Volume 235, number 1,2, 149–152 FEB 06139 August 1988

Phosphorylation of brush border myosin at threonine on its 20 kDa light chains by a calmodulin-independent kinase activates its ATPase

Christopher Borysenko, 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

Received 18 May 1988

A calmodulin-independent kinase isolated from chicken intestinal brush border phosphorylates brush border myosin mainly at an apparently single threonine on its 20 kDa light chains. Phosphorylation to 1.9 mol phosphate/mol myosin activated the myosin actin-activated ATPase about 12-fold, to about 100 nmol/min per mg. Brush border myosin ATPase can thus be activated by phosphorylation either at threonine, by calmodulin-independent kinase, or at serine, by calmodulin-dependent myosin light chain kinase, as previously shown [(1987) FEBS Lett. 223, 262–266].

Myosin; Myosin light chain kinase; Myosin actin-activated ATPase; Calmodulin; Cell motility; (Chicken intestinal brush border)

1. INTRODUCTION

Phosphorylation of myosin on its heavy and light chains is an important mechanism for regulation of the contractile and motile activities of many smooth and non-muscle cells. Phosphorylation of the regulatory light chains at a specific serine by calcium- and calmodulin (CaM)-dependent myosin light chain kinases activates the actin-activated Mg^{2+}-ATPase activity and promotes the bipolar filament assembly of many of these myosins [1], including myosin from chicken intestinal brush borders [2,3].

We have previously found that brush border myosin can also be phosphorylated on its 20 kDa light chains by a CaM-independent kinase identified by gel-filtration chromatography of a crude extract of brush border [4]. We now report, using a preparation free of CaM-dependent light [3] and heavy chain kinases [5], that the CaM-independent kinase activates the actin-activated ATPase activity of the myosin by phosphorylation mainly at threonine instead of at serine.

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

2. MATERIALS AND METHODS

CaM-independent kinase was isolated as follows from freshly prepared chicken intestinal brush borders. The preparation of brush borders from four chickens [6], that of a $100000 \times g$ brush border extract, and gel-filtration chromatography of the extract on Sepharose CL-4B [7] were performed as described. The extract (101.5 mg protein in 109 ml) contained 80–90% of the brush border CaM-independent myosin light chain kinase activity present in the extract. The kinase (79.6 mg in 306 ml) eluted at 1620–1926 ml from the Sepharose CL-4B column (5×90 cm) and was separated from earlier-eluting peaks of CaM-dependent myosin light chain and heavy chain kinases, with results similar to those shown previously for a Superose 6 gel-filtration column [4]. The kinase was dialyzed against 2×2-l of 10 mM imidazole chloride, 25 mM NaCl, 10% sucrose, 1 mM EDTA, 1 mM EGTA, 1 mM dithiotreitol, 0.02% NaN_3, 0.2 mM phenylmethylsulfonyl fluoride, pH 7.5 (buffer K), filtered through a Millipak 20 filter unit (Millipore) and applied at a flow rate of 1 ml/min to a Pharmacia Mono Q high-performance anion-exchange column (0.5×5 cm) equilibrated with buffer K. Following sample application the column was

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/88/$3.50

washed with 10 ml buffer K and eluted in 0.5-ml fractions with a 40 ml gradient of 25–400 mM NaCl in buffer K at a flow rate of 0.5 ml/min.

Fractions containing calcium- and CaM-independent myosin light chain kinase activity, but no Mg^{2+}-ATPase activity (about 20% of the kinase activity recovered from the column), eluted between 0.23 and 0.29 M NaCl and were pooled (3.8 mg in 5.5 ml) and dialyzed against buffer K. Brush border CaM-dependent myosin light chain kinase [3] and brush border myosin [5] were isolated as described. Rabbit skeletal muscle actin was purified according to Eisenberg and Kielley [8].

Myosin kinase assays were carried out at 30°C for 30 min, except where otherwise 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 phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, 0.25 mM dithiothreitol, 63 μg/ml brush border myosin, pH 7.5, and either 0.85 mM EGTA (plus EGTA), 0.71 mM $CaCl_2$ and 0.14 mM EGTA (plus calcium), or 0.71 mM $CaCl_2$, 0.14 mM EGTA and 2 μg/ml bovine brain CaM (Sigma) (plus calcium and CaM) and the amounts of kinase stated in the text. For assays conducted in the presence of cAMP (10 μM); cAMP-dependent protein kinase inhibitor (10 μg/ml) (Sigma); or phosphatidylserine (80 μg/ml), diolein (2.4 μg/ml) and calcium (0.1 mM), the extent of phosphorylation was less than 30% of maximal. Phosphate incorporation into myosin light chains isolated by SDS-polyacrylamide gel electrophoresis [9] on 5–20% gradient slab gels was determined as in [5].

For phosphoamino acid analysis and phosphopeptide mapping, bands containing ^{32}P-labeled myosin light chains were excised from SDS-polyacrylamide gels and extensively digested with trypsin, as described earlier [3]. Phosphoamino acid analysis was carried out according to [3], with an additional second-dimensional ascending chromatography step at room temperature in isobutyric acid/0.5 M ammonium hydroxide (5:3). Quantitation of the relative amounts of ^{32}P label in phosphoamino acids and phosphopeptides was performed either by liquid scintillation counting of radioactivity present in spots scraped from the cellulose plates or by two-dimensional densitometry using a Bio-Rad model 620 video densitometer.

ATPase assays were performed at 35°C for 60 min with 1–2 μCi/ml [γ-^{32}P]ATP [10]. 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.

Sedimentation coefficients ($s_{20,w}$) were determined by velocity sedimentation through calibrated 5–20% sucrose gradients [5]. Molecular masses and frictional coefficients were calculated from Stokes radii and sedimentation coefficients by assuming partial specific volumes of 0.725 ml/g as described by Siegel and Monty [11]. Protein concentrations were determined by the method of Bradford [12], using a Bio-Rad protein assay kit with bovine serum albumin as the standard.

3. RESULTS AND DISCUSSION

A kinase isolated from chicken intestinal brush border as described in section 2 is specific for the 20 kDa light chains on brush border myosin (fig.1). Unlike brush border CaM-dependent myosin light chain kinase [3], this enzyme does not require calcium and CaM. In the presence of EGTA, phosphate incorporation was 0.9 mol phosphate/mol light chain, and was reduced about 30% by calcium and CaM. The CaM-independent kinase also differs in its physical properties from brush border CaM-dependent myosin light chain kinase (table 1).

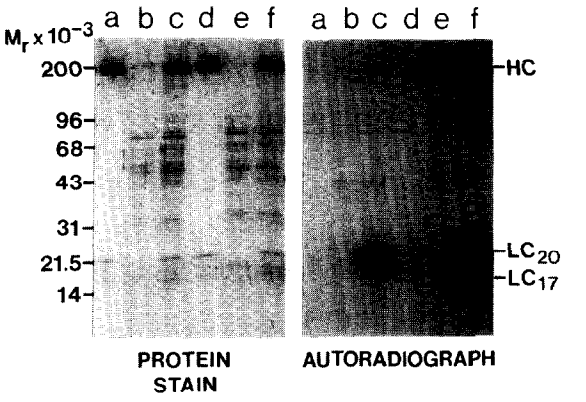

Fig.1. SDS-polyacrylamide gel electrophoretic patterns and autoradiographs of brush border myosin phosphorylated by the brush border CaM-independent kinase. Reaction mixtures containing 1.9 μg brush border myosin (lanes a,c,d,f), 1 μg kinase (b,c,e,f) and EGTA (a–c), or calcium and CaM (d–f) were incubated in a volume of 30 μl for 120 min and electrophoresed, as described in section 2. The sample for lane c was from the mixture described in fig.4 after 120 min incubation. 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.

Threonine was the major amino acid residue phosphorylated on the 20 kDa light chains, with only about 5% incorporation at serine (fig.2). In

Table 1

Physical properties of chicken brush border CaM-independent and CaM-dependent myosin light chain kinases

	CaM-independent kinase	CaM-dependent kinase
Stokes radius (Å)	25.5	52.5
Sedimentation coefficient ($s_{20,w}$) (S)	4.1	5.9
Molecular mass (Da)	44 000	130 000
Frictional ratio (f/f_0)	1.09	1.57

Values for the Stokes radii are from Rieker et al. [4]

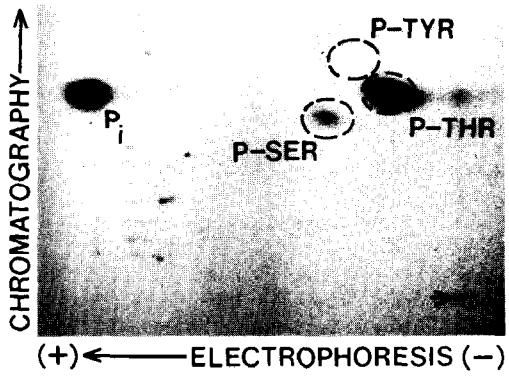

Fig.2. Phosphoamino acid analysis of 20 kDa light chains of brush border myosin phosphorylated by brush border CaM-independent kinase. The 20 kDa light chains in a slice from the gel shown in fig.1, lane c were exhaustively digested with trypsin. An aliquot containing one-half of the eluted peptides were subjected to partial acid hydrolysis, and the hydrolysates were analyzed on a thin-layer cellulose sheet, as described in section 2. The positions of the ninhydrin-stained marker phosphoamino acids, $^{32}P_i$ and the origin (X) are indicated.

contrast, phosphorylation of brush border myosin by brush border and chicken gizzard CaM-dependent myosin light chain kinases has been reported only at serine [3]. The specificity of the brush border enzyme for serine is retained after proteolysis to an active, but CaM-independent form (Rieker, J.P. and Collins, J.H., unpublished). Therefore, the strong preference of the CaM-independent kinase for threonine demonstrates that it is not derived from CaM-dependent myosin light chain kinase by possible proteolysis during purification. The activity of the CaM-independent kinase was not significantly affected by cAMP, cAMP-dependent protein kinase inhibitor, or by the protein kinase C activators calcium, diolein, and phosphatidylserine.

Two-dimensional mapping of a complete tryptic digest of brush border myosin shows that about

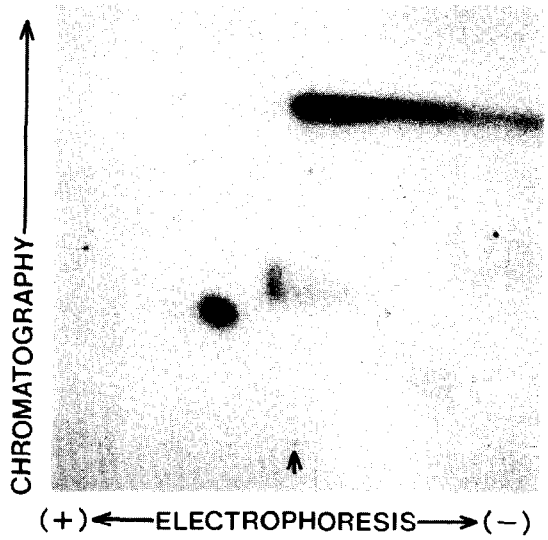

Fig.3. Two-dimensional mapping of the phosphopeptides from a tryptic digest of the 20 kDa light chains of brush border myosin phosphorylated by the CaM-independent kinase. One-half of the tryptic digest of the 20 kDa light chains from the gel shown in fig.1, lane c was analyzed in two dimensions and phosphopeptides visualized by autoradiography, as described in section 2. The origin is indicated (X).

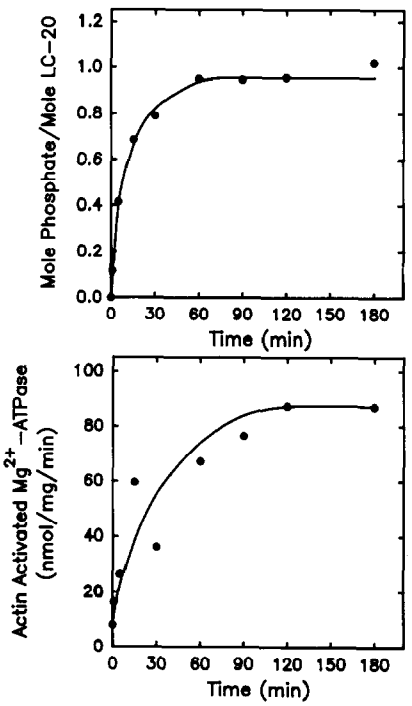

Fig.4. Time course of phosphorylation of brush border myosin by CaM-independent kinase and the effect of phosphorylation on the actin-activated ATPase activity. Myosin (38 μg) was incubated with CaM-independent light chain kinase (20 μg) in phosphorylation mixture (0.6 ml) containing EGTA but no added calcium. Incubations were carried out in the presence of [γ-^{32}P]ATP (upper panel) or unlabeled ATP (lower panel). Aliquots (30 μl) of the reaction mixtures were removed at the times indicated and assayed for either phosphate incorporation into myosin (upper panel) and actin-activated Mg^{2+}-ATPase activity (lower panel). The ATPase activity of non-phosphorylated myosin in the presence of actin is 8 nmol/min per mg. No activity was detected in assays containing kinase, but no myosin. Details are given in section 2.

80% of the phosphate incorporation catalyzed by the CaM-independent kinase occurs within a single peptide (fig.3). The remaining incorporation occurs within a second peptide, which may represent another site of phosphorylation.

Phosphorylation of brush border myosin increases its actin-activatable ATPase activity, as shown in the time course in fig.4. Incorporation of approx. 2 mol phosphate/mol myosin gave a specific activity of 100 nmol/min per mg, more than 12-fold higher than that of non-phosphorylated myosin. Phosphorylation of brush border myosin by CaM-dependent myosin light chain kinase from brush border [3] or gizzard [2,3] also activates its ATPase, and phosphorylation in both cases occurs at an apparently identical serine [3]. Therefore, brush border myosin ATPase can be activated through phosphorylation of either a threonine or a serine on its 20 kDa light chains.

Acknowledgements: We thank Brendon Lamperski for technical assistance. This research was supported by NIH grants GM32567 and GM35448 and by postdoctoral fellowship HL07557 to J.P.R.

REFERENCES

[1] Citi, S. and Kendrick-Jones, J. (1987) BioEssays 7, 155–159.
[2] Citi, S. and Kendrick-Jones, J. (1986) J. Mol. Biol. 188, 369–382.
[3] Rieker, J.P. and Collins, J.H. (1987) FEBS Lett. 223, 262–266.
[4] Rieker, J.P., Swanljung-Collins, H., Montibeller, J. and Collins, J.H. (1987) FEBS Lett. 212, 154–158.
[5] Rieker, J.P., Swanljung-Collins, H. and Collins, J.H. (1987) J. Biol. Chem. 262, 15262–15268.
[6] Collins, J.H. and Borysenko, C.W. (1984) J. Biol. Chem. 259, 14128–14135.
[7] Rieker, J.P., Swanljung-Collins, H., Montibeller, J. and Collins, J.H. (1987) Methods Enzymol. 139, 105–114.
[8] Eisenberg, E. and Kielley, W.W. (1974) J. Biol. Chem. 249, 4742–4748.
[9] Laemmli, U.K. (1970) Nature 227, 680–685.
[10] Korn, E.D., Collins, J.H. and Maruta, H. (1982) Methods Enzymol. 85, 357–363.
[11] Siegel, L.M. and Monty, K.J. (1966) Biochim. Biophys. Acta 112, 346–362.
[12] Bradford, M.M. (1976) Anal. Biochem. 72, 248–254.