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Brush border myosin heavy chain phosphorylation is regulated by calcium and calmodulin

James P. Rieker, Helena Swanljung-Collins, Judith Montibeller and Jimmy H. Collins

Department of Microbiology, Biochemistry and Molecular Biology, School of Medicine, University of Pittsburgh, Pittsburgh, PA 15261, USA

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Myosin from chicken intestinal brush borders is phosphorylated on its heavy chains at threonine by a kinase isolated from brush borders. In contrast to other heavy chain kinases, the brush border kinase activity is dependent on calcium and calmodulin. The partially purified preparation also phosphorylated myosin on its light chains at serine, but in a calmodulin-independent manner. Phosphorylation of the light chains in the absence of calmodulin or both heavy and light chains in the presence of calmodulin activated its actin-activated ATPase activity about 10-fold, to about 50 nmol/min per mg.

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

1. INTRODUCTION

Phosphorylation of myosin on its heavy chains has been found to occur in a variety of vertebrate [1–5] and invertebrate [6–8] non-muscle cells. Although the role of heavy chain phosphorylation in the regulation of vertebrate myosins is not known, heavy chain phosphorylation has been shown to regulate the actin-activated ATPase activity and filament assembly of some invertebrate myosins [6–8]. In most cell types, heavy chain phosphorylation occurs in addition to calcium- and CaM-dependent light chain phosphorylation, which is a well-established mechanism for regulation of smooth muscle and many non-muscle myosins [9], including brush border myosin [10].

The regulation of heavy chain kinases from both vertebrates [2–4] and invertebrates [6,11] is poorly understood. Here we describe the identification and regulation by calcium and CaM of myosin heavy chain kinase activity from chicken intestinal epithelial cell brush borders.

Correspondence address: J.H. Collins, Dept of Microbiology, Biochemistry and Molecular Biology, School of Medicine, University of Pittsburgh, Pittsburgh, PA 15261, USA

Abbreviations: CaM, calmodulin; PMSF, phenylmethylsulfonyl fluoride; DTT, dithiothreitol; PAGE, polyacrylamide gel electrophoresis; DATD, *N,N'*-diallyltartardiamide; FPLC, fast protein liquid chromatography; LC_{20}, myosin 20 kDa light chain

2. MATERIALS AND METHODS

The myosin kinases were fractionated as follows. Brush borders isolated as described in [12] from the small intestine of one chicken were suspended in 2 vols of buffer containing 10 mM imidazole-HCl, 0.2 M KCl, 5 mM $MgCl_2$, 5 mM ATP, 1 mM EGTA, 1 mM DTT, 0.2 mM PMSF, 1 mM diisopropyl fluorophosphate and 5 μg/ml aprotinin, pH 6.8 (buffer A). After incubation for 30 min on ice, this suspension was centrifuged for 30 min at 40000 rpm in a Beckman 50 Ti rotor (100000 × *g*). 2 ml of the supernate (1.1 mg/ml protein) was filtered through a Millipore HVLP filter (0.22 μm pore size) and chromatographed, in four successive runs of 0.5 ml each, on a Phar-

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macia Superose 6 FPLC gel filtration column (1 × 30 cm) equilibrated at 2–4°C with buffer A. Fractions containing calcium- and CaM-dependent myosin heavy chain kinase activity (eluting at 12–14 ml, fig.1) were pooled (0.39 mg protein in 12 ml), dialyzed in 10 mM imidazole-HCl, 25 mM NaCl, 10% sucrose, 1 mM DTT, 1 mM EDTA, 1 mM EGTA, 0.2 mM PMSF, pH 7.5 (buffer B), and applied to a Pharmacia Mono Q FPLC anion-exchange column (0.5 × 5 cm) equilibrated with buffer B at 2–4°C. The concentrated myosin kinase activity (0.3 mg protein in 2 ml) was eluted with buffer B adjusted to 0.25 M NaCl. The average recovery from five preparations was about 50% of the activity present in the extract. These had only trace amounts of CaM and ATPase, phosphatase and protease activities. Brush border myosin was prepared as described by Collins and Borysenko [12]. Rabbit skeletal muscle F actin was prepared according to Eisenberg and Kielly [13].

Kinase assays were carried out at 30°C for the times indicated in 20 mM imidazole-HCl, 60 mM NaCl, 6 mM $MgCl_2$, 0.5 mM [γ-^{32}P]ATP (New England Nuclear), 0.25 mM PMSF, 1 μg/ml leupeptin, 0.25 mM DTT, pH 7.5 and either 0.85 mM EGTA; 0.1 mM $CaCl_2$; or 0.1 mM $CaCl_2$ and 2 μg/ml bovine brain CaM (Sigma), as indicated. The reaction mixtures were heated at 100°C in 1% SDS for 2 min and electrophoresed on 5–20% SDS-polyacrylamide slab gels as described by Laemmli [14], except that the gels were made with the periodate-cleavable crosslinker DATD, at a DATD/acrylamide ratio of 1:10 (w/v). After protein staining and autoradiography, gel bands containing myosin heavy and light chains were excised, dissolved in 3% periodic acid and counted for radioactivity to determine the stoichiometry of phosphorylation. About 4000 cpm per gel band was obtained per mol phosphate incorporated into myosin. Two-dimensional phosphoamino acid analyses were performed [15] on the heavy and light chains eluted from gel slices [16].

ATPase assays were performed at 35°C for 60 min with 1–2 μCi/ml [γ-^{32}P]ATP [17]. 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. Values for the actin-activated Mg^{2+}-ATPase activity were corrected for the very low ATPase activity of myosin in the absence of F-actin and of F-actin in the absence of myosin. Protein concentrations were determined according to Bradford [18], with bovine serum albumin as the standard.

3. RESULTS AND DISCUSSION

A kinase that catalyzes the phosphorylation of the heavy chains of purified, native brush border myosin in a calcium- and CaM-dependent manner was identified by high-performance gel filtration of an extract of chicken intestinal epithelial cell brush borders (fig.1, lower panel). This kinase has several properties in common with the CaM-

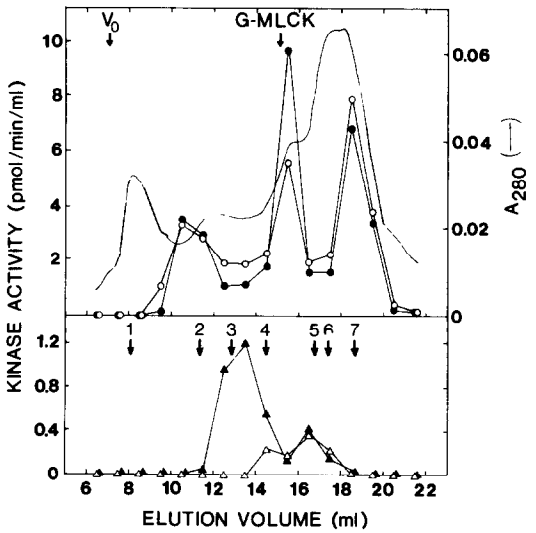

Fig.1. High-performance gel filtration chromatography of brush border extract. Freshly prepared brush border extract (100000 × *g* supernate) was chromatographed on a Superose 6 column at a flow rate of 12 ml/h. Fractions of 0.5 ml were collected and 5-μl aliquots were assayed for myosin light chain kinase (upper panel) or myosin heavy chain kinase (lower panel) in the presence of EGTA (○, △) or calcium and CaM (●, ▲) and myosin (1 μg) in a volume of 35 μl for 15 min. The void volume (V_0) and elution position of chicken gizzard myosin light chain kinase (G-MLCK) are indicated. Standards of (1) rabbit skeletal myosin (200 Å), (2) fibrinogen (107 Å), (3) thyroglobulin (85 Å), (4) ferritin (79 Å), (5) bovine serum albumin (35 Å), (6) ovalbumin (29 Å) and (7) myoglobin (21 Å) were used to estimate the Stokes' radii of the kinases by the method of Laurent and Killander [21].

dependent protein kinase II family of kinases present in brain, liver and other vertebrate tissues (review [19]). These are, in addition to CaM dependence, a high molecular mass as indicated by a Stokes' radius of 70–95 Å (70 Å estimated for the brush border kinase from the calibrated gel filtration column, fig.1) and the presence of one or more 50–60 kDa phosphorylatable polypeptides (see 50 kDa phosphorylated species in the heavy chain kinase fraction, fig.2, lane d). These results, and the recent finding that brain CaM-dependent protein kinase II phosphorylates the heavy and light chains of brain myosin [20], suggest that the brush border CaM-dependent myosin heavy chain kinase may be related to this family of kinases.

The brush border myosin heavy chain kinase was separated from the peak of calcium- and CaM-stimulated light chain kinase (Stokes' radius 52.5 Å) by high-performance gel filtration (fig.1). The later kinase eluted from the Superose 6 column in almost the same position as gizzard myosin light chain kinase (fig.1, upper panel) and, therefore, may belong to the widely distributed class of calcium- and CaM-dependent myosin kinases that are highly specific for the light chains of myosin [9]. The presence of both CaM-regulated myosin heavy and light chain kinases in brush border raises the possibility that heavy and light chain phosphorylation of myosin might occur concomitantly and regulate myosin in a coordinate manner in response to increases in the intracellular free calcium concentration.

In addition to the CaM-regulated myosin heavy and light chain kinases, a peak of heavy chain kinase activity (Stokes' radius 41.5 Å) and two peaks of light chain kinase activity (Stokes' radii 25.5 and 122 Å) that were not regulated by calcium and CaM were also obtained (fig.1). It is not yet known whether the calcium-independent enzymes phosphorylate the same sites as the CaM-regulated kinases and, if so, whether these represent different forms of the kinases.

Phosphorylation of purified brush border myosin in the presence of calcium and CaM with concentrated CaM-dependent heavy chain kinase-containing fraction resulted in incorporation of 3.8 mol P/mol myosin in 15 min (table 1). No further increase in phosphorylation occurred over a 2 h time course of incubation. Both the heavy chains and the LC_{20}s were phosphorylated to approx. 1 mol P/mol (table 1 and fig.2, lane g). The heavy chains were phosphorylated at an initial rate of 120 pmol/min per mg, approximately twice the rate of light chain phosphorylation. In the

Table 1

Effect of phosphorylation with brush border kinase on the actin-activatable ATPase activity of brush border myosin

Kinase	Phosphate incorporation (mol/mol)		Actin-activated ATPase (nmol/min per mg)
	LC_{20}	HC	
None	<0.05	<0.01	5
Brush border kinase			
$-Ca^{2+}$/CaM	1.14	<0.01	47
$+Ca^{2+}$/CaM	1.08	0.82	49

Brush border myosin (12.3 μg) was incubated with either buffer A or the brush border kinase fraction (2.8 μg) in the presence of EGTA or 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 (35 μl) of the mixtures containing radiolabeled ATP were assayed for phosphate incorporation into the 20 kDa light chains (LC_{20}) and heavy chains (HC). Aliquots (70 μl) of mixtures containing unlabeled ATP were assayed for actin-activated ATPase activity. Details are given in section 2

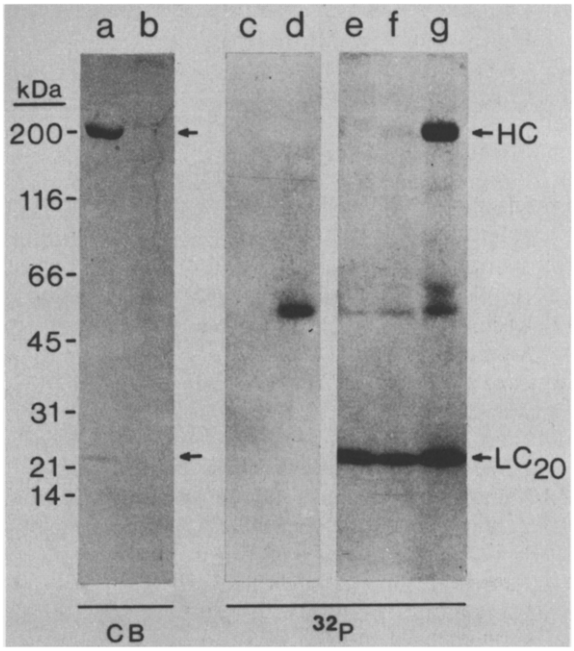

Fig.2. Autoradiography of SDS-polyacrylamide gel electrophoretic patterns of brush border myosin phosphorylated by the brush border kinase fraction. Reaction mixtures containing 1 μg brush border myosin (lanes a,c,e–g), 0.7 μg brush border kinase fraction (lanes b,d–g) and EGTA (lane e), calcium (lane f) or calcium and CaM (lanes c,d,g) were incubated in a volume of 35 μl for 15 min and analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography, as described in section 2. The Coomassie blue-stained gel patterns (CB) in lanes a,b correspond to the autoradiograms in lanes c,d. The positions of myosin heavy chains (HC), 20 kDa light chains (LC_{20}) and molecular mass standards [rabbit skeletal myosin heavy chain (200 kDa), β-galactosidase (116 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), soybean trypsin inhibitor (21 kDa) and lysozyme (14 kDa)] are indicated.

presence of EGTA or calcium without CaM (fig.2, lanes e,f), the heavy chains were phosphorylated to only a trace extent. LC_{20} phosphorylation did not require calcium and CaM (table 1 and fig.2) and, therefore, is apparently not catalyzed by the CaM-dependent heavy chain kinase. The presence of light chain kinase activity in this fraction may be due to overlap of the two adjacent peaks of light chain kinase with the peak of heavy chain kinase (see fig.1). Phosphoamino acid analyses demonstrated that threonine, but not serine or tyrosine, was the residue phosphorylated on the heavy chains and that light chain phosphorylation occurred exclusively at serine.

To study selectively the effects of heavy and light chain phosphorylation on the actin-activated ATPase activity of brush border myosin, advantage was taken of the different CaM dependences of these reactions. Phosphorylation of myosin with the brush border kinase fraction in the absence of calcium and CaM resulted in an almost 10-fold increase in the actin-activated ATPase activity (table 1). This activation must be due to phosphorylation of the LC_{20}s since the heavy chains were not phosphorylated under these conditions or during the ATPase assays conducted in the presence of EGTA. Similar activation of brush border myosin by phosphorylation of its LC_{20}s with chicken gizzard myosin light chain kinase has been reported [10]. In the presence of calcium and CaM, phosphorylation of the heavy chains, in addition to the light chains, resulted in essentially the same actin-activated ATPase activity as did light chain phosphorylation alone. Therefore, phosphorylation of the heavy chains is not required for actin-activated ATPase activity and does not alter the level of activation produced by phosphorylation of the LC_{20}s, in agreement with recent studies of brain myosin [20]. In contrast, heavy chain phosphorylation inhibits actin activation due to light chain phosphorylation of leukemic myeloblast myosin [3], and inhibits actin activation of myosins from the invertebrates *Acanthamoeba* [7] and *Dictyostelium* [6]. Further studies are needed to determine whether heavy chain phosphorylation of brush border myosin regulates filament formation or other properties of myosin, including its molecular conformation and association with the cytoskeleton.

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