Volume 334, number 1, 18–22 FEBS 13199 November 1993

Identification of casein kinase II as a major endogeneous caldesmon kinase in sheep aorta smooth muscle

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Received 13 September 1993

A caldesmon kinase activity was detected in an ATP extract of the myofibril-like pellet from sheep aorta. The enzyme was purified 745-fold and was identified as casein kinase II on the basis of molecular size, substrate specificity, and high sensitivity to heparin inhibition. Casein kinase II phosphorylated isolated caldesmon and caldesmon incorporated into native thin filaments, and transferred about 1 mol of phosphate per mol of caldesmon-*h*. Ser-73 was the main site phosphorylated by casein kinase II in chicken gizzard caldesmon. Phosphorylation of caldesmon reduced its affinity for smooth muscle myosin but had no effect upon the ability of caldesmon to inhibit the ATPase activity of actomyosin.

Caldesmon; Smooth muscle; Phosphorylation; Casein kinase II

1. INTRODUCTION

Caldesmon is a calmodulin- and actin-binding protein that is a component of the thin filaments in smooth muscles. This protein seems to play a role in Ca^{2+}-dependent regulation of smooth muscle contraction (for review see [1,2]), supplementing the well-established myosin phosphorylation mechanism.

It is well known that caldesmon can be phosphorylated in vitro by many protein kinases and that such phosphorylation can modulate caldesmon function [1,3]. However, work in vivo has only produced convincing evidence of a role for MAP kinase in phosphorylating caldesmon in arterial smooth muscle [4,5].

One way to determine which kinase is important in phosphorylating caldesmon in vivo is to assay the caldesmon phosphorylating activity in smooth muscle homogenates. We have investigated a crude extract of sheep aorta actomyosin for caldesmon phosphorylating activity. In initial work we identified kinase activity that precipitated in 65–80% saturated ammonium sulphate [6]. This activity has recently been identified as being due to a proteolytic fragment of protein kinase C [7]. When precautions were taken to exclude phosphatase activity it was found that a much larger caldesmon phosphorylating activity precipitates at 30 45% saturated ammonium sulphate. We have therefore purified this caldesmon phosphorylating enzyme and investigated its properties. We have found that this enzyme is casein kinase II and that it could be one of the endogenous protein kinases involved in caldesmon phosphorylation in vivo.

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2. MATERIALS AND METHODS

2.1. *Materials*

[γ-^{32}P]ATP was obtained from Amersham. Other reagents of analytical grade were from Sigma or BDH. α-Chymotrypsin type VII, histone III-S and α-casein were obtained from Sigma; okadaic acid from Kamiya Biomedical Co.

2.2. *Isolation of caldesmon phosphorylating activity*

Crude actomyosin was prepared from sheep aorta smooth muscle [8] and the thin filaments were sedimented from the actomyosin in the presence of 20% ethylene glycol as described in [9]. Kinase activity was left behind in the supernatant that was dialysed for 3 h against 2 l of 20 mM HEPES, pH 7.3, 0.1 M KCl, 5 mM $MgCl_2$, 1 mM EGTA, 10 mM 2-mercaptoethanol, 0.1 mM PMSF, and 1 μg/ml each of leupeptin and chymostatin to reduce the concentration of ethylene glycol, and subjected to ammonium sulphate fractionation. The fraction which precipitated at 30–45% of ammonium sulphate possessed the highest caldesmon kinase activity and was subjected to further purification by gel-filtration on Sepharose CL-4B followed by chromatography on Q-Sepharose and P-cellulose (Fig. 1).

2.3. *Purification of other proteins*

Sheep aorta and chicken gizzard caldesmon were purified by the Bretscher method [10] with slight modifications [6]. Sheep aorta myosin was purified and thiophosphorylated according to [11]. The method of Weeds and Taylor [12] was used for preparation of skeletal muscle HMM. Sheep aorta thin filaments, smooth and skeletal muscle actin and smooth muscle tropomyosin were isolated as described in [9]. The N-terminal fragment of chicken gizzard caldesmon containing

the first 128 amino acid residues (N-128) and 658C (C-terminal 99 amino acids) were expressed in *E. coli* [13]. Fragment H1 (human caldesmon C-terminal 288 amino acids) was supplied by Dr. P. Huber [14].

2.4. *Protein phosphorylation*

Phosphorylation was performed in the buffer containing 8 mM Tris, 12 mM KH_2PO_4, 6 mM $MgCl_2$, 0.1 mM EGTA, 30–100 mM NaCl, 5 mM 2-mercaptoethanol, 30–70 μM [γ-^{32}P]ATP (300–2,000 cpm/pmol), 0.1–0.4 mg/ml of protein substrate and 0.6–2 μg of the enzyme. After incubation at 30°C for appropriate times, 20–40 μl of incubation mixture was spotted on Whatman 3MM filters. Filters were washed and counted as in [15]. To obtain preparative quantities of phosphorylated proteins, 1–3 mg/ml of caldesmon or N-128 were incubated in the above mentioned buffer with a slightly increased concentration of ATP (70–150 μM) for 6–14 h at 30°C.

Native thin filaments were suspended in 20 mM Tris-HCl, pH 7.5, 70 mM KCl, 10 mM 2-mercaptoethanol and 0.1 mM PMSF and dialysed against the same buffer to remove ATP. Thin filaments (2 mg/ml) were incubated with the kinase in the presence of 5 mM $MgCl_2$, 5 nM okadaic acid and 2 mM [γ-^{32}P]ATP (150 cpm/pmol) at 30°C for 20 min.

2.5. *Binding of caldesmon and N-128 to myosin*

Caldesmon (3–4 μM) or N-128 peptide (8–12 μM) were co-sedimented with thiophosphorylated gizzard myosin filaments (0–12 μM) by centrifugation in a Beckman Airfuge at 30 psi for 30 min. The initial sample, supernatant and pellet were separated by electrophoresis on 8–30% polyacrylamide/0.1% SDS gels (Pharmacia Excelgel). The quantity of caldesmon or its peptide remaining in the supernatant after ultracentrifugation was determined by quantitative scanning of the Coomassie blue-stained gels. The radioactivity of phosphorylated caldesmon or its peptide remaining in the supernatant was determined either by Çerenkov's method or by quantitative scanning of the corresponding autoradiographs [16].

2.6. *Measurements of ATPase activity*

The acto-HMM ATPase activity was determined in buffer containing 5 mM K_2PIPES (pH 7.0), 2.5–5 mM $MgCl_2$, 1 mM dithiothreitol, and either 5 mM (low ionic strength) or 70 mM (high ionic strength) KCl at 37°C, according to our usual method [13].

2.7. *Determination of the sites of phosphorylation*

^{32}P-Phosphorylated caldesmon was precipitated by addition of TCA to the final concentration of 5% and the pellet was washed 5 times with 5% TCA followed by 3 washes with diethyl ether. The dried radioactive protein was suspended in 50 mM NH_4HCO_3, containing 100 μM $CaCl_2$ and 1 mM dithiothreitol, and hydrolyzed by TPCK-treated trypsin (weight ratio caldesmon:trypsin equal to 50:1) for 4.5 h. The resultant clear solution was loaded on 4.6 × 250 mm Vydac 218TP54C$_{18}$ reverse phase column and HPLC was performed as in [17]. Well-separated radioactive peptides were sequenced on an Applied Biosystems model 477A sequencer equipped with on-line model 120A phenylthiohydantoin (PTH) analyzer, as described earlier [17].

2.8. *Other methods*

Caldesmon concentration was determined from absorbance measurement at 280 nm. $E^{0.1\%}_{280}$ for caldesmon was taken to be equal to 0.33 and its molecular weight equal to 87 kDa [18]. Other protein concentrations were determined by the Lowry method.

RESULTS AND DISCUSSION

3.1. *Purification of endogenous caldesmon kinase from sheep aorta*

Sheep aorta was homogenised and washed twice in a low ionic strength, Triton X-100-containing buffer. The resulting pellet contained most of the contractile proteins which were then solubilised in 80 mM KCl/10 mM ATP solution [8]. Native thin filaments were separated from this crude actomyosin extract by ultracentrifugation in the presence of 20% glycol; the pellet contained actin tropomyosin and caldesmon in a ratio of 16:2:1 [9,19], but negligible caldesmon phosphorylating activity, as previously observed [11]. Caldesmon phosphorylating activity was abundant in the supernatant fraction; the bulk of the activity was precipitated in a 30–45% saturated ammonium sulphate fraction. About 10% of the activity precipitated in the range of 65–80% ammonium sulphate saturation, as previously found [6], and this activity was identified as being due to a constitutively active proteolytic fragment of protein kinase C [7].

The major endogenous caldesmon phosphorylating activity was purified from the P30–45% fraction (Fig. 1). On a Sepharose 4B column the caldesmon kinase activity was eluted in a single wide peak and the enzyme was separated from myosin. Fractions with the highest protein kinase activity were further purified by ion-exchange chromatography on Q-Sepharose (Fig. 1B). Although this step of purification was rather effective (yielding a 7.5-fold increase in specific activity) we were unable to separate protein kinase from tropomyosin. The final and most effective step of purification consisted of cation-exchange chromatography on phospho-cellulose in the presence of high NaCl concentrations. Under these conditions most of the proteins (including tropomyosin) did not interact with phospho-cellulose, whereas caldesmon kinase did (Fig. 1C) permitting a stepwise elution. Alternatively the phospho-cellulose column was developed with a linear (0.4–0.8 M) gradient of NaCl in buffer B (20 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1 mM EGTA, 5 mM mercaptoethanol, 0.1 mM PMSF). In this case protein kinase was eluted at around 0.6 M NaCl. The final product had a specific activity of 22 nmol P_i/min/mg caldesmon, 745-times greater than the crude actomyosin starting material. Fractions possessing caldesmon kinase activity were concentrated against 20% polyethyleneglycol in buffer B and stored at −20°C in 50% glycerol.

3.2. *Identification of the endogenous kinase as casein kinase II*

3.2.1. Chromatographic properties

This enzyme has a high molecular weight (Fig. 1A), is strongly bound to Q-Sepharose (Fig. 1B) and interacts extremely tightly with phospho-cellulose (Fig. 1C). All these properties are similar to those of casein kinase II that has a molecular weight of about 130–140 kDa, tends to aggregate at low ionic strength, and binds tightly to both anion- and cation-exchangers [20–22].

3.2.2. Substrate specificity

When we analyzed the substrate specificity of caldesmon kinase from aorta we found that of the sub-

strates tested, casein was phosphorylated at the highest rate whereas histone III-S, smooth muscle myosin, tropomyosin and actin were phosphorylated at a very low rate (Table I). It is known that casein kinase II preferentially phosphorylates acidic protein substrates such as casein and phosvitin [22]. The endogenous caldesmon kinase effectively phosphorylated both chicken gizzard and aorta caldesmon (Table I). This agrees with our demonstration that casein kinase II is able to phosphorylate caldesmon [15,16].

Casein kinase II is distinguishable from other protein kinases by its ability to use both ATP and GTP as substrates [21,22]. The K_m value for GTP is only 2- to 4-times higher than that for ATP [22,23]. Addition of non-radioactive GTP (in the micromolar range) to the incubation mixture containing [γ-^{32}P]ATP significantly decreased the rate of γ-^{32}P incorporation into caldesmon by the enzyme isolated from sheep aorta (Table I), suggesting that this enzyme can use GTP as an alternative substrate.

3.2.3. Inhibition by heparin

Heparin and other sulfated glycosaminoglycans are potent inhibitors of casein kinase II [24] at concentrations lower that 1 μg/ml which do not affect other protein kinases [21,22]. We found that heparin effectively inhibited aorta caldesmon kinase (Table I). The IC_{50} was found to be 0.35 μg/ml; this value agrees well with the IC_{50} determined for casein kinase II [22].

3.2.4. Phosphorylation site on caldesmon

There is a consensus sequence for casein kinase II in both gizzard and mammalian (human) caldesmon sequences: ^{73}SXXE (gizzard) and ^{73}SXXD (human) [21,25]. We have previously shown that casein kinase II from liver phosphorylates gizzard caldesmon uniquely at Ser-73 [16], whereas most other kinases phosphorylate at multiple sites or phosphorylate at the C-terminus of caldesmon [1,3]. We found that the endogenous caldesmon kinase mainly phosphorylated N-terminal caldesmon fragments in tryptic digests of phosphorylated caldesmon, as in [16]. The expressed caldesmon fragment N-128 (amino acids 1-128) was much better phosphorylated than the C-terminal frag-

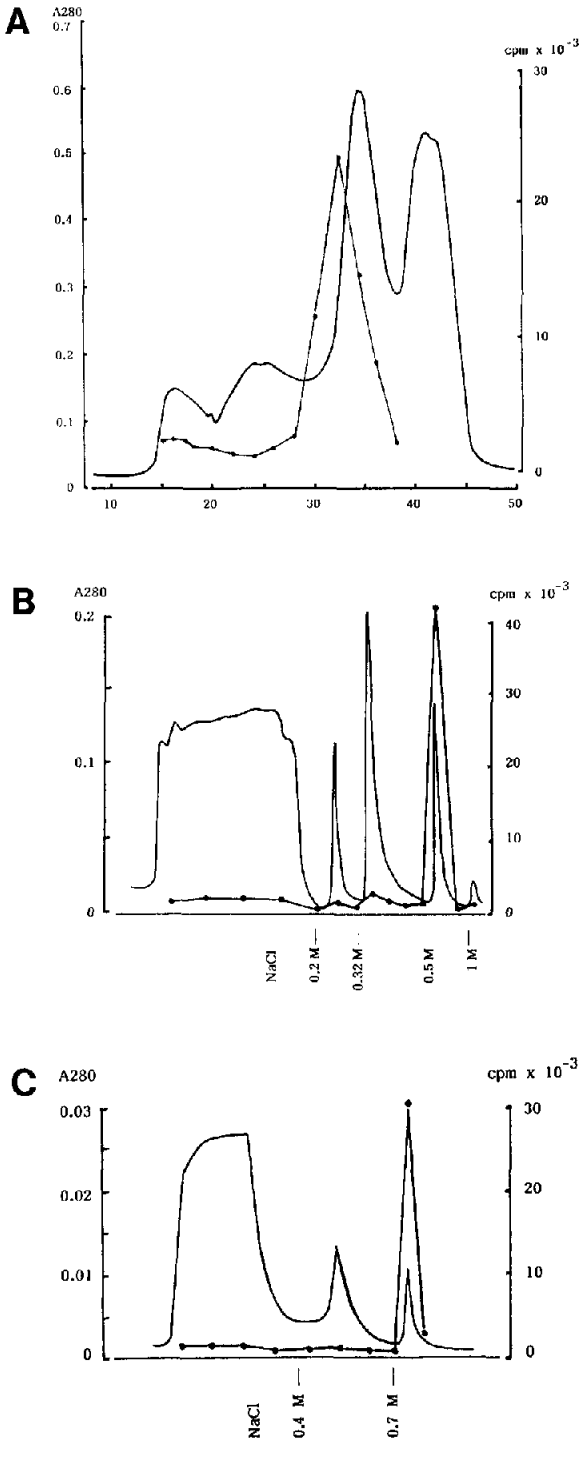

Fig. 1. Purification of caldesmon kinase. (A) Gel-filtration. The P30–45 fraction was dissolved in a small volume of buffer A containing 20 mM Tris-HCl, pH 7.5, 0.4 M KCl, 2.5 mM $MgCl_2$, 1 mM EGTA, 10 mM 2-mercaptoethanol and 0.1 mM PMSF and 5 ml sample was applied on the Sepharose 4B column (1.6 × 90 cm) equilibrated with the same buffer. 4.2 ml fractions were collected and the caldesmon kinase activity was determined. (B) Q-Sepharose chromatography. Fractions with the highest protein kinase activity were mixed, diluted twice with buffer B (20 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1 mM EGTA, 5 mM mercaptoethanol, 0.1 mM PMSF) and applied on the Q-Sepharose Fast Flow column (1.6 × 10 cm) equilibrated with buffer B, containing 0.2 M NaCl. After washing with buffer B proteins were eluted by stepwise increases in NaCl concentration (0.2, 0.32, 0.5 and 1 M). Caldesmon kinase was eluted with buffer B containing 0.5 M NaCl. (C) Phospho-cellulose chromatography. The fractions obtained after Q-Sepharose chromatography were diluted twice with buffer B and loaded on a phospho-cellulose (Whatman P-11) column (1.6 × 5 cm) equilibrated with buffer B containing 0.2 M NaCl. The column was washed with buffer B with 0.4 M NaCl, and caldesmon kinase was eluted with buffer B containing 0.7 M NaCl. (—) optical density at 280 nm; (●) caldesmon phosphorylating activity.

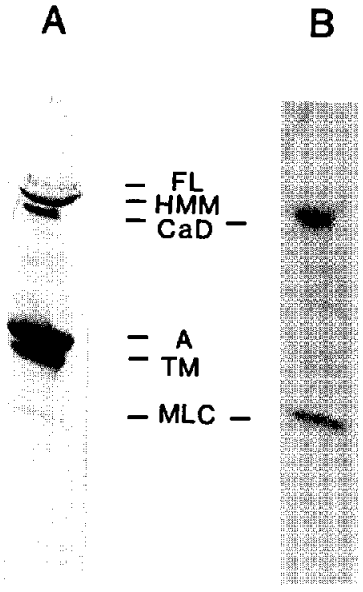

Fig. 2. Phosphorylation of aorta native thin filaments by smooth muscle casein kinase II. Phosphorylation was performed as described in section 2. (A) Coomassie blue-stained gel; (B) autoradiograph. FL, filamin; HMM, myosin heavy chains; CaD, caldesmon; A, actin; TM, tropomyosin; MLC, myosin light chain.

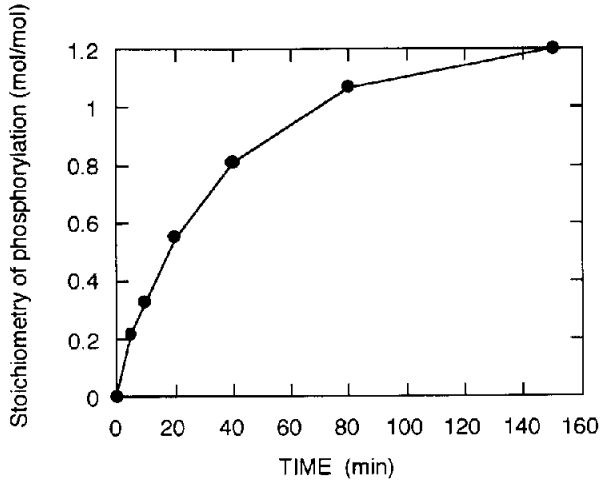

Fig. 3. Phosphorylation of caldesmon by smooth muscle casein kinase II. Time-course of aorta caldesmon phosphorylation. 0.4 mg/ml caldesmon, 50 μg/ml purified casein kinase II, 30°C. Conditions as described in section 2.

ments 658C and H1 (Table I). Finally the major phosphorylation site was positively identified as Ser-73 by sequencing tryptic peptides of phosphorylated gizzard caldesmon, as we previously showed with liver casein kinase II [17].

We were not able to determine the site of phosphorylation in sheep aorta caldesmon since the yield of phosphopeptides was very low and the tryptic phosphopeptides of aorta caldesmon had a blocked N-terminus. This is probably a consequence of sequence differences between sheep and chicken [25].

3.3. *Effects of casein kinase II phosphorylation upon caldesmon function*

Sheep aorta casein kinase II phosphorylated caldesmon equally well when pure and when incorporated into the native thin filaments (Figs. 2 and 3). However, phosphorylation had no detectable effect upon caldesmon inhibition of actin tropomyosin activation of myosin MgATPase activity, which is a sensitive indicator of the caldesmon-actin-tropomyosin interaction: these functions are known to be associated with the C-terminus of caldesmon [1,13].

Myosin binding involves both N- and C-terminal sites in caldesmon [13,14,26,27] and we found, as in our previous work with liver casein kinase II, that phosphorylated caldesmon bound smooth muscle myosin weaker [16]. This effect was particularly marked when myosin binding to the isolated N-terminus (N-128) containing Ser-73 was measured (Fig. 4).

3.4. *Possible physiological significance*

Whilst casein kinase II is reported to be abundant in spleen, testis, liver and kidney, much smaller quantities are present in striated muscle tissue [28–30]. Little has been published on casein kinase II from smooth muscle [31,32], but it has been assumed that the properties are similar to that of the enzymes purified from other tissues, and this is in accord with our results (Table I).

In the crude actomyosin which was the starting material for our isolation, casein kinase II was the most abundant caldesmon phosphorylating activity and cal-

Table I

Effects of substrate and inhibitors on activity of purified caldesmon phosphorylating enzyme

Substrate	Relative phosphorylation rate
Casein	100
Casein + 0.18 μg/ml heparin	61
Casein + 0.36 μg/ml heparin	26
Casein + 80 μM GTP	55
Caldesmon	82
Caldesmon N-terminus, gizzard 1–128 (N-128)	75
Caldesmon C-terminus, gizzard 658–756 (658C)	12
Caldesmon C-terminus, human 506–793 (H1)	21
Smooth muscle myosin	5
Smooth muscle myosin (thiophosphorylated)	6
Smooth muscle tropomyosin	3
Actin	4
Histone III-S	7

All protein substrates at concentration of 0.4 mg/ml were phosphorylated for 10 min under conditions described in section 2. The rate of casein phosphorylation was taken as 100%.

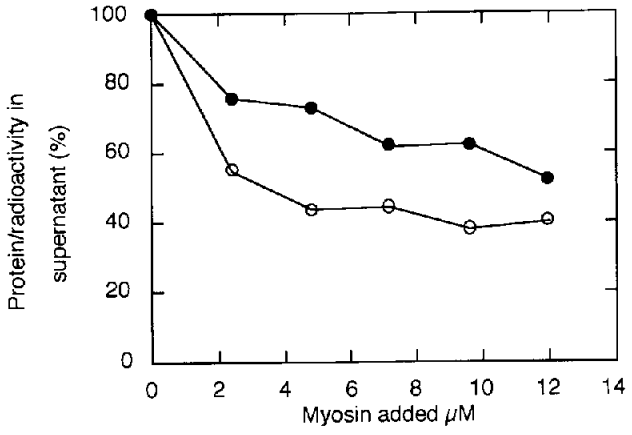

Fig. 4. Effect of phosphorylation of chicken gizzard caldesmon N-128 fragment on its binding to smooth muscle myosin. Caldesmon N-terminal fragment, phosphorylated at a level of 0.1 mol ^{32}P per mol N-128, was mixed with myosin under conditions described in section 2. After ultracentrifugation concentration (○) and radioactivity (●) in the supernatant were determined. The phosphorylated N-128 bound much weaker than the bulk of the protein, which was largely unphosphorylated.

desmon is almost as good a substrate for this enzyme as casein itself (Table I); however, we do not know whether phosphorylation by this enzyme occurs in vivo. As a rule, the turnover rate of phosphate incorporated by casein kinase II is rather low [22], so we may suppose that caldesmon phosphorylation is unlikely to change rapidly.

Measurements of caldesmon phosphorylation in vascular smooth muscle stimulated by phorbol dibutyrate has provided positive evidence that caldesmon is phosphorylated by MAP kinase and has excluded significant phosphorylation by Ca^{2+}–calmodulin-dependent kinase II and protein kinase C [4,5]. It is notable that an early report also indicated that the N-terminus of caldesmon became phosphorylated in vivo [33]. Since Ca^{2+}–calmodulin-dependent kinase II was excluded it seems possible that this phosphorylation was due to the casein kinase II present in vascular smooth muscle. If so this phosphorylation might be expected to modulate the ability of caldesmon to cross-link thick and thin filaments [26,34].

Acknowledgements: This work was supported by grants from the British Heart Foundation to S.B.M, the Muscular Dystrophy Association of America to J.H.C. and Russian Fund for Fundamental Research (320/6) to N.B.G. A.V.V was a Wellcome Visiting Fellow and N.B.G. was a BHF Overseas Visiting Fellow.

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