Identification, Phosphorylation, and Dephosphorylation of a Second Site for Myosin Light Chain Kinase on the 20,000-Dalton Light Chain of Smooth Muscle Myosin

Mitsuo Ikebe, David J. Hartshorne, and Marshall Elzinga

From the Muscle Biology Group, Departments of Biochemistry and of Nutrition and Food Science, University of Arizona, Tucson, Arizona 85721 and the Department of Biology, Brookhaven National Laboratory, Upton, New York 11973

At relatively high concentrations of myosin light chain kinase, a second site on the 20,000-dalton light chain of smooth muscle myosin is phosphorylated (Ikebe, M., and Hartshorne, D. J. (1985) J. Biol. Chem. 260, 10027–10031). In this communication the site is identified and kinetics associated with its phosphorylation and dephosphorylation are described. The doubly phosphorylated 20,000-dalton light chain from turkey gizzard myosin was hydrolyzed with \( \alpha \)-chymotrypsin and the phosphorylated peptide was isolated by reverse phase chromatography. Following amino acid analyses and partial sequence determinations the second site of phosphorylation is shown to be threonine 18. This site is distinct from the threonine residue phosphorylated by protein kinase C. The time courses of phosphorylation of serine 19 and threonine 18 in isolated light chains follow a single exponential indicating a random process, although the phosphorylation rates differ considerably. The values of \( k_{cat}/K_m \) for serine 19 and threonine 18 for isolated light chains are 550 and 0.2 min\(^{-1} \) \( \mu \)M\(^{-1} \), respectively. With intact myosin, phosphorylation of serine 19 is biphasic; \( k_{cat}/K_m \) values are 22.5 and 7.5 min\(^{-1} \) \( \mu \)M\(^{-1} \) for the fast and slow phases, respectively. In contrast, phosphorylation of threonine 18 in intact myosin is a random, but markedly slower process, \( k_{cat}/K_m = 0.44 \) min\(^{-1} \) \( \mu \)M\(^{-1} \). Dephosphorylation of doubly phosphorylated myosin (2 mol of phosphate/mol of myosin) and isolated light chains (2 mol of phosphate/mol of light chain) follows a random process and dephosphorylation of the serine 19 and threonine 18 sites occurs at similar rates.

Phosphorylation of the 20,000-dalton light chain of smooth muscle myosin is accepted as an important component of the regulatory mechanism in smooth muscle (1, 2). Under most experimental conditions the extent of incorporation is limited to 1 mol of phosphate/mol of light chain and the site of phosphorylation has been identified as serine 19 (3-5). Recently, however, phosphorylation at a second site has been found (6, 7). Cole et al. (6) showed that double phosphorylation of the 20,000-dalton light chain could be observed only if the chicken gizzard myosin was prepared under certain conditions involving an overnight settling period. The identity of the kinase responsible for the phosphorylation of the second site was not established and it was found also that the additional phosphorylation did not increase actin-activated ATPase activity (6). The results from our laboratory (7) differed from those of Cole et al. (6) in several respects. Phosphorylation of the second light chain site was achieved at high concentrations of myosin light chain kinase (MLC kinase), and no dependence on preparative procedures was observed. It was suggested that the enzyme responsible for the extra phosphorylation was in fact MLC kinase and in addition it was shown that phosphorylation of the second light chain site markedly increased the actin-activated ATPase activity of myosin (7). The second phosphorylation site was shown to be a threonine residue (7), in agreement with the results of Cole et al. (6), and threonines 9, 10, or 18 were suggested as possible locations.

In this article other results regarding the second phosphorylation site are presented. The second site of phosphorylation is identified as threonine 18 and it is shown that this threonine is distinct from that phosphorylated by protein kinase C (8). Kinetics of phosphorylation and dephosphorylation of threonine 18 for the isolated light chain and intact myosin also are presented and compared to those involving serine 19.

**MATERIALS AND METHODS**

The following procedures were used for the isolation of proteins: myosin (9), MLC kinase (10) and myosin light chain phosphatase (11) from frozen turkey gizzards; calmodulin from frozen beef testes (10); the 20,000-dalton light chain from gizzard myosin (12); and protein kinase C (partially purified) from cow brain (13). A spontaneously active preparation of bovine aorta phosphatase (14) was kindly supplied by Dr. J. DiSalvo (University of Cincinnati). \( \alpha \)-Chymotrypsin (Type III) was obtained from Sigma, and trypsin (treated with L-1-tosylamido-2-phenylethyl chloromethyl ketone) was obtained from Worthington.

Phosphorylation assays for MLC kinase and protein kinase C were carried out as described previously (10). Other conditions are given in the figure legends. Isolation of the peptide containing the second phosphorylation site was as follows: the isolated 20,000-dalton light chain (12 mg at 2 mg/ml) was phosphorylated in 50 mM KCl, 30 mM Tris-HCl (pH 7.5) to remove \( [\gamma-^3P]ATP \). Digestion of the phosphorylated light chain was carried out at 37 °C for 15 min with 0.1 mg/ml \( \alpha \)-chymotrypsin in 50 mM Tris-HCl (pH 7.5), 1 mM MgCl\(_2\), 0.6 mM \( [\gamma-^3P]ATP \) (approximately 50 cpm/pmol), 0.1 mM CaCl\(_2\), 30 \( \mu \)g/ml calmodulin, and 180 \( \mu \)g/ml MLC kinase at 25 °C for 30 min. The reaction was stopped by the addition of trichloroacetic acid to 0.5%. The extent of phosphate incorporation was 1.9 mol of phosphate/mol of light chain. The phosphorylated light chain was dialyzed against several changes of 50 mM KCl, 10 mM Tris-HCl (pH 7.5) to remove \( [\gamma-^3P]ATP \). Digestion of the phosphorylated light chain was carried out at 37 °C for 15 min with 0.1 mg/ml \( \alpha \)-chymotrypsin in 50 mM Tris-HCl (pH 7.5), 1 mM MgCl\(_2\), 0.1 mM CaCl\(_2\), 50 mM KCl. (After 5-min digestion, approximately 99% of the incorporated \( P \) was soluble in 5% trichloroacetic acid.) The proteolysis was stopped by the addition of diisopropylfluorophosphate to 1 mM (stock solution, 0.1 M in isopropyl alcohol). The sample was lyophilized, dissolved in 50 mM NH\(_2\)HCO\(_3\) (pH 8.3) and applied to a TSK G 2000 SW column (60 x 0.75 cm).

---

*This work was supported by National Institutes of Health Grants HL 23615 and HL 20984 (to D. J. H) and by National Institutes of Health Grant HL 21471 (to M. E.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

---

1The abbreviations used are: MLC kinase, myosin light chain kinase; EGTA, ethylene glycol bis(\( \beta \)-aminooethyl ether) N,N',N'-tetraacetic acid; HPLC, high performance liquid chromatography.
using a Perkin-Elmer Series 4 HPLC system. The radioactive fractions were pooled (94% of applied radioactivity was recovered), lyophilized, dissolved in 1.5 ml of 0.1% trifluoroacetic acid (in HzO), and eluted with a linear gradient, 0.1% trifluoroacetic acid to 65% CH₃CN, 0.1% trifluoroacetic acid. A peak containing ⁴¹P was eluted at approximately 28% CH₃CN (see Fig. 1A). This contained approximately 95% of the applied radioactivity. The radioactive fractions were combined, lyophilized, dissolved in 1.5 ml of 0.1% trifluoroacetic acid, and reapplied to the above C₁₈ column. Elution was achieved using two steps, initially of 10% CH₃CN, 0.1% trifluoroacetic acid and subsequently of 15% CH₃CN, 0.1% trifluoroacetic acid. The radioactivity was eluted in a single peak in the latter solvent (see Fig. 1B). The radioactive fractions were pooled, lyophilized, and submitted for amino acid analyses and sequence determinations (see Table I). A partial sequence was obtained for this α-chymotryptic peptide. The α-chymotryptic peptide was subjected to further hydrolysis with trypsin (to give the tryptic subpeptide, see Table I) as follows: 10 nmol of α-chymotryptic peptide in 0.5% NH₄HCO₃ (pH 8.5) was digested for 16 h at 25 °C with 1% (by weight) trypsin. The hydrolysate was dried, dissolved in 50 μl of 0.1% trifluoroacetic acid, applied to HPLC on a C₁₈ reverse phase column (ODS 120T, 25 × 0.46 cm, Toyo Soda, Japan), and eluted with a linear CH₃CN gradient. The radioactive peptide eluted at 14% CH₃CN. The amino acid composition of this peptide was determined (see Table I).

Amino acid analyses were carried out using an instrument that employs single-column ion exchange separation, with detection by postcolumn derivatization with ninhydrin. Its sensitivity limit is about 1 nmol. Sequences were determined using both gas-phase and spinning-cup sequencers. The gas-phase sequencer (Applied Biosystems) was used with a load of about 1 nmol of peptide, and the phenylthiohydantoin derivatives were identified on a Hewlett-Packard Model 1100 HPLC, using a Zorbax Cyanopropylsilane column (250 × 4.6 mm). In an effort to determine the site(s) of phosphorylation, one-half of each sample from the Applied Biosystems sequencer was subjected to liquid scintillation counting. After a run was complete, the disc to which the sample was applied was also counted. Since the radioactive material (the peptide as well as the phosphorylated ammonium and/or phosphoserine) remained bound to the disc in the gas-phase sequencer, the peptide (1 nmol) was also analyzed in a Beckman 890C sequencer using the 0.1 M 2-mercaptoethanol, 0.5% ammonium hydroxide solution, and comparison of this sequence with the

TABLE I

**Amino acid compositions and sequences of the phosphorylated peptides**

| Fraction | Amino Acid Sequence | Observed | Expected |
|----------|---------------------|----------|----------|
| 10       | Ac-S-S-K-R-A-K-A-K-T-K-K-R-P-Q-R-A-T-S-N-V-F | 1         | 1         |
| 20       | K-K-R-P-×-R-A-T-S-N-V-F | 1         | 1         |
| 20       | A-×-S-N-V-F | 1         | 1         |

(a) NH₃ sequence of the 20-kDa light chain: sequence established by Pearson et al. (5). (b) The α-chymotryptic peptide: the underlined residues and the locations of the phosphates (shown by ×) were determined by sequencer analysis. The order of the Thr and Ser in the α-chymotryptic and tryptic peptides was assumed to coincide with the assignments of Pearson et al. (5). (c) The tryptic subpeptide: the sequence of this peptide is assumed, based upon the correspondence of its composition and residues 17-22 of the light chain.

![A Second Site for Myosin Light Chain Kinase](image)

**RESULTS AND DISCUSSION**

Identification of the Second Site of Phosphorylation—The isolated 20,000-dalton turkey gizzard light chain was phosphorylated by MLC kinase approximately to 2.0 mol of phosphate/mol of light chain and digested with α-chymotrypsin (see "Materials and Methods"). Under the conditions of proteolysis, the light chain is degraded to a 16,000-dalton fragment and the ⁴¹P is quantitatively released in a trichloroacetic acid-soluble peptide (7). The latter was applied initially to a gel filtration column (to remove [γ-³²P]ATP) and subsequently to reverse phase chromatography. As shown in Fig. 1A, the radioactivity is found predominantly in one peak. To achieve further purification, this fraction was reapplied to reverse phase chromatography and the elution profile is shown in Fig. 1B. A single peak was associated with the ³²P label. The recovery of radioactivity at each step during this isolation procedure was greater than 90% and it is therefore reasonable to conclude that both phosphorylation sites are in the fraction shown in Fig. 1B. The amino acid composition of this fraction, shown in Table I, suggests that it is a pure peptide. The first 7 residues were identified as their phenylthiohydantoin derivatives, using the gas phase Applied Biosystems sequencer, and comparison of this sequence with the
sequence published by Pearson et al. placed this peptide unambiguously within the sequence of the light chain. The radioactive phosphate was expected in one or both of the next 2 residues, but no counts were observed when these (or any other steps) were counted, presumably because the phosphate groups bind to the sample disc in the gas-phase sequencer. Analysis with the Beckman 890C Sequencer yielded counts in both steps 8 and 9. The recovery was about 5%, and the relative amount in the two steps suggested that they were phosphorylated to approximately equal extent. This peptide was then digested with trypsin and the radioactive subpeptide was isolated by reverse phase chromatography (see “Materials and Methods”); its composition is shown in Table I. Based on the sequencer results, and the amino acid composition of two peptides, the complete sequence of the a-chymotryptic peptide must be as shown in Table I. It was previously determined that the second site of phosphorylation is a threonine residue (6, 7) and the combined data prove that this residue is threonine 18.

Phosphorylation Kinetics of Serine 19 and Threonine 18—Previously it was shown that the phosphorylation of threonine is slower than that of serine 19 (7) and at levels of phosphorylation below 1 mol of phosphate/mol of light chain only phosphoserine is detected. Since the two sites are kinetically so distinct, it is possible to determine the rate constants for phosphorylation of each site. Time courses of phosphorylation of serine 19 are shown in Fig. 2A for isolated light chains and intact myosin. (Note that different concentrations of MLC kinase were used in each case. See figure legend.) The inset shows a semilogarithmic plot of these data. For the isolated light chains, a single exponential is adequate to fit the data but for intact myosin two exponentials are required (16, 17). From the slope(s) of the semilogarithmic plot, V_max/K_m is obtained and kcat/K_m can be calculated assuming a molecular weight of 130,000 for MLC kinase. For the isolated light chains, kcat/K_m = 550 min^{-1} μM^{-1} and for intact myosin, values of kcat/K_m for the fast and slow phases are 22.5 and 7.5 min^{-1} μM^{-1}, respectively. The rate of phosphorylation of serine 19 in isolated light chains is at least 20 times faster than the rate of phosphorylation of this residue in intact myosin. Previously reported values for kcat/K_m for the fast phase of serine 19 phosphorylation in gizzard myosin ranged from 110 to 150 min^{-2} μM^{-1} (16). However, these earlier values were obtained at a higher MgCl2 concentration (4 mM total) than used for our present results and we have observed a marked dependence of the phosphorylation rate (fast phase) on MgCl2 concentration.2 At low ionic strength (85 mM KCl) increasing the MgCl2 concentration (total) from 1 to 4 mM causes a 3- to 4-fold increase in the phosphorylation rate. If this is taken into account then the values of kcat/K_m obtained previously and those reported here are similar.

Time courses of phosphorylation of threonine 18 for isolated light chains and intact myosin are shown in Fig. 2B. In contrast to the results shown in Fig. 2A, the two time courses can be fit by a single exponential indicating that phosphorylation of threonine 18 in both isolated light chains and intact myosin is a random process. Values of kcat/K_m for intact myosin and isolated light chains are 0.44 and 0.20 min^{-1} μM^{-1}, respectively. Both values are considerably lower than those obtained for the phosphorylation of serine 19, but the difference is more marked when the double phosphorylation of the isolated light chain is considered.

Dephosphorylation of Serine 19 and Threonine 18—If the phosphorylation of threonine 18 is to have any physiological role in the regulation of smooth muscle activity, it must be subject to irreversible phosphorylation and dephosphorylation. To test this possibility we examined the kinetics of dephosphorylation for doubly labeled light chains (~2 mol of P/mol of light chain) and intact myosin (~4 mol of P/mol of myosin). The time courses of dephosphorylation are shown in Fig. 3A for intact myosin and in Fig. 3B for isolated light chains. From these data it appears that both threonine and serine residues are dephosphorylated. Complete dephosphorylation of the light chains and intact myosin was obtained at higher concentrations of phosphatase (data not shown). The amount of phosphatase used was the same on a molar basis for the intact myosin and the isolated light chains. Dephosphorylation of intact myosin is approximately 3.8 times faster than the dephosphorylation of isolated light chains. The insets show semilogarithmic plots of the time course data and in both cases a random dephosphorylation process is indicated (i.e. a single exponential). During the dephosphorylation of intact myosin, samples were taken at different times and analyzed (by autoradiograms of acid hydrolysates (7)) for phosphoserine and phosphothreonine. For each time point the amounts of residual (i.e. attached to light chain) phosphoserine and phosphothreonine were approximately equal (data not shown). This confirms that the dephosphorylation

\[ \text{extinction} \]

\[ \text{trylation of intact myosin and iso-} \]

\[ \text{myosin; } \]

\[ \text{phosphorylation at time } \]

\[ \text{ogarithmic plots } \]

\[ \text{5 ml calmodulin (pH 7.5), 10 mM [γ-32P]ATP, 25 °C. A, } \]

\[ \text{1 mg/ml myosin, 3 μg/ml MLC kinase, 5 μg/ml calmodulin (O), and 0.1 mg/ml } \]

\[ \text{isolated light chain, 0.1 μg/ml MLC kinase, 5 μg/ml calmodulin (Δ). B, 1 mg/} \]

\[ \text{ml myosin, 60 μg/ml MLC kinase, 20 μg/ml calmodulin (O) and 0.1 mg/ml } \]

\[ \text{isolated light chain, 60 μg/ml MLC kinase, 20 μg/ml calmodulin (Δ).} \]

\[ \text{FIG. 2. Time courses of phospho-} \]

\[ \text{ylation of intact myosin and iso-} \]

\[ \text{lated 20,000-dalton light chain. A, } \]

\[ \text{myosin; } \]

\[ \text{rylation of threonine 18. Insets show semil-} \]

\[ \text{ogarithmic plots of time course data, where } \]

\[ \text{Pt, } \]

\[ \text{from the slope(s) of the semilogarithmic plot, } \]

\[ \text{and } \]

\[ \text{obtained previously and } \]

\[ \text{those reported here are similar.} \]

\[ \text{Time courses of phosphorylation of threonine 18 for isolated light chains and intact myosin are shown in Fig. 2B. In contrast to the results shown in Fig. 2A, the two time courses can be fit by a single exponential indicating that phosphorylation of threonine 18 in both isolated light chains and intact myosin is a random process. Values of kcat/K_m for intact myosin and isolated light chains are 0.44 and 0.20 min^{-1} μM^{-1}, respectively. Both values are considerably lower than those obtained for the phosphorylation of serine 19, but the difference is more marked when the double phosphorylation of the isolated light chain is considered.} \]

\[ \text{Dephosphorylation of Serine 19 and Threonine 18—If the phosphorylation of threonine 18 is to have any physiological role in the regulation of smooth muscle activity, it must be subject to irreversible phosphorylation and dephosphorylation. To test this possibility we examined the kinetics of dephosphorylation for doubly labeled light chains (~2 mol of P/mol of light chain) and intact myosin (~4 mol of P/mol of myosin). The time courses of dephosphorylation are shown in Fig. 3A for intact myosin and in Fig. 3B for isolated light chains. From these data it appears that both threonine and serine residues are dephosphorylated. Complete dephosphorylation of the light chains and intact myosin was obtained at higher concentrations of phosphatase (data not shown). The amount of phosphatase used was the same on a molar basis for the intact myosin and the isolated light chains. Dephosphorylation of intact myosin is approximately 3.8 times faster than the dephosphorylation of isolated light chains. The insets show semilogarithmic plots of the time course data and in both cases a random dephosphorylation process is indicated (i.e. a single exponential). During the dephosphorylation of intact myosin, samples were taken at different times and analyzed (by autoradiograms of acid hydrolysates (7)) for phosphoserine and phosphothreonine. For each time point the amounts of residual (i.e. attached to light chain) phosphoserine and phosphothreonine were approximately equal (data not shown). This confirms that the dephosphorylation} \]

\[ \text{2 M. Ikebe and D. J. Hartshorne, unpublished results.} \]

\[ \text{In NA} \]

\[ \text{Second Site for Myosin Light Chain Kinase} \]
Phosphorylation of intact myosin by protein kinase C—It was shown previously (8) that the 20,000-dalton light chains of intact turkey gizzard myosin can be phosphorylated by protein kinase C to the extent of 1 mol of phosphate/mol of light chain and that the major site of phosphorylation is a threonine residue. An obvious concern was that threonine 18 may serve as a target for both MLC kinase and protein kinase C. To assess this possibility we measured the sequential phosphorylation of intact myosin by protein kinase C and by MLC kinase. As shown in Fig. 4, phosphorylation of myosin by protein kinase C results in the incorporation of approximately 1.8 mol of P/mol of myosin. Addition of MLC kinase and calmodulin at the point indicated by the arrow results in further phosphorylation. At the lower MLC kinase concentration (5 μg/ml) approximately 2 additional mol of phosphate are incorporated, consistent with the phosphorylation of serine 19. At a higher MLC kinase concentration (100 μg/ml) an additional 3.6 mol of phosphate are incorporated, i.e. phosphorylation of both serine 19 and threonine 18. It is concluded, therefore, that the threonine residue phosphorylated by protein kinase C is not threonine 18. The identity of the protein kinase C phosphorylation site is not established but it might be located close to the MLC kinase sites in the N-terminal region of the light chain. This suggestion is based on observations made on the sequential α-chymotryptic degradation of the light chain which yields initially an 18,000-dalton fragment followed by a 16,000-dalton fragment. The transition from 18,000 to 16,000 involves the removal of N-terminal peptides (residues 1-22) and results in the loss of the two MLC kinase sites and the loss of the protein kinase C site. (A 17,000-dalton fragment is produced by tryptic hydrolysis and this retains all phosphorylation sites.) Possible phosphorylation sites for protein kinase C are therefore threonines 9 and 10. An interesting point of speculation is that phosphorylation of residues 18 and 19 by MLC kinase results in an increase of actin-activated ATPase activity (7) but the phosphorylation of adjacent residues, suggested as 9 or 10, causes a decrease in actin-activated ATPase activity (8).

REFERENCES
1. Walsh, M. P., and Hartshorne, D. J. (1982) in Calcium and Cell Function (Cheung, W. Y., ed) Vol. III, pp. 223-269, Academic Press, New York
2. Adelstein, R. S., and Eisenberg, E. (1980) Annu. Rev. Biochem. 49, 921-956
3. Jakes, R., Northrop, F., and Kendrick-Jones, J. (1976) FEBS Lett. 70, 229-234
4. Maia, T., Chee, J.-I., and Matsuda, G. (1981) Eur. J. Biochem. 117, 417-426
5. Pearson, R. B., Jakes, R., John, M., Kendrick-Jones, J., and Kep, B. E. (1984) FEBS Lett. 169, 101-112
6. Cole, H. A., Griffiths, H. S., Patchell, V. B., and Perry, S. V. (1985) FEBS Lett. 185, 165-168
7. Ikebe, M., and Hartshorne, D. J. (1982) J. Biol. Chem. 257, 1097-1099
8. Nishikawa, M., Sellers, J. R., Adelstein, R. S., and Hidaka, H. (1984) J. Biol. Chem. 259, 8808-8814
9. Ikebe, M., and Hartshorne, D. J. (1986) J. Biol. Chem. 260, 13146-13153
10. Walsh, M. P., Hinkins, S., Dabrowska, B., and Hartshorne, D. J. (1983) Arch. Biochem. 229, 276-288
11. Onishi, H., Umeda, J., Uchiwa, H., and Watanabe, S. (1982) J. Biol. Chem. (Tokyo) 11, 265-271
12. Hathaway, D. R., and Haeberle, R. J. (1983) Anal. Biochem. 135, 37-43
13. Walsh, M. P., Valentine, K. A., Ngai, P. K., Carnethers, C. A., and Hellingberg, M. D. (1984) Biochem. J. 224, 117-127
14. DiSalvo, J., and Gifford, D. (1983) Biochem. Biophys. Res. Commun. 111, 312-318
15. Walsh, M. P., Hinkins, S., Flink, I. L., and Hartshorne, D. J. (1982) Biochemistry 21, 6880-6886
16. Persichini, A., and Hartshorne, D. J. (1983) Biochemistry 22, 470-476
17. Sellers, J. R., Chock, P. B., and Adelstein, R. S. (1983) J. Biol. Chem. 258, 14181-14188

For the above experiments the phosphatase was prepared from turkey gizzard according to Onishi et al. (11); however, similar results were obtained using the spontaneously active bovine aorta phosphatase (14).

**FIG. 4. Sequential phosphorylation of myosin by protein kinase C and by MLC kinase.** Conditions for protein kinase C phosphorylation: 4 mM MgCl₂, 0.5 mM CaCl₂, 1 mM [γ-32P]ATP, 33 mM KCl, 30 mM Tris-HCl (pH 7.5), 0.2 mg/ml phosphatidyl serine, 25°C. The protein kinase C preparation was not homogeneous and therefore the amount added is not known. The two symbols (O and △) show duplicate experiments that also serve as controls for the subsequent MLC kinase phosphorylations. At the arrow, MLC kinase and calmodulin added to final concentrations of 5 μg/ml MLC kinase plus 5 μg/ml calmodulin (O) and 100 μg/ml MLC kinase plus 30 μg/ml calmodulin (△).

is random and that the phosphatase has no apparent preference for the phosphorylated forms of threonine 18 or serine 19.