Sequence of the Sites Phosphorylated by Protein Kinase C in the Smooth Muscle Myosin Light Chain*

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We have determined the sequence of the sites phosphorylated by protein kinase C in the turkey gizzard smooth muscle myosin light chain. In contrast to previous work (Nishikawa, M., Hidaka, H., and Adelstein, R. S. (1983) J. Biol. Chem. 258, 14069-14072), two-dimensional tryptic peptide maps of both heavy meromyosin and the isolated myosin light chain showed two major phosphopeptides, one containing phosphoserine and the other phosphothreonine. We have purified the succinylated tryptic phosphopeptides using reverse phase and DEAE high pressure liquid chromatography. The serine-containing peptide, residues 1-4 (AcSSKR), is the NH2-terminal peptide. The phosphorylated serine residue may be either serine 1 or serine 2. The threonine-containing peptide, residues 5-16, yielded the sequence AAKTTKRPQR. Analysis of the yields and radioactivity of the products from automated Edman degradation showed that threonine 9 is the phosphorylation site.

Protein kinase C is a Ca2+-activated phospholipid-dependent enzyme that is activated in vivo by diacylglycerol through phosphatidylinositol (PI) turnover and directly by tumor promoters such as 12-O-tetradecanoylphorbol-13-acetate which mimics diacylglycerol. Protein kinase C has been shown to phosphorylate a large number of substrates, the physiological significance of which is unclear. In platelets, protein kinase C phosphorylates two major proteins (1, 2), a 40,000-Da protein which is believed to be a phosphatase with activity against inositol trisphosphate (3) and also the 20,000-Da light chain subunit of myosin (4-6). It has been suggested by earlier work (7) that phosphorylation of the 20,000-Da light chain by protein kinase C may modulate the phosphorylation-dependent regulation of smooth muscle contraction.

The reversible phosphorylation of the 20,000-Da myosin light chain by the Ca2+-calmodulin-dependent enzyme myosin light chain kinase plays an important role in regulating smooth muscle contraction (8-12). This phosphorylation occurs at serine 19 in the light chain sequence (13, 14) and results in a large increase in the actin-activated MgATPase activity (12), the in vitro biochemical correlate of muscle contraction. Recently, it has become clear that a second site, threonine 18, can be phosphorylated when an excess of myosin light chain kinase is used (15, 16). Phosphorylation of this second site results in a further increase in the actin-activated MgATPase activity.

Protein kinase C has been shown to phosphorylate a threonine residue in the myosin light chain that is clearly distinct from the threonine 18 which is phosphorylated by myosin light chain kinase (16). This protein kinase C-mediated phosphorylation has been shown to have two effects. First, it decreases the actin-activated MgATPase activity of heavy meromyosin (HMM), the soluble two-headed subfragment of myosin, that has already been phosphorylated by myosin light chain kinase. This decrease is due to a 6- to 7-fold increase in the K_m of HMM for actin with only a small effect on V_max.

In addition, when HMM has been prephosphorylated by protein kinase C, there is a marked decrease in the rate of phosphorylation by myosin light chain kinase through an effect on the K_m of the light chain for myosin light chain kinase (7).

In this paper we have determined the sequence of the threonine site phosphorylated by protein kinase C. Although it has already been proposed by indirect methods that either threonine 9 or 10 is phosphorylated (17), we now give direct evidence that threonine 9 is the actual phosphorylation site. In addition, we also present evidence for a second major phosphorylation site, serine 1 or 2, at the acetylated NH2 terminus (13) of the light chain.

Our results are in agreement with the previously reported substrate specificity of protein kinase C in other proteins. That is, there is a requirement for a basic residue, usually arginine, that is separated from the phosphorylation site by 1 or 2 neutral amino acids. Interestingly, this basic residue may be NH2-terminal, as in myelin basic protein, or COOH-terminal, as in myosin light chain, to the phosphorylation site (17-20)

MATERIALS AND METHODS

Protein Purification—Protein kinase C was purified to near homogeneity from fresh human platelet as previously described by Nishikawa et al. (21). The preparation was free of myosin light chain kinase activity as determined by the absence of calmodulin-dependent activity. Myosin and HMM were prepared from turkey gizzards as previously described (22). The 20,000-Da myosin light chain was prepared from turkey gizzard according to the method of Stafford (23). Rabbit skeletal muscle actin was prepared using the Eisenberg and Kielley modification (24) of the method of Spudich and Watt (25). Myosin light chain kinase was prepared from turkey gizzard according to the method of Adelstein and Klee (26).

Phosphorylation of HMM and 20,000-Da Myosin Light Chain—Phosphorylation of purified HMM and 20,000-Da myosin light chain by protein kinase C was carried out in 20 mM Tris-HCl (pH 7.5), 5
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mm MgCl₂, 200 μM CaCl₂, 1 mM [γ-32P]ATP (192 Ci/mol), 25 μg/ml phosphatidylserine, and 1 μg/ml 1,3-diolein prepared as described by Nishikawa et al. (27) at 25 °C for 90 min. HMM was phosphorylated by myosin light chain kinase under identical conditions without phospholipid and in the presence of 0.2 μM calmodulin. HMM was sequentially phosphorylated by myosin light chain kinase and then protein kinase C for measurement of the MgATPase activity; phosphorylation was terminated by adding 25 mM EGTA to a final concentration of 1 mM and placing the samples in ice. The extent of phosphorylation was determined by applying 10 μl of reaction mixture to Whatman No. 3 filter paper discs which were immediately immersed in 10% trichloroacetic acid containing 8% sodium pyrophosphate at 0 °C and then washed by the method of Corbin and Reimann (28). The site molecular weight of the HMM was 167,000 Da and that of light chain 20,000 Da.

Phosphorylated HMM and 20,000-Da myosin light chain were then dialyzed extensively against 0.5 M NaCl, 10 mM MOPS (pH 7.0), 0.1 mM EGTA, 3 mM NaN₃, 1 mM dithiothreitol and then against 50 mM NH₄HCO₃ (pH 8.0).

**Two-dimensional Peptide Mapping**—Following dialysis, the HMM and 20,000-Da myosin light chain were digested with TPCK trypsin (Worthington) at a substrate to trypsin weight ratio of 80:1 in NH₄HCO₃ (pH 8.0) at 37 °C for 16 h. The samples were lyophilized to dryness and resuspended in 5–10 μl of electrophoresis buffer, acetic acid/formic acid:H₂O (15:5:80). Between 1 and 5 μl of this solution was spotted on Silica Gel 60 thin layer plates (0.25-mm thickness, 20 × 20 cm, EM Science). Peptides were electrophoresed (CAMAG Scientific, Model 67702) at 1000 V for 80 min (4 °C) in the above buffer in the first dimension and then subjected to ascending chromatography in n-butyl alcohol/pyridine:acetic acid:H₂O (195:30:30:120) in the second dimension. The location and number of phosphopeptides were determined by autoradiography using either Kodak X-Omat AR or TL film.

**Phosphoamino Acid Analysis**—The radioactive peptides from the two-dimensional gels were scraped from the thin layer plates and eluted with 50 mM NH₄HCO₃ (pH 8.0) and lyophilized. The peptides were then subjected to acid hydrolysis in 6 N HCl for 3 h at 100 °C *in vacuo*. The resultant amino acids were lyophilized to dryness and resuspended in a buffer containing acetic acid:formic acid:H₂O (78:25:897) and spotted on cellulose thin layer plates. The samples were then electrophoresed, along with phosphoamino acid standards (phosphoserine and phosphothreonine) at 1000 V (15 °C) for 90 min. The phosphoamino acid standards were visualized with ninhydrin, whereas the sample phosphoamino acids were determined by autoradiography as described above.

**Purification of Phosphorylated Peptides**—The 20,000-Da myosin light chain which had been phosphorylated by protein kinase C and exten-

**RESULTS**

**Peptide Mapping and Phosphoamino Acid Analysis**—Two-dimensional tryptic peptide maps of both HMM and isolated myosin light chain show that there are two major phospho-

**Purification of Tryptic Phosphorylated Peptides**—The 20,000-Da myosin light chain was phosphorylated with protein kinase C in the presence of [γ-32P]ATP. It was then succinylated and subjected to complete tryptic digestion. The resulting peptides were lyophilized, resuspended in 0.05% A. HMM

**A. HMM**

**B. LIGHT CHAIN**

**FIG. 1.** Two-dimensional tryptic peptide maps of smooth muscle HMM and myosin light chain phosphorylated with protein kinase C in the presence of [γ-32P]ATP. The site molecular weight of the HMM was 167,000 Da and that of light chain 20,000 Da. The sample filter on gas phase sequencing was cut into sections after applying the peptide, and a section then was removed at successive cycles around the presumed phosphorylated residue. The peptide and the free phosphate were eluted from the filter with 50% formic acid by sonication and then separated by reverse phase HPLC (μBondapak C18, Waters Associates) using an acetonitrile gradient from 0 to 40% over 1 h.

**Assay of the MgATPase Activity**—The actin-activated MgATPase activity was assayed by the method of Pollard and Korn (31) in 10 mM imidazole (pH 7.0), 2 mM MgCl₂, 1 mM ATP, 0.1 mM EGTA, 25 μM skeletal actin, and 0.12 μM HMM at 25 °C.

**Amino Acid Composition and Sequence Analysis**—Purified peptides were hydrolyzed *in vacuo* in 6 N HCl at 106 °C for 24 h and then applied to a Beckman System 6300 amino acid analyzer using ninhydrin detection. Automated Edman sequence analyses were carried out on an Applied Biosystems gas phase sequencer 470A equipped with an on-line 120A phenylthiodyantion analyzer.

**Determination of Phosphorylated Residues**—The phosphorylated threonine residue was determined by the method of Wang et al. (30).
trifluoroacetic acid, and run over a μBondapak C18 reverse phase column (Waters Associates) with a 0–40% acetonitrile gradient (Fig. 2). The presence of more than the predicted number of peaks probably results from incomplete succinylation and incomplete digestion. There are three peaks that contain radioactivity. The two peaks eluting early in the acetonitrile gradient, as detected by radioactivity and absorbance, probably correspond to the doublet in peptide 1 seen on two-dimensional tryptic peptide maps, as the separation in peptide mapping was predominantly in the chromatographic dimension.

The three peaks were then individually subjected to HPLC DEAE chromatography with a gradient from pH 7 to 3. In each case, a single radioactive peak was eluted. The major peptide eluting at 41 min on reverse phase chromatography eluted at fraction 64 (Fig. 3), whereas the smaller peptides which eluted at 13–15 and 17–19 min on reverse phase chromatography each eluted at fraction 48 on HPLC DEAE ion exchange chromatography (Fig. 4).

Determination of Phosphorylation Site Sequences—Each of the major peaks as detected by radioactivity from HPLC DEAE chromatography was then subjected to amino acid analysis. The two peptides which eluted at 13–15 min (Peptide A) and 18–20 min (Peptide B) on reverse phase chromatography co-chromatographed on the DEAE ion exchange column (Fig. 4). Peptide A yielded the amino acid composition Arg (1.0), Ser (1.72), and Lys (0.96) with the presence of Asp and Gly as contaminating amino acids that are present in our columns. Peptide B had an identical amino acid composition, Arg (1.0), Ser (2.06), Lys (1.10), with the same contaminating amino acids present. The two peptides thus have an amino acid composition that corresponds to the NH2-terminal peptide, Ac-Ser-Ser-Lys-Arg (13). There is no other tryptic peptide in the 20,000-Da light chain that would contain only these 4 residues. As the NH2 terminus is acetylated, we were unable to determine the phosphorylated serine residue by amino-terminal sequencing. An attempt to determine the phosphorylated serine residue by first digesting the peptide with carboxypeptidase B and Y and then submitting the digest for amino acid composition did not allow us to distinguish which of the two serine residues is phosphorylated.

The peptide which eluted at fraction 64 on HPLC DEAE chromatography yielded the sequence Ala-Lys-Ala-Lys-Thr-Lys-Lys-Arg-Pro-Gln-Arg, corresponding to residues 5–16 in the 20,000-Da light chain (Table I). The low yield from gas phase sequencing of phenylthiohydantoin-Thr at cycle 5 (5% of theoretical) compared to the normal yield at cycle 6 (40% of theoretical) suggested that threonine 9 (cycle 5) is the phosphorylation site. Approximately 90% of the applied radioactivity remained on the filter after automated Edman degradation with little or no radioactivity found in the fractions. The threonine site was then confirmed by performing a second Edman degradation in which a portion of the filter was removed after cycles 5 and 6. The free phosphate and remaining peptides were eluted from the filter pieces with formic acid and separated by reverse phase HPLC. The radioactivity remaining in the peptide and present in the free phosphate was determined by Cerenkov counting. Phosphate is cleaved from the phosphorylated residue when it becomes the NH2-terminal residue. This free phosphate binds to the filter and is thus seen on the cycle containing the phosphorylated amino acid as well as on subsequent cycles. The cycle containing the phosphorylated residue should also demonstrate radioactivity in the remaining peptide, as the cleavage reaction is not complete (30). Since cycle 5 has radioactivity in the free phosphate as well as in the remaining peptide, whereas cycle 6 only contains radioactivity in the free phosphate, one can conclude that threonine 9 is the phosphorylated residue (Table II).

Effect of Two Site Phosphorylation by Protein Kinase C on the Actin-activated MgATPase Activity—The actin-activated MgATPase of HMM phosphorylated with myosin light chain kinase alone to 1 mol of Pi/mol of light chain, with myosin light chain kinase (1 mol of Pi/mol of LC) and protein kinase C (2 mol of Pi/mol of LC) to a total of 3 mol of Pi/mol of light chain, and with protein kinase C alone to 2 mol of Pi/mol of light chain was assayed at 25 μM actin. HMM phosphorylated with myosin light chain kinase at one site and protein kinase C at two sites caused approximately a 50% inhibition of the actin-activated MgATPase activity when compared to HMM phosphorylated with myosin light chain kinase alone (Table III).
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FIG. 4. HPLC DEAE ion exchange chromatography of fractions 13–15 (A) and 18–20 (B) from reverse phase. Column conditions were as described in the legend to Fig. 3 and under "Materials and Methods."

TABLE I

Sequence analysis of DEAE fraction 64

| Cycle | Residue | Amino acid | Yield pmol |
|-------|---------|------------|------------|
| 1     | 5       | Ala        | 558        |
| 2     | 6       | Suc-Lys    | 406        |
| 3     | 7       | Ala        | 451        |
| 4     | 8       | Suc-Lys    | 333        |
| 5     | 9       | Thr        | 16.5       |
| 6     | 10      | Thr        | 95.7       |
| 7     | 11      | Suc-Lys    | 100.1      |
| 8     | 12      | Suc-Lys    | 136.1      |
| 9     | 13      | Arg        | 63.5       |
| 10    | 14      | Pro        | 114        |
| 11    | 15      | Gln        | 156.2      |
| 12    | 16      | Arg        | 27.8       |

TABLE II

Radioactivity eluted from gas phase sequencer filter

7200 counts were applied to the gas phase sequencer filter, and approximately one-quarter of the filter was removed at each cycle.

| Cycle | Amino acid | cpm* as P<sub>i</sub> | cpm* in peptide |
|-------|------------|----------------------|-----------------|
| 5     | Thr        | 1029                 | 906             |
| 6     | Thr        | 723                  | 0               |

*cpm reflect subtraction of background counts.

TABLE III

Effect of protein kinase C phosphorylation on the actin-activated MgATPase activity of HMM

|                          | Phosphate incorporation mol P/mol LC | Actin-activated MgATPase activity s<sup>-1</sup> |
|-------------------------|-------------------------------------|-----------------------------------------------|
| Unphosphorylated HMM    | 0                                   | 0.035                                         |
| HMM phosphorylated with myosin light chain kinase | 1.2                                | 0.427                                         |
| HMM phosphorylated with myosin light chain kinase and protein kinase C | 3.0                                | 0.226                                         |
| HMM phosphorylated with protein kinase C | 1.9                                | 0.071                                         |

DISCUSSION

Phosphorylation of the 20,000-Da light chain subunit of myosin on serine 19 by myosin light chain kinase plays an important role in regulating the actin-activated MgATPase activity of smooth muscle myosin (8–12). It has been demonstrated that the myosin light chain may also be phosphorylated by protein kinase C and that this phosphorylation may play a role in modulating the MgATPase activity of myosin that has already been phosphorylated by myosin light chain kinase. Phosphorylation by protein kinase C has also been shown to decrease the rate of serine 19 phosphorylation by myosin light chain kinase (7).

We have determined the sequence of the protein kinase C phosphorylation sites in the 20,000-Da light chain subunit of myosin. This sequence is in agreement with what is known about the substrate specificity of protein kinase C (17–20). We have demonstrated that there are two major tryptic phosphopeptides, one containing a phosphorylated threonine at residue 9 and the other a phosphorylated serine at either residue 1 or 2. This is in contrast to a previous study (27) where only one major protein kinase C phosphorylation site was found when HMM was used as a substrate. This site corresponds to the threonine-containing site observed in the present study. The difference in our data and the earlier report may be due to differential rates of phosphorylation of the two sites. In this regard, preliminary data suggest that threonine 9 is phosphorylated at a faster rate than the serine site. The effect of protein kinase C phosphorylation at two sites on the actin-activated MgATPase activity of HMM that has been phosphorylated with myosin light chain kinase does not appear to be different from that reported earlier for single site phosphorylation by protein kinase C (27).

It is interesting to note that the protein kinase C phosphorylation sites are in close proximity to the myosin light chain kinase sites and that all the phosphorylation sites are near the NH<sub>2</sub> terminus. This proximity of the phosphorylation sites of the two kinases may help to explain the modulating role of protein kinase C in the regulation of smooth muscle myosin MgATPase activity. Phosphorylation of threonine 9 and serine 1 or 2 may alter the conformation of the NH<sub>2</sub>-terminal portion of the smooth muscle myosin light chain, thus making the light chain a poorer substrate for myosin light chain kinase. In the case where the light chain has already been phosphorylated with myosin light chain kinase, the conformational changes induced by protein kinase C phosphorylation may decrease the activity of the myosin.

The phosphorylation of a serine residue and a threonine residue by protein kinase C is an important finding in light of the fact that myosin light chain kinase can also phosphorylate both of these residues. In looking at the effects of myosin phosphorylation on cellular function in vivo, one will not be able to differentiate between myosin light chain kinase and protein kinase C-mediated phosphorylation by merely looking at whether a serine or threonine residue is phosphorylated. It will be important to actually determine the site phosphorylated by two-dimensional peptide mapping. These data, then, provide an important basis for looking at the effects of myosin phosphorylation by myosin light chain kinase and protein kinase C in vivo.
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Acknowledgments—We would like to acknowledge Angela Murphy for her contribution in performing the amino acid compositions. We thank Catherine S. Magruder for her help in preparing the manuscript.

REFERENCES

1. Haslam, R. J., and Lynham, J. A. (1977) Biochem. Biophys. Res. Commun. 77, 714–722
2. Lyons, R. M., Stanford, N., and Majerus, P. W. (1975) J. Clin. Invest. 56, 924–936
3. Controll, T. M., Lawing, W. J., and Majerus, P. W. (1986) Cell 46, 951–958
4. Daniel, J. L., Holmen, H., and Adelstein, R. S. (1977) Thromb. Haemostasis 38, 984–989
5. Naka, M., Nishikawa, M., Adelstein, R. S., and Hidaka, H. (1983) Nature 306, 490–492
6. Endo, T., Naka, M., and Hidaka, H. (1982) Biochem. Biophys. Res. Commun. 105, 942–948
7. Nishikawa, M., Sellers, J. R., Adelstein, R. S., and Hidaka, H. (1984) J. Biol. Chem. 259, 8808–8814
8. Chacko, S., Conti, M. A., and Adelstein, R. S. (1977) Proc. Natl. Acad. Sci. U. S. A. 74, 129–133
9. Sherry, J. M. F., Gorecka, A., Aksoy, M. O., Desbrowska, R., and Hartshorne, D. J. (1978) Biochemistry 17, 4411–4418
10. Sobieszek, A., and Small, J. V. (1977) J. Mol. Biol. 112, 559–576
11. Ikeye, M., Aiba, T., Onishi, H., and Watanabe, S. (1978) J. Biochem. (Tokyo) 83, 1643–1655
12. Sellers, J. R., Pato, M. D., and Adelstein, R. S. (1981) J. Biol. Chem. 256, 13137–13142
13. Pearson, R. B., Jakes, R., John, M., Kendrick-Jones, J., and Kemp, B. E. (1984) FEBS Lett. 168, 108–112
14. Maita, T., Chen, J., and Matsuda, G. (1981) Eur. J. Biochem. 117, 417–424
15. Ikebe, M., and Hartshorne, D. J. (1985) J. Biol. Chem. 260, 10027–10031
16. Ikebe, M., Hartshorne, D. J., and Elzinga, M. (1986) J. Biol. Chem. 261, 36–39
17. Hassel, T. C., Kemp, B. E., and Masaracchia, R. A. (1986) Biochem. Biophys. Res. Commun. 134, 240–247
18. O’Brian, C. A., Lawrence, D. S., Kaizer, E. T., and Weinstein, I. B. (1984) Biochem. Biophys. Res. Commun. 124, 296–302
19. Ferrari, S., Marchiori, F., Borin, G., and Pinna, L. A. (1985) FEBS Lett. 184, 72–77
20. Turner, R. S., Kemp, B. E., Su, H., and Kuo, J. F. (1985) J. Biol. Chem. 260, 11503–11507
21. Nishikawa, M., Shirakawa, S., and Adelstein, R. S. (1985) J. Biol. Chem. 260, 8978–8983
22. Sellers, J. R., Eisenberg, E., and Adelstein, R. S. (1982) J. Biol. Chem. 257, 13880–13888
23. Stafford, W. F. (1983) Biophys. J. 41, 93a
24. Eisenberg, E., and Kielley, W. W. (1974) J. Biol. Chem. 249, 4742–4748
25. Spudich, J. A., and Watt, S. (1971) J. Biol. Chem. 246, 4866–4871
26. Adelstein, R. S., and Klee, C. B. (1981) J. Biol. Chem. 256, 7501–7509
27. Nishikawa, M., Hidaka, H., and Adelstein, R. S. (1983) J. Biol. Chem. 258, 14069–14072
28. Corbin, J. D., and Reinmann, E. M. (1975) Methods Enzymol. 38, 287–290
29. Klotz, I. M. (1967) Methods Enzymol. 11, 576–580
30. Wang, Y., Bell, A. W., Hernochson, M. A., and Roach, P. J. (1986) in Proceedings of the 6th International Conference on Methods in Protein Sequence Analysis (Walsh, K. A., ed) in press
31. Pollard, T. D., and Korn, E. D. (1973) J. Biol. Chem. 248, 4682–4690