared according to Sellers and co-workers (8, 9). The 20,000-Da light chains of both proteins contained negligible phosphate, as shown by glycerol-urea gel electrophoresis. The heavy meromyosin used in the experiments described in Table I, Fig. 1, and Fig. 2 was contaminated with a very small amount of both Ca\(^{2+}\)/calmodulin-dependent and Ca\(^{2+}\)-independent light chain kinase activities. After a 20-min incubation of heavy meromyosin alone (30 °C), approximately 0.1–0.15 mol of phosphate was incorporated/mol of heavy meromyosin in the presence of Ca\(^{2+}\)/calmodulin and 0.05 mol of phosphate/mol of heavy meromyosin in the presence of EGTA. The heavy meromyosin used in the experiments described in Table II, which was prepared by chymotryptic digestion in the presence of EGTA, was contaminated with a very small amount of Ca\(^{2+}\)/calmodulin-dependent light chain kinase activity only (a 20-min incubation of heavy meromyosin alone at 30 °C yielded less than 0.1 mol of phosphate/mol of heavy meromyosin). Smooth muscle myosin was essentially devoid of myosin kinase activity. Both myosin and heavy meromyosin were devoid of phosphatase activity, since the phosphorylated enzymes did not lose detectable amounts of protein-bound phosphate after 1 week of storage at 4 °C. Phosphate-free turkey gizzard total smooth muscle light chains (0.4 ratio, 20,000- to 17,000-Da light chain) were prepared from purified myosin as described by Sellers et al. (10). Ca\(^{2+}\)/calmodulin-dependent turkey gizzard smooth muscle myosin light chain

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1 The abbreviations used are: SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; EGTA, [ethylene bis(oxyethylenetriitol)]tetraacetic acid; MOPS, 4-morpholinopropanesulfonic acid.
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kinase, purified by the method of Aldeleiten and Klee (11), and porcine brain calmodulin, prepared according to Klee (12), were generous gifts of Dr. Elizabeth Payne (National Institutes of Health). Rabbit skeletal muscle heavy meromyosin, prepared by the method of Weeds and Pope (13) using a 2-min digestion with a 1:2000 (w/w) ratio of chymotrypsin to myosin, was a generous gift of Dr. Joseph Chait (National Institutes of Health). SDS-PAGE of skeletal muscle heavy meromyosin revealed greater than 90% intact light chains.

Assay of Actin-activated Mg\(^{2+}\)-ATPase of Gizzard Smooth Muscle Myosin and Heavy Meromyosin—Myosin and heavy meromyosin were phosphorylated by myosin I heavy chain kinase or smooth muscle myosin light chain kinase in a reaction preceding the ATPase assay (see below). For heavy meromyosin, the ATPase assay was performed at 30 °C in a mixture containing 2 mM imidazole (pH 7.0), 1.8 mM MgCl\(_2\), 0.1 mM EGTA, 1 mM \(\gamma\text{-}^3\text{P}\)ATP (5 μCi/μmol), and 0.1 mM dithiothreitol in a final volume of 0.6 μl. Where indicated, skeletal muscle F-actin was added to a final concentration of 1 mg/ml. The assay was initiated by the addition of heavy meromyosin (120 μg in 25 μl) to the assay mixture pre-equilibrated at 30 °C. At 3 and 6 min, 250-μl aliquots were removed and 32P, released from \(\gamma\text{-}^3\text{P}\)ATP was measured as described by Pollard and Korn (14). In all cases, the rate of 32P release was the same at the two time points. For myosin, the ATPase assay was performed at 25 °C in an assay mixture containing 50 mM KC\(_1\), 15 mM Tris (pH 7.0), 5 mM MgCl\(_2\), 0.1 mM EGTA, 1 mM \(\gamma\text{-}^3\text{P}\)ATP (50 μCi/μmol), and 0.1 mM dithiothreitol in a final volume of 1 ml. Myosin and actin were pre-mixed in 0.5 mM KC\(_1\) and diluted 1:10 by dilute dithiothreitol into the assay mixture (without ATP) to final concentrations of 0.16 and 0.5 mg/ml, respectively. After gentle stirring for 30 s, the reaction was initiated by the addition of ATP. At 30-s intervals, 100-μl aliquots were removed and assayed for 32P, release. The rate was obtained from the linear portion of the time course (usually from time 0 to 3 min). Both myosin I kinase and smooth muscle light chain were devoid of Mg\(^{2+}\)-ATPase activity.

Comparison of the Specific Activities of Myosin I Heavy Chain Kinase and Smooth Muscle Myosin Light Chain Kinase Toward Isolated Smooth Muscle Myosin Light Chains, Smooth Muscle Heavy Meromyosin, and Intact Smooth Muscle Myosin

Kinase specific activities toward the indicated substrates were estimated as described under “Experimental Procedures.” These results were obtained using a single preparation of myosin I heavy chain kinase. Several different kinase preparations gave values within approximately 25% of those shown.

| Substrate | Substrate specific activity | MIHCK\(^a\) specific activity | SMLCK\(^a\) specific activity |
|-----------|----------------------------|-----------------------------|-----------------------------|
| SMLC      | 1.14                       | 1.72                        | ND                          |
| HMM       | 1.79                       | 2.36                        | ND                          |
| SMM       | 3.19                       | 3.90                        | ND                          |
|            | 3.75                       | 5.35                        | ND                          |

*The concentrations shown are for smooth muscle myosin, smooth muscle heavy meromyosin, and, in the case of isolated myosin light chains, the concentration is that of the 20,000-Da light chain which is present in a mixture of 20,000- and 17,000-Da light chains (64 ratio of 20,000- to 17,000-Da light chain).

**MIHCK, myosin I heavy chain kinase; SMLCK, smooth muscle myosin light chain kinase; SMLC, isolated smooth muscle myosin light chains; ND, not determined; HMM, smooth muscle heavy meromyosin; SMM, smooth muscle myosin.

Fig. 1. Stoichiometry of phosphate incorporation into gizzard smooth muscle heavy meromyosin. The maximal extent of phosphorylation of heavy meromyosin by myosin I heavy chain kinase alone, smooth muscle myosin light chain kinase alone, and both kinases together was measured by filter paper assay as described under “Experimental Procedures.” All incubations contained 6 μM heavy meromyosin, 30 mM Tris (pH 7.4), 4 mM magnesium acetate, 0.2 mM CaCl\(_2\), 0.2 mM CaCl\(_2\), 9.25 mM dithiothreitol, and 2.0 mM \(\gamma\text{-}^3\text{P}\)ATP (75 μCi/μmol) in a final volume of 55 μl. The solid lines indicate incubations done in the presence of 0.1 μM calmodulin. The dashed lines indicate incubations done with 0.5 mM EGTA present. Heavy meromyosin was incubated with no added kinase (○), 62 nm smooth muscle myosin light chain kinase alone (●), 75 nm myosin I heavy chain kinase together (■). Kinases were added at time 0 and 7-μl aliquots were assayed at the indicated times for protein-bound 32P. At 12.5 min (arrow), the kinase concentrations were doubled by addition of fresh kinase aliquots to the remainder of the incubations so as to ensure maximal phosphate incorporation into heavy meromyosin. Essentially identical results were obtained using a different preparation of myosin I heavy chain kinase.
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Eastman Kodak) along with 1 µl of methyl green marker. Electrophoresis was performed on a Camag TLC unit at 1000 V (4 °C). The run was stopped when the methyl green had migrated to within 1 cm of the cathodic wick. Following air-drying, ascending chromatography was performed in 1-butanol/pyridine/acetate acid/water (50:33:10:40, v/v). Radioactive peptides were visualized by autoradiography.

Miscellaneous Methods—The protein concentrations of the following proteins were determined from their extinction coefficients, E₂₈₀:
gizzard smooth muscle myosin, 5.4 cm⁻¹; gizzard heavy meromyosin, 6.5 cm⁻¹; gizzard myosin light chain kinase, 11.4 cm⁻¹; actin 11.5 cm⁻¹. The protein concentrations of Acanthamoeba myosin I isoenzymes and myosin I heavy chain kinase were determined using the colorimetric assay of Bradford (17), with bovine serum albumin as a standard. Discontinuous SDS-PAGE was performed as described by Laemmli (18), followed by Coomassie blue staining according to Fairbanks et al. (19). Gels were destained in 10% acetic acid. Glycerol-urea gel electrophoresis was performed as described by Perrie and Perry (20). For autoradiography of ³²P-labeled proteins and peptide maps, dried gels and thin layer sheets were exposed to x-ray film (Kodak X-OMat AR-2) beneath an intensifying screen (DuPont, Cronex Lightning Plus). ATP, imidazole, and EGTA were purchased from Sigma and [γ-³²P]ATP was from New England Nuclear. All other chemicals were reagent grade.

RESULTS

Initial Rates of Phosphorylation of Isolated Smooth Muscle Myosin Light Chains, Smooth Muscle Heavy Meromyosin, and Intact Smooth Muscle Myosin by Myosin I Heavy Chain Kinase—The specific activities of purified myosin I heavy chain kinase and purified Ca²⁺/calmodulin-dependent smooth muscle myosin light chain kinase toward smooth muscle myosin light chains and heavy meromyosin were compared directly (Table I). Myosin I heavy chain kinase was approximately 70% as active as smooth muscle myosin light chain kinase toward isolated smooth muscle myosin light chains. With heavy meromyosin as substrate, myosin I heavy chain kinase was approximately 95% as active as the myosin light chain kinase. In addition, myosin I heavy chain kinase phosphorylated intact smooth muscle myosin, which was present in the assay in a filamentous state, at an appreciable rate. As shown previously (1), myosin I heavy chain kinase is fully active in the absence of free Ca²⁺. In separate experiments (data not shown), smooth muscle myosin light chain kinase did not phosphorylate purified Acanthamoeba myosin IB (1.7 µM) and myosin I heavy chain kinase did not phosphorylate rabbit skeletal muscle heavy meromyosin (3 µM).

Stoichiometry of Phosphate Incorporation into Smooth Muscle Heavy Myosin Meromyosin and Smooth Muscle Myosin by Myosin I Heavy Chain Kinase—Fig. 1 shows that myosin I heavy chain kinase alone incorporated a maximum of 2 mol of phosphate/mol of heavy meromyosin. Smooth muscle myosin light chain kinase alone incorporated a maximum of 1.6 mol of phosphate/mol of heavy meromyosin. When the same amounts of myosin I kinase and myosin light chain kinase that caused maximal phosphorylation alone were incubated together with heavy meromyosin, 2 mol of phosphate/mol of heavy meromyosin was obtained. These results suggest that at least 80% of the phosphate incorporated by myosin I heavy chain kinase was incorporated into the same site within the 20,000-Da myosin light chain that is phosphorylated by

![Fig. 2. SDS-PAGE and autoradiography of smooth muscle myosin and heavy meromyosin phosphorylated by myosin I heavy chain kinase and smooth muscle myosin light chain kinase. Lanes 1-9, heavy meromyosin (HMM) (6 µM) was incubated with and without kinases for 10 min at 30 °C (95% of maximal phosphorylation) in 30 mM Tris (pH 7.4), 4 mM magnesium acetate, 0.2 mM CaCl₂, 0.25 mM dithiothreitol, and 2 mM [γ-³²P]ATP (50 µCi/µmol). Reactions were stopped by addition of an equal volume of a boiling solution containing 10% SDS and 10% mercaptoethanol and 17.5 µg of protein were loaded per lane of a 14% polyacrylamide gel. Lanes 2-5, Coomassie blue-stained gel. Lanes 6-9, autoradiogram of the same gel. The samples contained in addition to the above reaction mix the following additions: 0.5 mM EGTA (lanes 2 and 6); 0.5 mM EGTA and 75 nM myosin I heavy chain kinase (lanes 3 and 7); 0.1 µM calmodulin (lanes 4 and 8); 0.1 µM calmodulin and 62 nM smooth muscle myosin light chain kinase (lanes 5 and 9). Lane 1 contained the following molecular mass standards: phosphorylase b, 92,500 Da; bovine serum albumin, 66,200 Da; ovalbumin, 45,000 Da; carbonic anhydrase, 31,000 Da; soybean trypsin inhibitor, 21,500 Da; lysozyme, 14,400 Da. The two high molecular mass bands seen in lanes 2-5 are the 130,000-Da heavy meromyosin heavy chain and a 65,000-Da doublet, which results from a second proteolytic cleavage of the myosin heavy chain. This second cleavage does not affect the activity of heavy meromyosin, which dissociates into the two 65,000-Da polypeptides only under denaturing conditions. Lanes 10-15, the maximally phosphorylated smooth muscle myosin (SMM) samples shown here are the same as samples E, F, and G in Table II, Lanes 10-12, Coomassie blue-stained gel (5 µg/lane). Lanes 13-15, autoradiogram of the same gel. The samples contained, in addition to the reaction mix described in Table II, the following additions: 0.5 mM EGTA and 260 nM myosin I heavy chain kinase (lanes 10 and 13); 0.1 µM calmodulin (lanes 11 and 14); 0.1 µM calmodulin and 14 nM smooth muscle myosin light chain kinase (lanes 12 and 15).
smooth muscle myosin light chain kinase. In addition, myosin I heavy chain kinase may have incorporated as much as 0.4 mol of phosphate/mol of heavy meromyosin into site(s) not phosphorylated by smooth muscle myosin light chain kinase. The fact that the myosin light chain kinase incorporated only 1.6 mol of phosphate/mol of heavy meromyosin, instead of the expected 2 mol of phosphate/mol of heavy meromyosin, was not due to the presence of phosphate on the 20,000-Da light chain of heavy meromyosin used as substrate, since glycerol-urea gel electrophoresis showed negligible phosphorylated 20,000-Da light chain in the purified heavy meromyosin. Rather, the lower stoichiometry with smooth muscle myosin light chain kinase was probably due to partial proteolytic cleavage of the 20,000-Da light chain during chromotryptic digestion of intact myosin to generate heavy meromyosin, rendering about 20% of the 20,000-Da light chains nonphosphorylatable by the myosin light chain kinase.

Fig. 2 (lanes 1–9) shows an SDS-polyacrylamide gel and autoradiogram of heavy meromyosin phosphorylated to greater than 95% of maximum by myosin I heavy chain kinase alone and smooth muscle myosin light chain kinase alone. In both cases, essentially 100% of the radioactivity incorporated into the 20,000-Da myosin light chain. A small amount of radioactivity was incorporated by both kinases into an apparent proteolytic product of the 20,000-Da myosin light chain which migrated at about 18,500 Da. The Acanthamoeba myosin heavy chain kinase did not incorporate any phosphate into the heavy meromyosin heavy chain, indicating that the additional 0.4 mol of phosphate/mol of heavy meromyosin incorporated by myosin I heavy chain kinase over and above that incorporated by smooth muscle myosin light chain kinase was within the 20,000-Da myosin light chain.

In experiments described below (see Table II), incubation of smooth muscle myosin with high concentrations of either myosin I heavy chain kinase or smooth muscle myosin light chain kinase resulted in incorporation of 2 mol of phosphate/mol of myosin, as determined by filter paper assay. In Fig. 2 (lanes 10–15), an SDS-polyacrylamide gel and autoradiogram of these myosin samples reveals that essentially 100% of the radioactive phosphate incorporated by myosin I heavy chain kinase, as well as by smooth muscle myosin light chain kinase, was into the 20,000-Da myosin light chain.

Activation of the Actin-activated Mg$^{2+}$-ATPase of Smooth Muscle Myosin and Heavy Meromyosin by Myosin I Heavy Chain Kinase—Since myosin I heavy chain kinase appeared to phosphorylate both gizzard myosin and heavy meromyosin at the same site that smooth muscle myosin light chain kinase does, the ability of myosin I kinase to activate the actin-activated Mg$^{2+}$-ATPase activity of myosin and heavy meromyosin was tested. Myosin and heavy meromyosin were preincubated with myosin I heavy chain kinase alone and smooth muscle myosin light chain kinase alone under conditions giving maximal phosphorylation and the Mg$^{2+}$-ATPase activities of myosin and heavy meromyosin were assayed in the presence of F-actin (Table II). Myosin I heavy chain kinase activated the actin-activated Mg$^{2+}$-ATPase of heavy meromyosin to about 85% of the rate achieved by smooth muscle myosin light chain kinase-activated heavy meromyosin. The actin-activated Mg$^{2+}$-ATPase of intact myosin was activated by myosin I kinase to about 94% of the rate achieved by smooth muscle myosin light chain kinase-activated myosin. Myosin I kinase activation of myosin and heavy meromyosin did not require Ca$^{2+}$. These results, therefore, provided additional evidence that myosin I heavy chain kinase phosphorylated the 20,000-Da myosin light chain at the same site as smooth muscle myosin light chain kinase.

### Table II

**Activation of the actin-activated Mg$^{2+}$-ATPase of smooth muscle myosin and heavy meromyosin by myosin I heavy chain kinase**

| Preincubation | Phosphate incorporation by myosin I heavy chain kinase | Specific activity (mol mol$^{-1}$ ATPase/mg protein) |
|---------------|-------------------------------------------------------|---------------------------------------------|
| A. HMM + EGTA | 0.428                                                  | 11.6                                       |
| B. HMM + EGTA + MIHCK | 1.95                                                | 42.22                                      |
| C. HMM + Ca$^{2+}$/calmodulin | 0.26                                                | 14.1                                       |
| D. HMM + Ca$^{2+}$/calmodulin + SMLCK | 1.98                                                | 419.5                                      |
| E. SM + EGTA + MIHCK | 1.98                                                | 4.4                                       |
| F. SM + Ca$^{2+}$/calmodulin | 0.01                                                | 2.02                                       |
| G. SM + Ca$^{2+}$/calmodulin + SMLCK | 2.02                                                | 43.9                                       |

*HMM, heavy meromyosin; SM, smooth muscle myosin; MIHCK, myosin I heavy chain kinase; SMLCK, smooth muscle myosin light chain kinase.

Light Chains Phosphorylated by Myosin I Heavy Chain Kinase and Smooth Muscle Myosin Light Chain Kinase—Isolated smooth muscle myosin light chains were phosphorylated to near maximal extent by myosin I heavy chain kinase alone, smooth muscle myosin light chain kinase alone, and both kinases together. Phosphorylated myosin light chains were digested with trypsin, the peptides separated by two-dimensional cellulose thin layer mapping, and the radioactive peptides identified by autoradiography (Fig. 3). Myosin light chains phosphorylated by smooth muscle myosin light chain kinase alone (Fig. 3A) yielded one major radioactive peptide as expected. Two very minor radioactive peptides were also seen. Myosin light chains phosphorylated by myosin I heavy chain kinase alone (Fig. 3B) yielded one major radioactive peptide with a migration similar to the peptide in Fig. 3A. Two other radioactive peptides of lower intensity were also seen. These light chains were phosphorylated simultaneously by both myosin I heavy chain kinase and smooth muscle myosin light chain kinase, again only one major radioactive peptide was observed (Fig. 3C). These results, therefore, support the conclusion that most of the phosphate incorporated by myosin I heavy chain kinase was in the same tryptic peptide as the phosphate incorporated by smooth muscle.
FIG. 3. Tryptic peptide maps of isolated smooth muscle myosin light chains phosphorylated by myosin I heavy chain kinase and smooth muscle myosin light chain kinase. Myosin light chains were phosphorylated in reactions containing 100 μM total gizzard myosin light chains (60 μM 20,000-Da light chains), 10 mM imidazole (pH 7.4), 25 mM KCl, 1.2 mM MgCl$_2$, 0.2 mM CaCl$_2$, and 1 mM [γ-32P]ATP (50 μCi/μmol) in a final volume of 36 μl. The myosin I heavy chain kinase reaction contained 0.5 mM EGTA and 50 nM myosin I heavy chain kinase. The smooth muscle myosin light chain kinase reaction contained 0.1 μM calmodulin and 42 nM smooth muscle myosin light chain kinase. The reaction containing both kinases contained 0.1 μM calmodulin, 50 nM myosin I heavy chain kinase, and 42 nM smooth muscle myosin light chain kinase. Only the 20,000-Da myosin light chain was phosphorylated by the two kinases (data not shown). Reactions were incubated for 10 min at 30 °C and stopped by addition of 50% trichloroacetic acid to a final concentration of 10%. Tryptic digestion and two-dimensional peptide mapping were performed as described under “Experimental Procedures.” The sections are peptide maps of light chains phosphorylated by: (A) smooth muscle myosin light chain kinase, (B) myosin I heavy chain kinase, and (C) smooth muscle myosin light chain kinase and myosin I heavy chain kinase simultaneously. The methyl green marker (X) and the chromatography front (arrow) are indicated. The origin of the peptide map is just to the left of the C. The radioactive material at the origin of the peptide map in B indicates perhaps that this sample was either not completely digested or solubilized prior to mapping.

myosin light chain kinase. In addition, myosin I heavy chain kinase also incorporated a small but significant amount of radioactivity into two peptides not phosphorylated by smooth muscle myosin light chain kinase.

DISCUSSION

Previous work has shown that purified Acanthamoeba myosin I heavy chain kinase phosphorylates a single site within the heavy chain of Acanthamoeba myosin I isoenzymes, resulting in a 20-fold increase in their actin-activated Mg$^{2+}$-ATPase activities (1–3). Measurements of phosphorylation rates at subsaturating myosin I concentrations indicate that the $V_{max}$ for myosin I heavy chain kinase toward myosin I would probably exceed 10 μmol/min·mg (1). More importantly, myosin I heavy chain kinase phosphorylates only the heavy chain of the myosin I isoenzymes of Acanthamoeba (1–3). Myosin I isoenzymes which have been maximally phosphorylated by myosin I heavy chain kinase do not contain detectable levels of light chain phosphate (1). However, the results reported here demonstrate that Acanthamoeba myosin I heavy chain kinase phosphorylates isolated turkey gizzard smooth muscle myosin 20,000-Da light chains, and the 20,000-Da light chains of smooth muscle heavy meromyosin and intact smooth muscle myosin, all in the absence of Ca$^{2+}$ and at high rates. While insufficient kinetic data were obtained to determine $K_m$ and $V_{max}$ values, at the substrate concentra-
tions tested, myosin I heavy chain kinase demonstrated specific activities which were very close to those measured for purified Ca\(^{++}\)/calmodulin-dependent smooth muscle myosin light chain kinase.

Myosin I heavy chain kinase appears to phosphorylate the same site within the 20,000-Da myosin light chain as does smooth muscle myosin light chain kinase. This conclusion is based on the fact that 1) phosphorylation of heavy meromyosin by myosin I heavy chain kinase and smooth muscle myosin light chain kinase is largely mutually exclusive, 2) that myosin I heavy chain kinase fully activates the actin-activated Mg\(^{++}\)/ATPase of both myosin and heavy meromyosin, and 3) that tryptic digestion of isolated smooth muscle myosin light chains phosphorylated by both kinases yields a single major phosphopeptide. While myosin light chains phosphorylated by smooth muscle myosin light chain kinase alone yielded only one tryptic phosphopeptide as expected, and myosin light chains phosphorylated by myosin I heavy chain kinase primarily yielded the same phosphopeptide, the peptide maps of the latter also showed two other significant radioactive peptides. These peptides may contain secondary sites phosphorylated by myosin I heavy chain kinase and could explain the additional 0.2 mol of phosphate incorporated/mol of light chain which myosin I heavy chain kinase incorporates into heavy meromyosin over and above smooth muscle myosin light chain kinase. The secondary radioactive peptides might have been derived from incomplete digestion, secondary cleavages, or peptides with variable oxidation states, but these explanations would require that the event occurred specifically in the myosin I kinase-phosphorylated peptides and not in the smooth muscle myosin light chain kinase-phosphorylated peptides. The amount of myosin I heavy chain kinase in the light chain phosphorylation reactions was too low to contribute significant amounts of radioactive peptides by way of myosin I kinase autophosphorylation (1).

In addition to myosin I heavy chain kinase, cAMP-dependent protein kinase has been reported (21) to phosphorylate isolated gizzard smooth muscle myosin 20,000-Da light chains in the same site as does smooth muscle myosin light chain kinase. However, unlike myosin I heavy chain kinase and smooth muscle myosin light chain kinase, cAMP-dependent protein kinase does not phosphorylate the 20,000-Da light chain in intact smooth muscle myosin (22).

The substrate specificity of protein kinases is thought to depend at least in part upon the primary sequence around the phosphorylation site, with specific amino acid side chains near the phosphorylated residue playing an essential role in recognition of the substrate by the kinase (for reviews, see Refs. 23 and 24). The fact that purified Acanthamoeba myosin I heavy chain kinase readily phosphorylates smooth muscle myosin as well as Acanthamoeba myosin I isoenzymes indicates a sequence homology around the phosphorylation site in the Acanthamoeba myosin I heavy chain and the smooth muscle myosin light chain. However, Ca\(^{++}\)/calmodulin-dependent smooth muscle myosin light chain kinase did not phosphorylate Acanthamoeba myosin IB, perhaps because of steric hindrance. At present, neither the myosin I heavy chain nor the turkey gizzard smooth muscle myosin light chain phosphorylation site sequence is known, although the sequence of the 20,000-Da myosin light chain from turkey gizzard is probably very similar to the known sequence for the chicken gizzard myosin light chain, Lys-Ala-Thr-Ser(P)-Asn-Val-Phe-Ser (25). Smooth muscle myosin light chain kinase has been shown to phosphorylate readily a heptadecapeptide with a similar sequence around the phosphorylated serine: Ser-Ser-Lys-Thr-Thr-Lys-Arg-Pro-Gln-Ala-Thr-Ser(P)-Asn-Val-Phe-Ser (26). Future work, including sequencing the Acanthamoeba myosin I heavy chain phosphorylation site and studies with synthetic phosphorylatable peptide substrates, should provide direct information concerning the specificity determinants for Acanthamoeba myosin I heavy chain kinase.

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