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The functional effects of mutations Thr^{673}→Asp and Ser^{702}→Asp at the Pro-directed kinase phosophorylation sites in the C-terminus of chicken gizzard caldesmon

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We expressed the C-terminal 99 amino acids of chicken gizzard caldesmon (658C) and two point mutants in which the preferred phosphorylation sites of MAP kinase and p34^{cdc2} kinase, Ser^{702} and Thr^{673} were altered to aspartic acid The T673D mutant was indistinguishable from 658C but S702D was not phosphorylated by MAP kinase, was significantly less potent as an inhibitor of actin–tropomyosin activation of myosin MgATPase, and bound less actin–tropomyosin at low concentrations Thus Ser^{702} is involved in the tropomyosin-dependent inhibitory mechanism of caldesmon, and its phosphorylation by MAP kinase or p34^{cdc2} kinase could modulate caldesmon function.

Caldesmon; Smooth muscle; MAP kinase; p34^{cdc2} kinase; Thin filament; Regulation

1. INTRODUCTION

In smooth muscle and non-muscle cells Ca^{2+} controls myosin filament activity via the Ca–calmodulin-dependent myosin light-chain kinase which activates myosin by phosphorylation. In addition, the thin filaments, which contain actin, tropomyosin, caldesmon and a calcium binding protein, are also Ca^{2+} regulated. In vitro both native thin filaments and synthetic systems, consisting of actin, tropomyosin, caldesmon and calmodulin at ratios similar to native thin filaments, are effectively regulated by Ca^{2+} in a manner formally analogous to the regulatory function of troponin in striated muscles [1,2]. Caldesmon is a very potent inhibitor of actomyosin ATPase activity; inhibition of actin activation of myosin MgATPase activity by over 90% is commonly observed with fewer than one caldesmon molecule bound to every ten actin monomers and Ca^{2+} and calmodulin, or a calcium binding protein can reverse this inhibition [1,3,4].

In order to understand the molecular mechanism of caldesmon regulation we obtained a chicken gizzard caldesmon cDNA [5] and expressed intact caldesmon and a series of mutants in *E. coli* [6]. We found that the entire regulatory function of caldesmon resides in an expressed fragment, 658C, consisting of the C-terminal 99 amino acids (domain 4b) [3,6–8].

Further modulation of caldesmon function may occur as a consequence of phosphorylation. Caldesmon is a substrate for many kinases in vitro; however, the only kinases for which there is evidence of caldesmon phosphorylation in vivo are the proline-directed kinases, p34^{cdc2} in non-muscle tissue [9,10] and MAP kinase in smooth muscle [11,12].

The consensus sequence for p34^{cdc2} kinase is (Ser/Thr)-Pro-X-Z in which X is a polar amino acid and Z is normally a basic residue [13]; inspection of the caldesmon sequence located only one such site occurring at residues 673–676 (Thr-Pro-Asn-Lys) which is within the 658C fragment (the numbering is from the chicken gizzard CD$_h\beta$ sequence of Bryan et al [5,7]). Experimentally five in vitro p34^{cdc2} kinase sites have been identified: Ser^{582}, Ser^{667}, Thr^{673}, Thr^{696} and Ser^{702}; all of these amino acids are adjacent to proline, however, the Thr^{673} site was found to have at least twice as much phosphate incorporated as any other [14]. Phosphorylation of caldesmon by p34^{cdc2} kinase has been shown to inhibit actin and calmodulin binding to both non-muscle [15] and smooth muscle [16] caldesmon.

In vitro the predominant MAP kinase phosphorylation site observed experimentally was Ser^{702} [12]. Such phosphorylation results in a small decrease in actin binding and has no effect on calmodulin binding [17]. Caldesmon phosphorylation in intact smooth muscle has been shown to occur at the same sites as those phosphorylated by MAP kinase, and MAP kinase (but

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Abbreviations: MAP kinase, mitogen activated protein kinase; p34^{cdc2} kinase, 34 kDa protein kinase encoded by the *S. pombe* cell division control 2 gene and its functional homologues in other species; PIPES, piperazine *N*,*N*′-bis (2-ethanesulphonic acid)

not p34^{cdc2}) has been detected in normal smooth muscle cells [12,17].

Both Thr^{673} and Ser^{702} are in the C-terminal domain of caldesmon (domain 4b) which we have shown contains all its regulatory functions [6,18], and Thr^{673} is close to a putative binding site for calmodulin at 658–666 [7,19]. To test the possible role of phosphorylation of these residues in modulating caldesmon function we constructed and expressed mutants of the C-terminal 99 amino acids of gizzard caldesmon in which the threonine or serine had been changed to aspartic acid. If these residues were the natural sites of phosphorylation, the mutants would not be phosphorylated. Moreover introduction of the negatively charged aspartic acid has been shown to mimic the effects of phosphorylation in a number of enzymes [20–22]. Thus it might be expected to alter caldesmon function in the same way as phosphorylation of these sites. In fact we found that modification of Ser^{702} (mutant S702D) had significant effects on actin binding, inhibition of actomyosin MgATPase, and phosphorylation by MAP kinase, but modification of Thr^{673} (mutant T673D) did not.

2. MATERIALS AND METHODS

2.1. *Construction and expression of mutants*

The construction of the pMW172 expression plasmid encoding the C-terminal 99 amino acids of chicken gizzard caldesmon (658C) was described in [6]. This was used as a template for the production of point mutants by the inverse PCR method [23] Back-to-back primers of 5′-TGCTGGACTGAAAGTTGGTGT-3′ and 5′-GTTTCCTTATTTGGATCTCCTGTTCCCCCAGGTGATG-3′ (mutated nucleotides underlined) were used to change threonine to aspartic acid at position 673 (all numbering refers to the full-length 756 amino acid sequence [5]). Similarly 5′-TCTGATTTAAGACCAGGAGATGTA-3′ and 5′-AGGTTTTGGAGCAGGATCTTTGTTACCCTCTGG-3′ were used to change serine to aspartic acid at position 702. Linear products from the total plasmid PCR were re-circularised using T4 DNA ligase and used to transform Ca^{2+}-competent JM101 cells. Expression plasmids containing the required mutation, named, respectively, T673D and S702D, were identified by double-stranded sequencing.

Constructs were expressed in BL21(DE3) cells and the fragments purified as previously described for 658C [6]

2.2. *Phosphorylation by MAP kinase*

Recombinant MAP kinase, activated by thiophosphorylation, was kindly supplied by Dr. Chris Marshall, Institute of Cancer research, London. 0.2–0.3 mg/ml caldesmon 658C and the two mutants were incubated with 0.004 mg/ml MAP kinase for 30–60 min in 5 mM Tris-HCl, pH 7.5, 11 mM $MgCl_2$, 0.18 mM EGTA, 0.27 mM Na_3VO_4, 0.1% mercaptoethanol, 0.01% Brij 35, 0.36 mg/ml bovine serum albumin and 110 μM [γ^{32}P]ATP. An aliquot of the reaction mixture was spotted onto Whatman P-81 paper and, after washing in 25 mM phosphoric acid, incorporation of $^{32}P_i$ into protein was determined by liquid scintillation counting. Another aliquot of the sample was separated by SDS gel electrophoresis.

2.3. *Functional assays*

ATPase activity was determined by assaying P_i released following 10 min incubation of smooth muscle actin, tropomyosin and skeletal muscle myosin (or heavy meromyosin). The reaction was initiated by adding MgATP to 2 mM, and terminated with 10% trichloroacetic acid [6]. Binding to calmodulin–Sepharose (Pharmacia) was performed as in [6]. The expressed proteins were covalently labelled with *N*-succinimidyl[2,3,^{3}H]propionate (Amersham) and their binding to actin–tropomyosin was assayed by co-sedimentation, as described in [1] and [6].

3. RESULTS

3.1. *Phosphorylation by MAP kinase*

The parent C-terminal domain of caldesmon, 658C, and the point mutants, T673D and S702D, were expressed at approximately equal yield and purified. On SDS gel electrophoresis they migrated with identical apparent molecular weights of 10,500 Da (Fig. 1). When incubated for 60 min with MAP kinase and [γ^{32}P]ATP, 658C and T673D incorporated similar amounts of ^{32}P whilst there was no measurable ^{32}P incorporation into S702D using the filter technique. This is also shown in autoradiographs of the reaction mixtures following incubation with MAP kinase (Fig. 1, bottom panel); 658C and T673D were strongly radioactive whereas the band of S702D was much less radioactive.

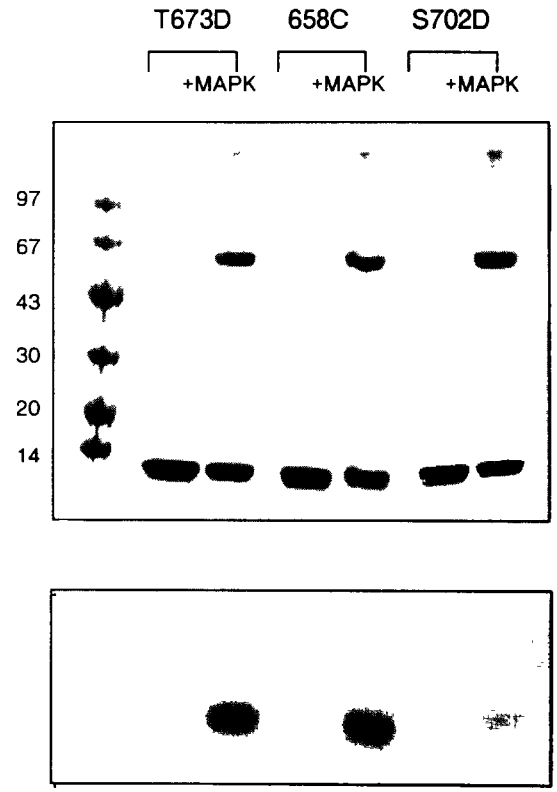

Fig. 1. Phosphorylation of 658C, T673D and S702D by MAP kinase (Top panel) Proteins were separated by electrophoresis on 0.1% SDS/8–18% polyacrylamide gradient gels (Pharmacia Excelgel) and stained in PAGE Blue 83 (BDH/MERCK) The quantities loaded on the gel were 5 μg T673D, 3.8 μg 658C and 3.3 μg S702D. The pure protein and the reaction mixture (containing BSA) following incubation with MAP kinase and [γ^{32}P]ATP are shown in adjacent lanes (Bottom panel) Autoradiograph of the lower part of this gel. Following incubation with MAP kinase, 658C and T673D were phosphorylated but S702D was not.

3.2. *Inhibition of actin-activated ATPase*

The C-terminal 99 amino acids of caldesmon contained in 658C have been shown to contain the tropomyosin-dependent inhibitory site of caldesmon [2,6,7]. None of the caldesmon fragments inhibited actin activation under our experimental conditions, but in the presence of tropomyosin, 658C inhibited actin activation at both low (5 mM KCl) and moderate (50 mM KCl) ionic strengths (Fig. 2), in agreement with previous work using 658C and whole caldesmon [2,6]. The mutant T673D also inhibited actin–tropomyosin activation and was indistinguishable from 658C, but the mutant S702D was significantly less effective as an inhibitor in 50 mM KCl (Fig. 2A). At lower ionic strength (5 mM KCl, Fig. 2B) S702D became practically ineffective as an inhibitor.

3.3. *Calmodulin and actin–tropomyosin binding*

658C, T673D and S702D bound to calmodulin–Sepharose in the presence of Ca^{2+} and the three expressed proteins were not distinguishable (Fig. 3).

We compared the binding of 658C, T673D and S702D to 11 μM actin saturated with tropomyosin in the concentration range 5–50 μM using quantitative scanning of gel electrophoresis to determine the quantity of protein co-sedimenting with actin. We could not see any differences between the mutants under these conditions. This method is reliable for measuring weak binding between the caldesmon peptides and actin–tropomyosin, but is insufficiently sensitive to enable us to assay the high affinity binding sites which have a stoichiometry of 0.07 per actin and which have been identified in whole caldesmon and 658C [1,6,15]. Therefore we also compared 658C and S702D binding to actin–tropomyosin after labelling them with *N*-succinimidyl[2,3,^{3}H]propionate at a more extended range of concentrations (Fig. 4). S702D bound significantly less than 658C at all concentrations up to 20 μM, but this was particularly pronounced at the lowest concentrations, below 1 μM, consistent with a reduction in the affinity of the tight binding sites (Fig. 4, inset).

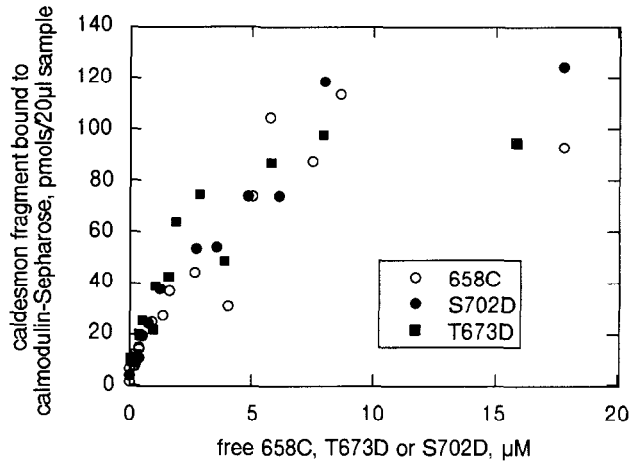

Fig. 3. Binding to calmodulin–Sepharose. Calmodulin–Sepharose (10 μl of 50% suspension in 50 μl total volume) was mixed with 0–20 μM 658C (○), T673D (■) and S702D (●) labelled with *N*-succinimidyl[2,3^{3}H]propionate in the buffer used in Fig. 2 plus 0.1 mM $CaCl_2$. Binding was determined from the radioactivity co-sedimenting with the calmodulin–Sepharose as described in [6].

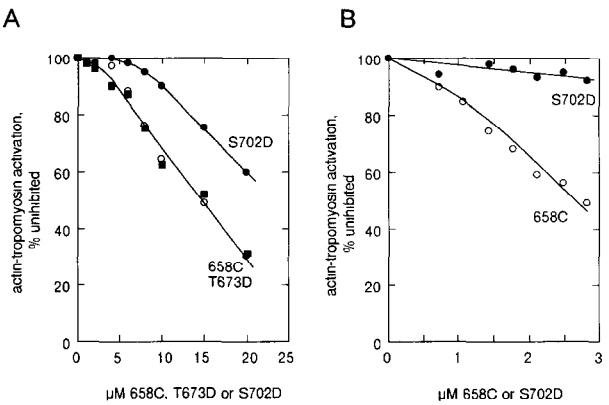

Fig. 2 Inhibition of actin and actin–tropomyosin activation of myosin MgATPase. (A) Effect of 658C (○), T673D (■) and S702D (●) on actin–tropomyosin-activated myosin MgATPase activity. Conditions: 0.125 mg/ml skeletal muscle myosin, 0.5 mg/ml smooth muscle actin, 0.2 mg/ml smooth muscle tropomyosin in 5 mM PIPES, 5 mM $MgCl_2$, 1 mM dithiothreitol, 50 mM KCl, pH 7.0, 37°C. Uninhibited ATPase activity = 1,100 nmol P_i/mg myosin/min. (B) Effect of 658C (○) and S702D (●) on actin–tropomyosin-activated heavy meromyosin MgATPase activity at lower ionic strength. Conditions: 0.04 mg/ml heavy meromyosin, 0.15 mg/ml actin, 0.04 mg/ml tropomyosin, 5 mM PIPES, pH 7.0, 5 mM KCl, 2.5 mM $MgCl_2$, 37°C. Uninhibited ATPase activity = 2,775 nmol/mg heavy meromyosin/min

4. DISCUSSION

4.1. *Confirmation of MAP kinase site at Ser^{702} in caldesmon*

Under the conditions of our measurements, mutation of Ser^{702} almost completely abolished MAP kinase phosphorylation whilst mutation of Thr^{673} had hardly any effect (Fig. 1). Thus it appears that Ser^{702} is the main site for the enzyme in the C-terminus of gizzard caldesmon. This is compatible with previous work using MAP kinase. Adam et al. [11] found that two sites in caldesmon were phosphorylated in canine aortas following phorbol dibutyrate stimulation, Ser^{702} and a site VTS*PTKV which is at the extreme C-terminus of mammalian caldesmon but is absent in the gizzard caldesmon we studied [5,7]. The same sites were phosphorylated by MAP kinase in vitro [12]. Childs et al. [17] noted that Thr^{673} was poorly phosphorylated by MAP kinase in vitro and suggested that the preferred sites in the C-terminus were at Thr^{696} and/or Ser^{702}.

4.2. *Involvement of Ser^{702} in the tropomyosin-dependent inhibitory site*

Mutation of Ser^{702} to aspartate significantly reduced

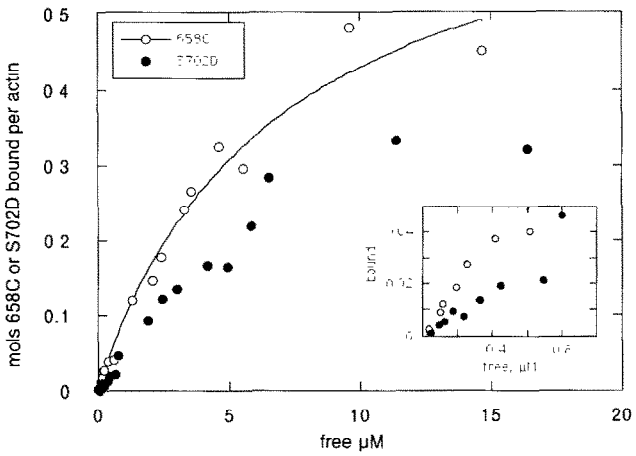

Fig. 4. 658C and S702D binding to actin–tropomyosin. Up to 20 μM 658C (○) or S702D (●) labelled with *N*-succinimidyl[2,3-^{3}H]propionate (50–250 cpm/pmol) were mixed with a constant 11 μM F-actin in the presence of tropomyosin (A:tm 0.4 w/w) at 25°C in 5 mM K_2- PIPES, 2.5 mM $MgCl_2$, 1 mM DTT, pH 7.0, and centrifuged for 20 min in a Beckman Airfuge. The amount of each caldesmon fragment in the supernatant before and after centrifugation was measured by liquid scintillation counting. The 'free' and 'bound' concentration of each fragment was calculated as described by Smith et al. [1]. Pooled data from at least two experiments are shown. The line is a best-fit of the 658C data to a single class of binding sites: K_d = 6.6 ± 1.1 μM, B_m = 0.71 ± 0.05 658C bound per actin. (Inset) Low concentration range of the same experiments. The lines are derived from binding measurements on 658C in [6]. The upper line represents the two classes of binding sites in 658C; strong, B_{max} = 0.027/actin, K_d = 0.11 μM plus weak, B_{max} = 0.69/actin, K_m = 14.9 μM. The lower line represents weak binding with the strong binding component absent. Note how this corresponds to the data for S702D.

the ability of 658C to inhibit actin–tropomyosin activation of myosin MgATPase, especially at low ionic strength, whereas it had little effect on inhibition in the absence of tropomyosin (Fig. 2). It has been shown that inhibition of actin–tropomyosin activation is directly coupled to caldesmon binding to a class of high affinity actin–tropomyosin sites with a stoichiometry of 1 per 14 actins [1–3]; these high affinity sites are located in the 658C C-terminal fragment of caldesmon [2,6]. The effect of mutation at Ser^{702} was to reduce actin–tropomyosin binding and this was most pronounced at the lowest concentrations (Fig. 4, inset). This is consistent with a substantial decrease in the tight binding component (stoichiometry 0.07 per actin), with rather little effect upon the low affinity actin binding (stoichiometry 0.7–1 per actin) which is not coupled to inhibition [3,6].

Ser^{702} is thus implicated in the tropomyosin-dependent inhibitory mechanism of caldesmon [2]. Current evidence on the location of the sites important for inhibition is entirely compatible with this assignment [6,18]. The mutation of serine to aspartate introduces a negative charge at neutral pH, and this has been shown to have the same effect as phosphorylation in a number of different enzymes [20–22]. The effect of changing Ser^{702} of caldesmon to aspartic acid upon its actin–tropomyosin binding and inhibition (Figs. 2 and 4) is similar to the effect of phosphorylating caldesmon following both MAP kinase and $p34^{cdc2}$ kinase phosphorylation [15,17]. A small effect on binding was observed in measurements using high concentrations of caldesmon [15] whilst a larger effect was noted under conditions where only the high affinity sites would be occupied [17]. This further emphasises the importance of Ser^{702} as a site where caldesmon may be regulated by phosphorylation.

4.3. *No evidence for a role of Thr^{673}*

Thr^{673} is supposed to be the preferred site of phosphorylation by $p34^{cdc2}$ kinase [14]; however mutation of this amino acid to aspartate did not alter the properties of 658C (Figs. 2 and 3). $p34^{cdc2}$ kinase has been reported to introduce up to 4 P_i per caldesmon [12]. The effect of $p34^{cdc2}$ kinase on actin binding and caldesmon inhibition may therefore be a consequence of phosphorylation at Ser^{702} rather than Thr^{673}, or more sites may need to be phosphorylated to produce the observed effect [15].

Thr^{673} is close to a sequence, MWEKGNVFS (amino acids 658–666), which had been identified as a calmodulin binding site from work with isolated peptides and expressed fragments [7,19,24]. An effect of mutation of Thr^{673} upon calmodulin binding might be predicted, especially since $p34^{cdc2}$ kinase phosphorylation was reported to affect calmodulin binding [15].

Using calmodulin–Sepharose we found that binding was not affected by mutation at Thr^{673} or Ser^{702} (Fig. 3). It should be noted, however, that the parent sequence of 658C is slightly modified in the region of the calmodulin binding site (Trp^{659} in the wild-type changed to glycine [6]) and so may not have bound calmodulin normally anyway. We have recently obtained evidence for another calmodulin binding region around 670–710, hence the calmodulin binding we observed (Fig. 3) may not reflect the properties of the 658–666 sequence.

4.4. *Physiological significance*

This work has demonstrated that the introduction of a negative charge at a single amino acid, Ser^{702} of caldesmon, significantly impairs its regulatory function. Caldesmon is phosphorylated in vivo and the level of phosphorylation increases in smooth muscle following stimulation by contractile agonists, particularly endothelin [25] and phorbol dibutyrate [11,26], and in non-muscle cell at mitosis [9,10]. In the smooth muscle the primary phosphorylation site is Ser^{702} [12]; in the mitotic cell Ser^{702} is the second most highly phosphorylated site [14]. Phosphorylation of serine introduces a negative charge to an equal or greater extent than mutation of serine to aspartic acid. Thus phosphorylation will impair caldesmon function (both inhibition and actin–myosin crosslinking [7]) by the mechanism we have

demonstrated, and consequently may increase contractility.

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