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Phosphorylation of brush border myosin by brush border calmodulin-dependent myosin heavy and light chain kinases

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Calmodulin-dependent myosin light chain kinase isolated from chicken intestinal brush border phosphorylates brush border myosin at an apparently single serine identical to that phosphorylated by smooth muscle myosin light chain kinase. Phosphorylation to 1.8 mol phosphate/mol myosin activated the myosin actin-activated ATPase about 10-fold, to about 50 nmol/min per mg. Myosin phosphorylated on its light chains could then be further phosphorylated to a total of 3.2 mol phosphate per mol by brush border calmodulin-dependent heavy chain kinase. Heavy chain phosphorylation did not alter the actin-activated ATPase of either myosin prephosphorylated on its light chains or of unphosphorylated myosin.

Brush border myosin; Myosin heavy chain kinase; Myosin light chain kinase; Calmodulin; (Chicken)

1. INTRODUCTION

Phosphorylation of the heavy chains, in addition to the light chains, of myosin has recently been found to occur in many types of non-muscle cells (review [1]). In intestinal brush borders, both myosin heavy [2] and light chain kinases [2,3] have been identified that are dependent on calcium and CaM. Thus, in brush border at least, CaM-dependent phosphorylation by distinct heavy and light chain kinases might occur concomitantly in response to intracellular increases in calcium. Light chain phosphorylation activates the actin-activated ATPase activity and promotes the filament assembly of many [4,5], but not all [6-9], non-muscle and smooth muscle myosins. Heavy chain phosphorylation has been found to inhibit [10-12], activate [13] or have no effect [14] on ATPase activity and filament formation of the few myosins for which studies have been carried out. Also, the effects of heavy and light chain phosphorylation may be interdependent. Activation of brush border myosin actin-activated ATPase activity [15,16] and filament assembly [16] by CaM-dependent light chain phosphorylation alone has been found using light chain kinase purified from smooth muscle [16]. For the studies reported here, we isolated myosin light chain kinase from brush border and show that the site of phosphorylation and the effect on brush border myosin ATPase activity of phosphorylation by light chain kinases from smooth muscle and brush border are apparently identical. CaM-dependent myosin heavy chain kinase has also been purified from brush borders [17,18] and shown to be a type II CaM-dependent protein kinase [18]. Here, we use brush border heavy chain and smooth muscle light chain kinases to study the effects of CaM-dependent

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Abbreviations: CaM, calmodulin; PMSF, phenylmethylsulfonyl fluoride; DTT, dithiothreitol; PAGE, polyacrylamide gel electrophoresis; LC_{20}, myosin 20 kDa light chain

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heavy and light chain phosphorylation on the ATPase activity of brush border myosin.

2. MATERIALS AND METHODS

Brush border CaM-dependent myosin light kinase was isolated as follows. Intestinal epithelial brush border cells from the small intestines of one chicken were isolated [19] and suspended in 15 vols of buffer containing 10 mM imidazole-HCl, 4 mM EDTA, 1 mM EGTA (pH 7.3) and protease inhibitors consisting of aprotonin (5 μg/ml), leupeptin (2 μg/ml), pepstatin (5 μg/ml), PMSF (0.2 mM) and DFP (1 mM). The cells were homogenized in a Waring blender at 15 000 rpm, then centrifuged at 16 000 $\times$ g for 30 min. The supernate was made 25 mM in NaCl and 48 ml was applied to a column of DEAE-Sephacel (Pharmacia, 1.5 $\times$ 2.7 cm) equilibrated with 10 mM imidazole-HCl, 10% sucrose, 25 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1.0 mM DTT, 0.25 mM PMSF, 0.02% NaN_3, pH 7.5 (buffer K), at a flow rate of 42 ml/h. The column was washed with 10 ml buffer K and eluted with a 25 ml linear gradient of NaCl (0.025–0.6 M) in buffer K at 60 ml/h. Calcium- and CaM-dependent myosin light chain kinase activity eluted between 0.19 and 0.33 M NaCl and was pooled (4.1 mg in 7 ml) and dialyzed against buffer K.

Brush border CaM-dependent myosin heavy chain kinase was purified according to Rieker et al. [17] with an additional affinity chromatography step on CaM-Sepharose [18]. The kinase showed one major band of 50 kDa on SDS-PAGE [18]. Brush border myosin was prepared by the procedure of Rieker et al. [18]. Chicken gizzard myosin light chain kinase [20] and rabbit skeletal muscle F-actin [21] were purified as described.

Myosin kinase assays were carried out at 30°C for the times indicated in 20 mM imidazole-HCl, 60 mM NaCl, 0.5 mM [γ-^{32}P]ATP (New England Nuclear), 6 mM $MgCl_2$, 0.25 mM PMSF, 1 μg/ml leupeptin, 0.25 mM DTT (pH 7.5) and either 1 mM EGTA, 0.71 mM $CaCl_2$, or 0.71 mM $CaCl_2$ and 2 μg/ml bovine brain CaM (Sigma), and either brush border light chain kinase (82.8 μg/ml) or brush border heavy chain kinase (6.4 μg/ml) and/or gizzard myosin light chain kinase (2.9 μg/ml). Quantitation of phosphate incorporation was performed after separation of myosin into myosin heavy and light chains by SDS-PAGE [22] on 5–20% gradient slab gels as in [17].

For phosphoamino acid analysis and phosphopeptide mapping, stained gel bands containing ^{32}P-labeled myosin light chains were excised and incubated with 100 μg trypsin (TPCK-treated, Worthington) in 50 mM NH_4HCO_3 for 20 h at 35°C. 100 μg trypsin was then added, and digestion was continued for 4 h. Samples for phosphoamino acid analysis were incubated in 6 N HCl at 110°C for 2 h and analyzed by electrophoresis in acetic acid/formic acid/water (8:2:90; pH 1.9) at 1000 V for 2 h at 2°C.

Two-dimensional peptide mapping of tryptic digests was performed by electrophoresis in pyridine/acetic acid/water (10:0.4:90; pH 6.4) for 2 h at 400 V at 2°C, followed by ascending chromatography at room temperature in n-butanol/pyridine/acetic acid/water (50:30:10:40).

ATPase assays were performed at 35°C for 60 min with 1–2 μCi/ml [γ-^{32}P]ATP [23]. The Mg^{2+}-ATPase medium contained 10 mM imidazole-HCl, 2 mM $MgCl_2$, 1 mM EGTA, 1 mM ATP (pH 7.5) and when present, F-actin at 0.25 mg/ml.

3. RESULTS AND DISCUSSION

Myosin light chain kinase isolated from chicken intestinal epithelial cells as described in section 2 is specific for the 20 kDa light chains of intact brush border myosin and is stimulated 8-fold by calcium and CaM (fig.1). The maximum phosphate incorporation by this kinase was about 0.9 mol phosphate/mol light chain (table 1), and this occurred exclusively at serine (fig.1B, lane 1). Two-dimensional peptide mapping (fig.2) shows that phosphorylation occurred within a single tryptic peptide and possibly at a single site in the LC_{20}s. Maximal light chain phosphorylation converted myosin to a highly actin-activatable form, with a specific activity of 48 nmol/min per mg (table 1, expt A). These results, together with the previous finding that phosphorylation of myosin light chains during contraction of isolated brush borders [15,24] is stimulated by calcium [15], provide strong evidence for a role of CaM-dependent phosphorylation of the light chains in the function of brush border myosin. The CaM-dependent light chain kinases from brush border and smooth mus-

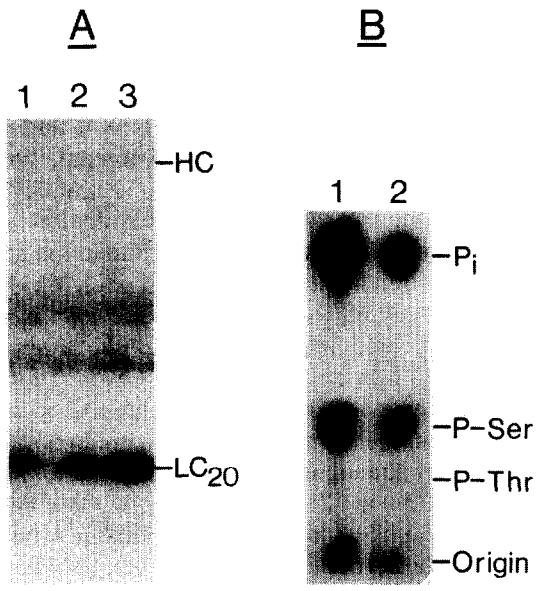

Fig.1. Autoradiography of SDS-polyacrylamide gel electrophoretic patterns and phosphoamino acid analyses of brush border myosin phosphorylated by the brush border myosin light chain kinase fraction. (A) Reaction mixtures containing 1 μg myosin, the brush border myosin kinase fraction and EGTA (lane 1), calcium (lane 2), or calcium and CaM (lane 3) were incubated in a volume of 35 μl for 15 min and analyzed by SDS-PAGE, as described in section 2. An autoradiograph of the gel is shown. The positions of myosin heavy chains (HC) and the 20 kDa light chains (LC_{20}) are indicated. (B) Myosin (2 μg) ws phosphorylated to approx. 1.8 mol phosphate/mol myosin in 60 min in the presence of calcium and CaM by either the brush border myosin kinase fraction (lane 1) or chicken gizzard myosin light chain kinase (lane 2). Heavy and light chains were separated by SDS-PAGE and gel slices containing LC_{20} were incubated with trypsin. The eluted peptides were subjected to limited acid hydrolysis, and the hydrolysates analyzed by electrophoresis as described in section 2. Less than 15% of the radioactivity applied remained at the origin. The positions of the ninhydrin-stained marker phosphoamino acids, $^{32}P_i$, and the origin are indicated on the autoradiograph.

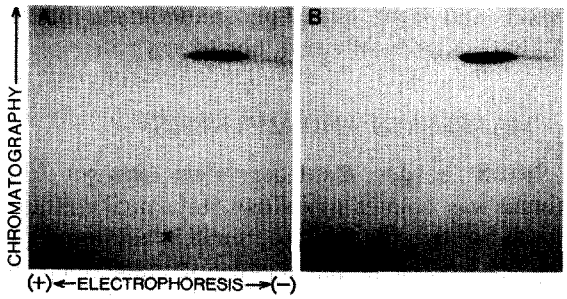

Fig.2. Analyses of the phosphopeptides formed by tryptic digestion of ^{32}P-labeled brush border myosin. Myosin (2 μg) was phosphorylated to approx. 1.8 mol phosphate/mol myosin in 60 min in the presence of calcium and CaM by the brush border myosin light chain kinase (panel A) or chicken gizzard myosin light chain kinase (panel B) and complete tryptic digests of the ^{32}P-labeled myosin LC_{20} were prepared as in fig.1. The peptides were separated in two dimensions on thin-layer cellulose sheets and phosphopeptides visualized by autoradiography, as described in section 2. The origin is indicated (X).

cle apparently phosphorylate identical sites on brush border myosin, as determined by phosphoamino acid analyses (fig.1) and peptide mapping (fig.2), and activate the actin-activated ATPase activity to the same extent (table 1, expt A). In addition, we have shown previously [2] that the Stokes radii of these kinases are very similar. These results also demonstrate that highly purified smooth muscle myosin light chain kinase [20] can be used instead of the less highly purified light chain kinase from brush border in studies of the effects of phosphorylation on brush border myosin, as described below.

To determine next whether heavy chain phosphorylation in the absence of light chain phosphorylation affects the actin-activated ATPase activity of brush border myosin, heavy chains of brush border myosin were specifically phosphorylated using brush border CaM-dependent myosin heavy chain kinase (table 1, expt B and [18]). The results show that the Mg^{2+}-ATPase activity of myosin phosphorylated on its heavy chains is not activated by F-actin. Therefore, heavy chain phosphorylation alone is not sufficient to activate brush border myosin ATPase.

To study the effects of phosphorylation of both its heavy and light chains, brush border myosin was phosphorylated with gizzard light chain kinase, and then with brush border heavy chain kinase, as shown in the time course in fig.3. After the incorporation of 0.9 mol phosphate/mol light chain, addition of heavy chain kinase resulted in

Table 1

Effect of phosphorylation with myosin heavy and light chain kinases on the actin-activatable activity of brush border myosin

Kinase	Phosphate incorporation (mol/mol)		Actin-activated ATPase (nmol/min per mg)
	LC_{20}	HC	
Expt A			
None	<0.01	<0.01	5
Brush border MLCK	0.91	<0.01	48
Gizzard MLCK	0.90	<0.01	50
Expt B			
None	<0.01	<0.01	8
Brush border MHCK	<0.04	0.80	9
Gizzard MLCK	0.90	<0.01	54
Gizzard MLCK then brush border MHCK	0.92	0.70	56

Expt A: Brush border myosin (5.0 μg) was incubated with either buffer K, the brush border myosin light chain kinase (MLCK) fraction or gizzard MLCK in the presence of calcium and CaM as indicated in a volume of 0.14 ml. Each incubation was carried out in parallel in the presence of [γ-^{32}P]ATP or unlabeled ATP for 60 min. Aliquots of the mixtures containing radiolabeled ATP were assayed for phosphate incorporation into LC_{20} and heavy chains. 70 μl of the mixtures containing unlabeled ATP were assayed for actin-activated ATPase activity. Expt B: Brush border myosin (4 μg) was incubated with gizzard MLCK in a volume of 0.14 ml for 60 min in the presence of [γ-^{32}P]ATP or unlabeled ATP. Then brush border myosin heavy chain kinase (MHCK) or an equal volume of buffer K was added. After a total incubation period of 120 min, aliquots were assayed for phosphate incorporation (40 μl) and ATPase activity (80 μl). The MG^{2+}-ATPase activity of myosin in the absence of actin was 5 nmol/min per mg. The K^+-, EDTA-ATPase and Ca^{2+}-ATPase activities of the myosin, which were 0.75 and 0.36 μmol/min per mg, respectively, were unchanged

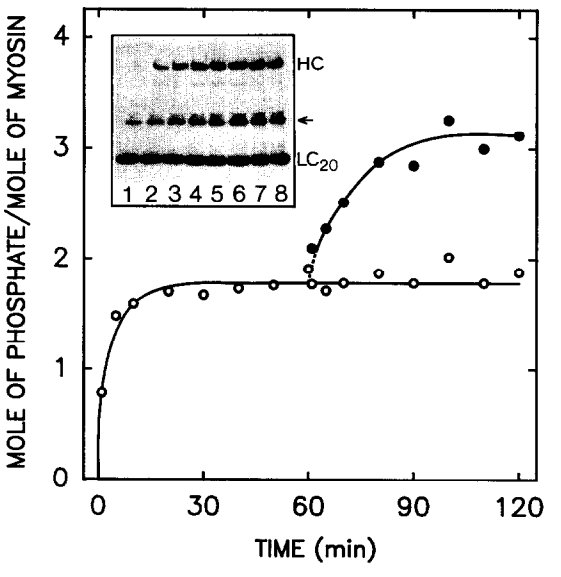

Fig.3. Sequential phosphorylation of brush border myosin by chicken gizzard myosin light chain kinase and brush border myosin heavy chain kinase. Brush border myosin (24 μg) was incubated with gizzard myosin light chain kinase in the presence of calcium and CaM in a volume of 0.84 ml (○). After 60 min the reaction mixture was divided equally and brush border myosin heavy chain kinase was added to one portion (●) and an equal volume of buffer K was added to the other portion (○). Each reaction mixture was incubated for an additional 60 min. Aliquots (40 μl) were taken at the times indicated and analyzed for myosin heavy and light chain kinase activity. The figure shows the sum of phosphate incorporation into the heavy and light chains. (Inset) Autoradiograph of the SDS-PAGE patterns of the reaction mixture at 1 (1), 5 (2), 10 (3), 20 (4), 30 (5), 40 (6), 50 (7) and 60 (8) min after the addition of the brush border heavy chain kinase fraction. The small arrow shows autophosphorylation of the 50 kDa subunit of myosin heavy chain kinase.

the incorporation of an additional 1.4 mol phosphate/mol myosin (see fig.3, inset and table 1, expt B). About 95% of the additional phosphorylation occurred in the heavy chains. These results establish that brush border myosin can be phosphorylated at two sites, on the light chains and on the heavy chains, by distinct brush border CaM-dependent myosin kinases. The results also show that the level of activation of actin-activated ATPase activity due to CaM-dependent light chain phosphorylation is not affected by subsequent CaM-dependent heavy chain phosphorylation. This is consistent with the finding that phosphorylation of brain myosin on its heavy and light chains with brain CaM-dependent type II kinase activates the actin-activated Mg^{2+}-ATPase activity of brain myosin [14]. However, they are in contrast with those reported for leukemic fibroblast myosin [12], whose actin-activated ATPase was inhibited by heavy chain phosphorylation, as is found for some invertebrate myosins [10,11].

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REFERENCES

[1] Kuznicki, J. (1986) FEBS Lett. 204, 169-176.
[2] Rieker, J.P., Swanljung-Collins, H. and Collins, J.H. (1987) FEBS Lett. 212, 154-158.
[3] Keller, T.C.S. and Mooseker, M.S. (1982) J. Cell Biol. 95, 943-959.
[4] Adelstein, R.S. and Eisenberg, E. (1980) Annu. Rev. Biochem. 49, 921-956.
[5] Kendrick-Jones, J. and Scholey, J.M. (1981) J. Muscle Res. Cell Motil. 2, 347-372.
[6] Barylko, B., Tooth, P. and Kendrick-Jones, J. (1986) Eur. J. Biochem. 158, 271-282.
[7] Wagner, P.D., Vu, N.-D. and George, J.N. (1985) J. Biol. Chem. 260, 8084-8089.
[8] Wagner, P.D. and Vu, N.-D. (1986) J. Biol. Chem. 261, 7778-7783.
[9] Collins, J.H., Kuznicki, J., Bowers, B. and Korn, E.D. (1982) Biochemistry 21, 6910-6915.
[10] Collins, J.H. and Korn, E.D. (1980) J. Biol. Chem. 255, 8011-8014.
[11] Kuczmarski, E.R. and Spudich, J.A. (1980) Proc. Natl. Acad. Sci. USA 77, 7292-7296.
[12] Sagara, J., Nagata, K. and Ichikawa, Y. (1983) Biochem. J. 214, 839-843.
[13] Ogihara, S., Ikebe, M., Takahashi, K. and Tonomura, Y. (1983) J. Biochem. 93, 205-223.
[14] Tanaka, E., Fukunaga, K., Yamamoto, H., Iwasa, T. and Miyamoto, E. (1986) J. Neurochem. 47, 254-262.
[15] Keller, T.C.S., Conzelman, K.A., Chasan, R. and Mooseker, M.S. (1985) J. Cell Biol. 100, 1647-1655.
[16] Citi, S. and Kendrick-Jones, J. (1986) J. Mol. Biol. 188, 369-382.
[17] Rieker, J.P., Swanljung-Collins, H., Montibeller, J. and Collins, J.H. (1987) Methods Enzymol. 139, 105-114.
[18] Rieker, J.P., Swanljung-Collins, H. and Collins, J.H. (1987) J. Biol. Chem., in press.
[19] Collins, J.H. and Borysenko, C.W. (1984) J. Biol. Chem. 259, 14128-14135.
[20] Ngai, P.K., Carruthers, C.A. and Walsh, M.P. (1984) Biochem. J. 218, 863-870.
[21] Eisenberg, E. and Kielley, W.W. (1974) J. Biol. Chem. 249, 4742-4748.
[22] Laemmli, U.K. (1970) Nature 227, 680-685.
[23] Korn, E.D., Collins, J.H. and Maruta, H. (1982) Methods Enzymol. 85, 357-363.
[24] Broschat, K.O., Stidwell, R.P. and Burgess, D.R. (1983) Cell 35, 561-571.