Role of the N-terminal Region of the Regulatory Light Chain in the Dephosphorylation of Myosin by Myosin Light Chain Phosphatase

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Myosin regulatory light chain (RLC) is phosphorylated at various sites at its N-terminal region, and heterotrimeric myosin light chain phosphatase (MLCP) has been assigned as a physiological phosphatase that dephosphorylates myosin in vivo. Specificity of MLCP toward the various phosphorylation sites of RLC was studied, as well as the role of the N-terminal region of RLC in the dephosphorylation of myosin by MLCP. MLCP dephosphorylates phosphoserine 19, phosphothreonine 18, and phosphothreonine 9 efficiently with almost identical rates, whereas it failed to dephosphorylate phosphorylated serine 1/serine 2. Deletion of the N-terminal seven amino acid residues of RLC markedly decreased the dephosphorylation rate of phosphoserine 19 of RLC incorporated in the myosin molecule, whereas this deletion did not significantly affect the dephosphorylation rate of isolated RLC. On the other hand, deletion of only four N-terminal amino acid residues showed no effect on dephosphorylation of phosphoserine 19 of incorporated RLC. The inhibition of dephosphorylation by deletion of the seven N-terminal residues was also found with the catalytic subunit of MLCP. Dephosphorylation at serine 1/serine 2 and threonine 9 did not influence the dephosphorylation rate of serine 19 and threonine 18 by MLCP. These results suggest that the N-terminal region of RLC plays an important role in substrate recognition of MLCP.

The motor function of conventional myosins expressed in smooth muscle as well as nonmuscle cells is regulated by phosphorylation of the regulatory light chain subunit (1–5). A calmodulin-dependent myosin light chain-specific protein kinase, myosin light chain kinase (MLCK), 1 phosphorylates serine 19 and threonine 18 of the regulatory light chain (RLC), and the phosphorylation of these sites activates the motor activity of myosin (1–5). Serine 19 is the preferred site and is important for the activation of the actomyosin contractile apparatus under physiological conditions. Other protein kinases can phosphorylate RLC at various sites. Phosphorylation of these sites activates the motor activity of myosin (1–5). Serine 19 is the preferred site and is important for the activation of the actomyosin contractile apparatus under physiological conditions. Other protein kinases can phosphorylate RLC at various sites. Phosphorylation of these sites activates the motor activity of myosin (1–5).

1 The abbreviations used are: MLCK, myosin light chain kinase; DTT, dithiothreitol; RLCP, regulatory light chain phosphatase of myosin; PKC, protein kinase C; MLCP, myosin light chain phosphatase; TCA, trichloroacetic acid; HMM, heavy meromyosin; SI, myosin subfragment 1.

MATERIALS AND METHODS

Preparation of Proteins and Construction of RLC Expression Vector—Smooth muscle myosin (20) and myosin light chain kinase (21) were prepared from turkey gizzards as described. Myosin was washed several times with high MgCl2 (50 mM) containing buffer to remove residual MLCP activity. HMM and SI were prepared from gizzard myosin as described (22). Calmodulin was prepared from frozen bull testes according to Walsh et al. (23). MLCP was prepared from turkey gizzard as described (17). The catalytic subunit of smooth muscle MLCP was prepared as described (16). CDNA of smooth muscle myosin RLC was prepared.
obtained from a chicken gizzard Agt11 cDNA library and subcloned into a P77-7 Escherichia coli expression vector (24) as described (25). Truncation of RLC cDNA was done using a PCR-based method as described previously (25). The expression of recombined RLCs and its mutants in the E. coli strain BL21 (DE3) was performed according to Kamisoyama et al. (26). The expressed RLC in E. coli cells was extracted in urea-containing buffer and purified with a series of liquid chromatography steps according to the method of Ikebe et al. (27).

Protein Biochemical Procedures—Phosphorylation of RLC at serine 19 was achieved by incubating RLC (4 mg/ml) with 2 μg/ml MLCK and 5 μg/ml calmodulin in buffer containing 0.5 mM [γ-32P]ATP, 150 mM KCl, 1 mM MgCl2, 1 mM CaCl2, 1 mM EGTA, and 30 mM Tris-HCl, pH 7.5 (buffer A), at 25 °C for 20 min. The extent of phosphorylation was 0.9 mol of phosphate/mol of RLC, and no phosphorylation at threonine residues was detected based upon phosphoamino acid analysis, indicating that only serine 19 was phosphorylated. Phosphorylation at both serine 19 and threonine 18 was achieved by incubating RLC (4 mg/ml) with 50 μg/ml MLCK and 30 μg/ml calmodulin in modified buffer A containing 1 mM [γ-32P]ATP and 30 mM KCl at 25 °C for 40 min. Incorporation of 1.8 mol of phosphate/mol of RLC was obtained, and an equal amount of phosphoserine and phosphothreonine was detected using phosphoamino acid analysis, indicating that both serine 19 and threonine 18 were phosphorylated (27). Phosphorylation of RLC (2 mg/ml) at serine 19 and threonine 9 by PKC was done by incubating with a solution containing 3 μg/ml PKC, 100 mg/ml phosphor 12-myristate 13-acetate, 0.1 mg/ml phosphatidyserine, 1 mM [γ-32P]ATP, 30 mM KCl, 1 mM MgCl2, and 30 mM Tris-HCl, pH 7.5 at 24 °C for 50 min. Two mol of phosphate/RLC was incorporated, and both serine and threonine were phosphorylated as judged by phosphoamino acid analysis (Ref. 7, also see Fig. 6). The phosphorylated RLCs were precipitated with 5% trichloroacetic acid and dissolved in and dialyzed against buffer B (30 mM KCl, 1 mM DTT, and 30 mM Tris-HCl, pH 7.5).

RLC-deficient gizzard myosin was prepared according to the method of Trybus et al. (28) with modification (29). Phosphorylated RLC (2 molar excess) was added to RLC-deficient myosin in buffer C (30 mM KCl, 1 mM DTT, 2 mM MgCl2, and 30 mM Tris-HCl, pH 7.5) at 0 °C. After 10 min, the myosin was centrifuged for 2 min at 10,000 × g. The pellets were suspended with buffer C and centrifuged again. This step was repeated three times. The pellets were then dissolved with 0.4 μl KCl, 5 mM DTT, and 30 mM Tris-HCl, pH 7.5, and used for experiments. The obtained myosin contained phosphorylated RLC as judged by SDS-polyacrylamide gel electrophoresis analysis followed by autoradiography (not shown). The extent of phosphorylation of the obtained myosin was stable at least for 6 h at 25 °C.

Phosphoamino acid analysis was done as described previously (27). The extent of phosphorylation of RLC was determined as described (27).

RESULTS

Dephosphorylation of Myosin by Smooth Muscle MLCP—Smooth muscle myosin containing various truncated RLCs (Fig. 1) was prepared as described under “Materials and Methods.” Dephosphorylation of native smooth muscle myosin phosphorylated at serine 19 of RLC by smooth muscle MLCP was described by single exponential kinetics (Fig. 2). The dephosphorylation time course of myosin incorporating exogenous serine 19 phosphorylated RLC was identical to that of native myosin, suggesting that the exogenous RLC was properly incorporated into myosin. The rate of dephosphorylation was somewhat reduced with ND-7 RLC-incorporated myosin, but the decrease was not significant. On the other hand, when the N-terminal seven residues of RLC were deleted (NDL-7), the dephosphorylation by smooth muscle MLCP was markedly and significantly inhibited. The rate of dephosphorylation was 1/10 of that of myosin incorporating wild type RLC. Further deletion of three amino acid residues did not affect the rate of dephosphorylation. These results suggest that Ala5-Lys6-Ala7 is critical for dephosphorylation of RLC at serine 19 by MLCP.

Dephosphorylation of Isolated RLC—Because many protein phosphatases can dephosphorylate isolated RLC but not intact myosin, we wished to assay whether or not the effect of deletion of the N-terminal amino acid residues observed above is also found with isolated RLC as a substrate. To address this notion, the dephosphorylation reaction by smooth muscle MLCP was carried out with isolated truncated mutants of RLC. As shown in Fig. 3, the rate of dephosphorylation was not markedly affected by the deletion of any N-terminal residues of RLC. The rates of dephosphorylation of ND-4 and NDL-7 were slightly lower than that of wild type RLC, but the decrease was not significant (Fig. 3). These results indicate that the deletion of Ala5-Lys6-Ala7 diminished dephosphorylation of serine 19 of RLC only in the presence of myosin heavy chain but not in its absence. These results also suggest that myosin heavy chain plays a role in the smooth muscle MLCP-RLC interaction.

Dephosphorylation of Myosin by the Catalytic Subunit of Smooth Muscle MLCP—Smooth muscle MLCP is composed of three subunits, i.e. the myosin binding subunit, catalytic subunit, and small subunit (17–19). It was shown that phosphatase activity against myosin is enhanced in the presence of the myosin binding subunit (30), suggesting a difference in the nature of the dephosphorylation reaction between apoenzyme and holoenzyme. To examine whether the decrease in phosphatase activity of smooth muscle MLCP by the deletion of the N-terminal region of myosin-incorporated RLC is because of the function of the myosin binding subunit or because of the characteristics of the catalytic subunit of smooth muscle MLCP, we used catalytic subunit of smooth muscle MLCP to dephosphorylate truncated phosphorylated RLC incorporated into myosin. As shown in Fig. 4, dephosphorylation of myosin by the catalytic subunit of smooth muscle MLCP was also significantly attenuated by deletion of the N-terminal residues. The rate of dephosphorylation of NDL-7-containing myosin was 9% of the rate of wild type RLC-containing myosin. This was also confirmed using a different myosin fragment which con-
tained truncated RLC generated by a brief tryptic proteolysis of myosin which cleaves off the N-terminal 16 residues of RLC (31). Myosin was first phosphorylated with MLCK, then subjected to tryptic digestion and used as a substrate for smooth muscle MLCP holoenzyme and apoenzyme as described under “Materials and Methods.” The rate of dephosphorylation of myosin incorporating the truncated RLC which lacked the N-terminal 16 residues was significantly decreased (not shown).

Dephosphorylation of Myosin Phosphorylated at Various Sites on RLC—It is known that RLC of smooth muscle and nonmuscle myosins are phosphorylated at various sites in the N-terminal region (7, 8, 32) (see Fig. 1). Serine 19 and threonine 18 are phosphorylated by MLCK (32). On the other hand, serine 1/serine 2 and threonine 9 are phosphorylated by PKC (7, 8) and cdc2 kinases (9). Myosin phosphorylated at these different sites was prepared (see “Materials and Methods”), and the rate of dephosphorylation by smooth muscle MLCP was determined. The dephosphorylation of myosin phosphorylated at both serine 19 and threonine 18 showed a single exponential decay curve, and the rate of dephosphorylation was identical to that for dephosphorylation of myosin phosphorylated at serine 19 alone (Fig. 5). This suggests that the rate of dephosphorylation of serine 19 and threonine 18 of RLC by smooth muscle MLCP is indistinguishable. Consistent with this notion, phosphoamino acid analysis revealed that both phosphoserine and phosphothreonine were decreased to the same extent after 10 min of the dephosphorylation reaction where approximately 70% of the total incorporated phosphate was removed by the phosphatase (Fig. 6). On the other hand, the dephosphorylation of myosin phosphorylated at PKC sites (i.e. serine 1/serine 2 and threonine 9) by smooth muscle MLCP showed dual phases. Myosin was initially dephosphorylated by smooth muscle MLCP with a rate similar to that for the dephosphorylation of the serine 19/threonine 18 sites but then became resistant to dephosphorylation by MLCP. To determine the sites resistant to smooth muscle MLCP, a sample was taken at 30 min after the addition of smooth muscle MLCP and subjected to phosphoamino acid analysis. As shown in Fig. 6, both phosphoserine and phosphothreonine were detected before MLCP addition, whereas only phosphoserine was observed at 30 min after the addition of MLCP, indicating that phosphorylation at serine 1/serine 2 is resistant to dephosphorylation by smooth muscle MLCP. This difference in the MLCP susceptibility of serine and threonine sites was also found using isolated RLC as a substrate (not shown).

Effect of PKC Phosphorylation on the Dephosphorylation of MLCK Sites—The results described above indicate that the N-terminal region of RLC is important for the dephosphorylation of RLC at serine 19/threonine 18 when it is incorporated into myosin heavy chain. Because PKC and cdc2 kinases phosphorylate RLC in the N-terminal region, it is of interest to examine whether or not the phosphorylation of RLC by these kinases can affect the dephosphorylation of serine 19/threonine 18. RLCs were first phosphorylated by PKC under conditions incorporating 2.0 mol of phosphate/mol of RLC with nonradioactive ATP as a substrate. RLCs were then precipitated with 5% TCA, dissolved, and dialyzed against neutral pH buffer. RLC was then phosphorylated with MLCK using radioactive ATP to incorporate 32P into both serine 19 and threonine 18. RLCs were then hybridized with RLC-deficient myosin as described under “Materials and Methods.” As shown in Fig. 7, the dephosphorylation of serine 19/threonine 18 by smooth muscle MLCP was not significantly affected by phosphorylation at
RLC was first phosphorylated by PKC as described under “Materials and Methods” with nonradioactive ATP and then phosphorylated with radioactive [γ-32P]ATP. The phosphorylated RLC was then phosphorylated at PKC sites by smooth muscle MLCP with the N-terminal region of RLC increases the accessibility of the MLCP catalytic site to the phosphate moieties of phosphorylated RLC.

Previously, it was found that the dephosphorylation of myosin at serine 19 by smooth muscle MLCP is significantly affected by the conformation of myosin, i.e., a folded conformation of myosin is highly resistant to dephosphorylation by MLCP (33). It was suggested that the myosin tail interacts with the N-terminal region of RLC, thus stabilizing the folded conformation, because the mutation of the basic residues at the N-terminal region of RLC abolishes the folded conformation (25). That result is consistent with the present finding that the N-terminal region of RLC plays a role in the substrate recognition of smooth muscle MLCP. It is plausible that in the folded conformation smooth muscle MLCP cannot access the substrate site because of obstruction by the myosin tail binding to the N-terminal region of RLC.

It is known that the rates of phosphorylation of serine 19 and threonine 18 by MLCK are significantly different from each other. In contrast, the rate of dephosphorylation of these sites by smooth muscle MLCP is practically the same. The results suggest that the difference in the phosphorylation level at serine 19/threonine 18 of RLC is influenced by the conformation of myosin and the interaction of RLC with smooth muscle MLCP. The present result provides a clear answer to this apparent discrepancy. The dephosphorylation at threonine 18 by MLCP in cells but the phosphorylated serine 1/serine 2 would be resistant to dephosphorylation by MLCP. Other protein phosphatases may slowly dephosphorylate the serine 1/serine 2 sites in cells because a spontaneously active aorta phosphatase was shown to dephosphorylate both threonine and serine sites in cells after phorbol ester treatment. A possible scenario to account for this finding is that the decrease in smooth muscle MLCP-dependent dephosphorylation is found only when RLC associates with myosin heavy chain. A possible scenario to account for this finding would be that the N-terminal region of RLC is involved in the interaction of RLC with smooth muscle MLCP and that the serine 19/threonine 18 of RLC are somewhat occluded from the smooth muscle MLCP catalytic site in the presence of the heavy chain. It is plausible that the interaction of smooth muscle MLCP with the N-terminal region of RLC increases the accessibility of the MLCP catalytic site to the phosphate moieties of phosphorylated RLC.

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ester stimulation provides further evidence that MLCP is the physiological myosin light chain phosphatase. The slow dephosphorylation of the serine 1/serine 2 sites was previously shown with crude gizzard phosphatase (7) so the present results are consistent with the earlier results.

The time course of dephosphorylation of myosin at serine 19 by smooth muscle MLCP is explained by a single rate constant. Furthermore, the rate of dephosphorylation of RLC at serine 19 is virtually the same for HMM and S1 (not shown). These results indicate that the dephosphorylation process of RLC at serine 19 is random and independent relative to the other head of myosin. In phosphorylation reactions, it has been reported that myosin is phosphorylated sequentially by MLCK, i.e. phosphorylation of the first head is faster than that of the second head (35–38), although this is still controversial. If one accepts the sequential phosphorylation of myosin by MLCK, it would be expected that significantly higher MLCK activity would be required for phosphorylation of the second head and that the majority of phosphorylated myosin population at lower overall phosphorylation levels in cells would be singly phosphorylated myosin. To date, it is controversial whether or not the motor activity of phosphorylated myosin head is dependent on the phosphorylation of the other head of myosin, but the actomyosin contractile activity as a function of overall myosin phosphorylation in cells could be complex.

While the N-terminal region of RLC is important for determining the dephosphorylation rate of myosin at serine 19 by smooth muscle MLCP, phosphorylation at this region, i.e. serine 1/serine 2 and threonine 9, failed to influence the dephosphorylation rate of myosin at serine 19. This is in contrast to the MLCK reaction in which the rate of phosphorylation at serine 19 is decreased by phosphorylation at serine 1/serine 2 and threonine 9 (7, 8). This difference might be because of a difference in the manner of substrate recognition between the two enzymes because MLCK requires basic residues at the N-terminal side of the phosphorylation sites. This charge interaction may not be critical for the MLCP reaction.

REFERENCES

1. Hartshorne, D. J. (1987) in *Physiology of the Gastrointestinal Tract* (Johnson, L. R., ed), 2nd Ed., Vol. 1, pp. 425–482, Raven Press, New York
2. Sellers, J. R., and Adelstein, R. S. (1987) in *The Enzymes* (Broyer, P., and Krebs, E. G., eds), Vol. 18, pp. 381–418, Academic Press, San Diego, CA
3. Kamm, K. E., and Stull, J. T. (1989) *Annu. Rev. Physiol. 51*, 299–313
4. Sellers, J. R. (1991) *Curr. Opin. Cell Biol.* 3, 98–104
5. Tan, J. L., Ravid, S., and Spudich, J. A. (1992) *Annu. Rev. Biochem. 61*, 721–759
6. Amano, M., Ito, M., Kimura, K., Fukata, Y., Chihara, K., Nakao, T., Matsuzira, Y., and Kurihara, K. (1996) *J. Biol. Chem.* 271, 20234–20240
7. Ikehata, M., Hartshorne, D. J., and Elzinga, M. (1987) *J. Biol. Chem.* 262, 9569–9573
8. Bengur, A. R., Robinson, E. A., Appella, E., and Sellers, J. R. (1987) *J. Biol. Chem.* 262, 7613–7617
9. Satterthwaite, L. L., Lokha, M. J., Wilson, K. L., Scherson, T. Y., Cisek, J. L., Corden, J. L., and Pollard, T. D. (1992) *J. Cell Biol.* 118, 585–605
10. Cohen, P. (1989) *Annu. Rev. Biochem.* 58, 453–508
11. Mumby, M. C., and Walter, G. (1993) *Physiol. Rev.* 73, 673–699
12. Mayer-Jaekel, R. E., and Hemmings, B. A. (1994) *Trends Cell Biol.* 4, 287–291
13. Paton, M. D., and Adelstein, R. S. (1983) *J. Biol. Chem.* 258, 7047–7054
14. Paton, M. D., and Kerc, E. (1985) *J. Biol. Chem.* 260, 12559–12366
15. Tullio, A. G., and Paton, M. D. (1981) *J. Biol. Chem.* 266, 20168–20174
16. Mitzi, T., Inagaki, M., and Ikehata, M. (1992) *J. Biol. Chem.* 267, 16727–16735
17. Allessi, D., MacDougall, L. D., Sola, M. M., Ikehata, M., and Cohen, P. (1992) *Eur. J. Biochem.* 210, 1023–1035
18. Shimizu, H., Ito, M., Miyahara, M., Ichikawa, K., Okuno, S., Komishii, T., Naka, M., Tanaka, T., Hirano, K., Hartshorne, D. J., and Nakano, T. (1994) *J. Biol. Chem.* 269, 30407–30411
19. Shirazi, A., Lizzuka, K., Fadden, P., Metso, C., Somlyo, A. P., and Somlyo, A. V., and Haystead, T. A. (1994) *J. Biol. Chem.* 269, 31588–31606
20. Ikehata, M., and Hartshorne, D. J. (1985) *J. Biol. Chem.* 260, 13146–13153
21. Ikehata, M., Stepinska, M., Kemp, B. E., Means, A. R., and Hartshorne, D. J. (1987) *J. Biol. Chem.* 262, 13828–13834
22. Ikehata, M., and Hartshorne, D. J. (1985) *Biochemistry* 24, 2380–2387
23. Walsh, M. P., Hinkins, S., Babrowska, R., and Hartshorne, D. J. (1983) *Methods Enzymol.* 99, 279–288
24. Tabor, S., and Richardson, C. C. (1985) *Proc. Natl. Acad. Sci. U. S. A.* 82, 1074–1078
25. Ikehata, M., Reardon, S., Schwonek, J. P., Sanders, C. R., Ii, and Ikehata, R. (1994) *J. Biol. Chem.* 269, 28165–28172
26. Kamisoyama, H., Araki, Y., and Ikehata, M. (1993) *Biochemistry* 30, 9539–9545
27. Ikehata, M., and Hartshorne, D. J. (1985) *J. Biol. Chem.* 260, 10027–10031
28. Trybus, K. M., Waller, G. S., and Chatman, T. A. (1994) *J. Cell Biol.* 124, 963–969
29. Ikehata, M., Kambara, T., Stafforow, F. W., Sata, M., Katayama, E., and Ikehata, R. (1998) *J. Biol. Chem.* 273, 1777–17707
30. Gong, M. C., Fuglsang, A., Allessi, D., Kobayashi, S., Cohen, P., Somlyo, A. V., and Somlyo, A. P. (1992) *J. Biol. Chem.* 267, 21492–21498
31. Ikehata, M., and Marita, J. (1991) *J. Biol. Chem.* 266, 21338–21342
32. Ikehata, M., Hartshorne, D. J., and Elzinga, M. (1986) *J. Biol. Chem.* 261, 36–39
33. Ikehata, M., Inagaki, M., Nakao, M., and Hidaka, H. (1988) *J. Biol. Chem.* 263, 10698–10704
34. Kawamoto, S., Bengur, A. R., Sellers, J. R., and Adelstein, R. S. (1989) *J. Biol. Chem.* 264, 2255–2265
35. Persechini, A., and Hartshorne, D. J. (1981) *Science* 213, 1383–1385
36. Persechini, A., and Hartshorne, D. J. (1983) *Biochemistry* 22, 4144–4150
37. Sellers, J. R., Chock, P. B., and Adelstein, R. S. (1983) *J. Biol. Chem.* 258, 14181–14188
38. Ikehata, M., Ogihara, S., and Tonomura, Y. (1982) *J. Biochem. (Tokyo)* 91, 1809–1812