The functional properties of full length and mutant chicken gizzard smooth muscle caldesmon expressed in *Escherichia coli*

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Wild type chicken gizzard caldesmon (756 amino acids) was expressed in a T7 RNA polymerase-based bacterial expression system at a yield of 1 mg pure caldesmon per litre bacterial culture. A mutant composed of amino acids 1–578 was also constructed and expressed. The wild type and mutant caldesmon were purified and compared with native chicken gizzard caldesmon. Native and wild type expressed caldesmon were indistinguishable in assays for inhibition of actin-tropomyosin activation of myosin ATPase, reversal of inhibition by Ca^{2+}-calmodulin and binding to actin, actin-tropomyosin, Ca^{2+}-calmodulin, tropomyosin and myosin. The mutant missing the C-terminal 178 amino acids had no inhibitory effect and did not bind to actin or Ca^{2+}-calmodulin. It bound to tropomyosin with a 5-fold reduced affinity and to myosin with a greater than 10-fold reduced affinity.

Caldesmon; Bacterial expression; Domain mapping

1. INTRODUCTION

Caldesmon is an actin binding protein present in smooth muscle and non-muscle cells. In smooth muscle, caldesmon is specifically located on the thin filaments within the contractile domain [1,2]. In vitro, caldesmon binds to actin, tropomyosin, calcium binding proteins such as calmodulin, S100 and troponin C [3–5] and to smooth muscle myosin [6]. Caldesmon is a potent inhibitor of actin-tropomyosin activation of myosin ATPase activity and this inhibition is reversed by Ca^{2+} and calcium binding proteins [3]. These observations suggest that caldesmon may provide a calcium-sensitive thin filament-based means of regulation of smooth muscle contractility, complementary to that provided by the reversible phosphorylation of the myosin regulatory light chain. Caldesmon may also be involved in the formation of the 'latch bridge' in smooth muscle via reversible attachment to myosin [6,7], thus cross-linking the thick and thin filaments.

Proteolytic cleavage of caldesmon has localised its inhibitory activity and actin and calmodulin binding domains to a 40 kDa C-terminal chymotryptic fragment while the myosin binding site has been localised towards the N-terminus [8–10]. The cloning and sequencing of a caldesmon cDNA encoding 756 amino acids from a chicken gizzard library has recently been reported [11]. As a first step towards localising more precisely the functional regions of caldesmon we have expressed this cDNA in a bacterial expression system and produced wild type caldesmon and a mutant encoding the first 578 amino acids. We have developed a method for the purification of milligram quantities of these expressed proteins and compared their activities with those of the native protein purified from chicken gizzard in a range of in vitro assays.

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Abbreviations: DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; EGTA, ethyleneglycol-bis-(β-aminoethyl ether)*N,N,N',N'*-tetraacetic acid; PIPES, piperazine-*N,N'*-bis-2-ethanesulphonic acid; SDS-PAGE, sodium dodecyl sulphate polyacrylamide electrophoresis; TAME, *p*-tosyl-L-arginine methyl ester; TPCK, *N*-tosyl-L-phenylalanine chloromethyl ketone

2. MATERIALS AND METHODS

2.1. *Expression*

The full length chicken gizzard CDM57 sequence [11] and a *NcoI/DraI* fragment encoding the N-terminal 578 amino acids were subcloned into a T7 RNA polymerase-based expression vector derived from the pET vector [12] (gift from Dr. M. Way, LMB, Cambridge). BL21 (DE3) cells containing the additional plasmid pLysE were used for expression as previously described [11] and induced cells were harvested by centrifugation.

2.2. *Purification*

Cell pellets were resuspended in 25% sucrose, 50 mM Tris-HCl, 2 mM EGTA, 2 mM EDTA, 2 mM DTT, pH 8.0, in the presence of 10 μg/ml each of soy bean trypsin inhibitor, TAME and TPCK. The cells were lysed with lysozyme and treated with DNAseI [13]. Sodium chloride was added to 600 mM and the lysed cells were heated to boiling in a microwave oven and then rapidly cooled. After centrifugation at 36 000 × *g* for 20 min the supernatant was taken and the pH lowered to 4.0. The solution was centrifuged (36 000 × *g*, 10 min) and

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the pellet which contained the caldesmon was resuspended in minimal volume. Final purification of caldesmon was by gel filtration on Sephacryl S300 in 25 mM Tris-HCl, 200 mM NaCl, 2 mM EDTA, 1 mM $MgCl_2$, 2 mM DTT pH 7.5. Caldesmon-containing fractions were pooled and concentrated by precipitation in 50% ammonium sulphate. Both the wild type and mutant proteins were purified by this method.

2.3. *Purification of native proteins*

Chicken gizzard caldesmon was prepared by a variation of Bretscher's technique [14] described in [15]. Sheep aorta tropomyosin, F-actin and thiophosphorylated myosin were prepared as in our previous work [3,16]. Skeletal muscle myosin and bovine brain calmodulin were prepared by standard techniques [17,18].

3. RESULTS

3.1. *Expression of caldesmon*

Using the full length chicken gizzard caldesmon cDNA, CDM57 [11], a low level of expression of caldesmon was obtained. The protein was identified by SDS-PAGE (Fig. 1) and on Western blots using an anti-chicken gizzard caldesmon antiserum. Expressed wild type caldesmon comprised less than 1% of total cell protein after induction; a similar level of protein was obtained using the mutant *NcoI/DraI* construct. Centrifugation of boiled, lysed cells yielded a supernatant enriched in caldesmon (Fig. 1, lane b) which was concentrated with some further purification by isoelectric precipitation at pH 4.0 (Fig. 1, lane c). Final purification was achieved by gel filtration on Sephacryl S300 (Fig. 1, lane d). The yield was approximately 1 mg/l of bacterial culture; a similar yield and purity was obtained with the mutant (Fig. 1, lane e). The wild type was expressed as the full 756 amino acids plus a Met-Gly-Ser N-terminal fusion peptide while the mutant had the same N-terminal tripeptide, amino acids 1-578 of caldesmon plus a Pro-Leu-Val C-terminal fusion peptide.

3.2. *Functional properties of native and expressed caldesmon*

Native and wild type caldesmon inhibited the actin-tropomyosin activation of skeletal muscle myosin ATPase with similar potencies (Fig. 2). The same results were obtained with both skeletal and smooth muscle actins. The inhibition of skeletal muscle actin-sheep aorta tropomyosin activation of myosin ATPase by both native and wild type caldesmon was reversed by high concentrations of Ca^{2+}-calmodulin (Fig. 2, inset).

Wild type and native caldesmon bound to actin (Fig. 3) or actin-tropomyosin with the same relative affinity. A similar level of binding by both native and wild type caldesmon to Ca^{2+}-calmodulin was detected by non-dissociating gel electrophoresis [5]. Caldesmon-tropomyosin binding was assayed by monitoring the fluorescence of pyrene maleimide-labelled tropomyosin which is enhanced up to 50% by caldesmon binding [19]. Native and wild type caldesmon bound to tropo-

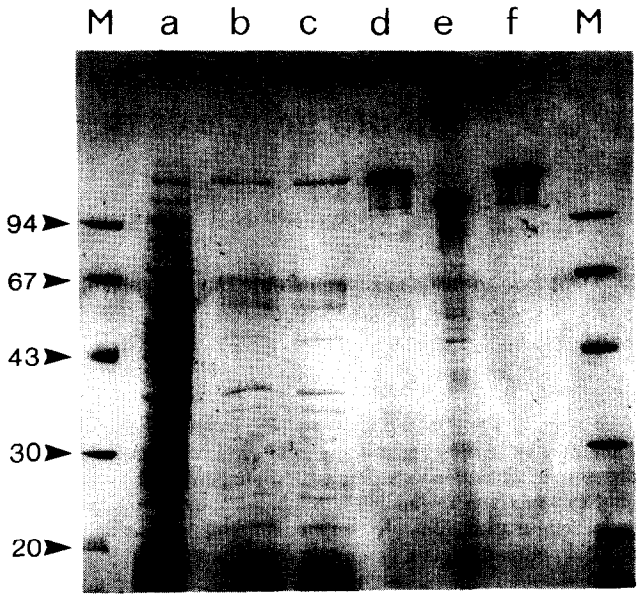

Fig. 1. 5-20% gradient polyacrylamide, 0.1% SDS gel electrophoresis stained with Coomassie Blue. Lanes: (a) total protein from induced cells carrying the full length caldesmon construct (10 μg); (b) supernatant following heating and centrifugation (1 μg); (c) pH 4.0 pellet (1 μg); (d) purified wild type caldesmon after Sephacryl S300 gel filtration (1 μg); (e) purified mutant (1 μg); (f) purified native chicken gizzard caldesmon (1 μg). M = molecular weight markers.

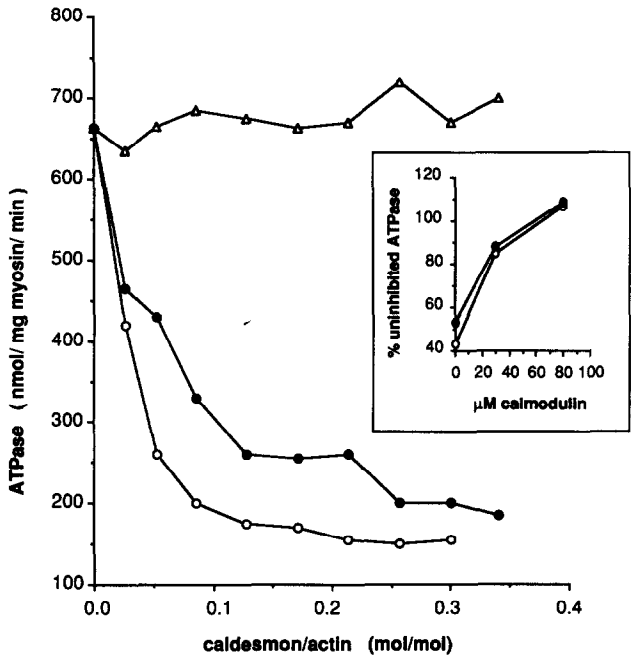

Fig. 2. Inhibition of actin-tropomyosin activated myosin ATPase by caldesmon and its reversal by Ca^{2+}-calmodulin (inset). Legend: (—○—), native; (—•—), wild type; (— ▵ —), mutant caldesmon. Rates were measured at 25°C by our standard method [3] using 0.125 mg/ml skeletal muscle myosin, 0.5 mg/ml actin, 0.2 mg/ml tropomyosin and caldesmon/actin molar ratios of between 0 and 0.34 in 5 mM K_2 · PIPES, 60 mM KCl, 5 mM $MgCl_2$, 1 mM DTT pH 7.0. The reversal of inhibition (inset) was measured using 0.22 caldesmon/skeletal muscle actin and 0, 30 and 80 μM Ca^{2+}-calmodulin.

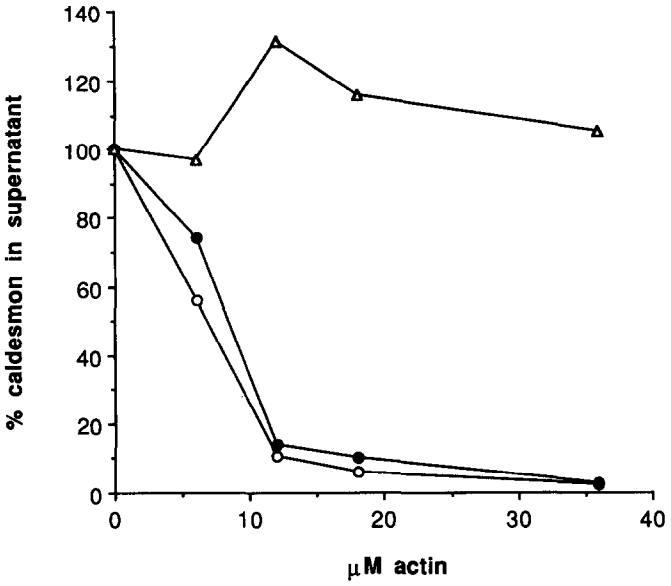

Fig. 3. Cosedimentation of caldesmon with aorta F-actin. Legend as in Fig. 2. 1 μM caldesmon was mixed with 0–36 μM actin in ATPase buffer. F-actin was pelleted in an airfuge and the supernatants were analysed by SDS-PAGE. The caldesmon bands were quantified by scanning densitometry.

myosin with the same apparent binding constant of around 2×10^6 M^{-1} (Fig. 4). Caldesmon binding to smooth muscle myosin was determined by cosedimentation at low ionic strength (Fig. 5); native and wild type caldesmon showed essentially identical binding properties with affinities greater than 10^6 M^{-1}.

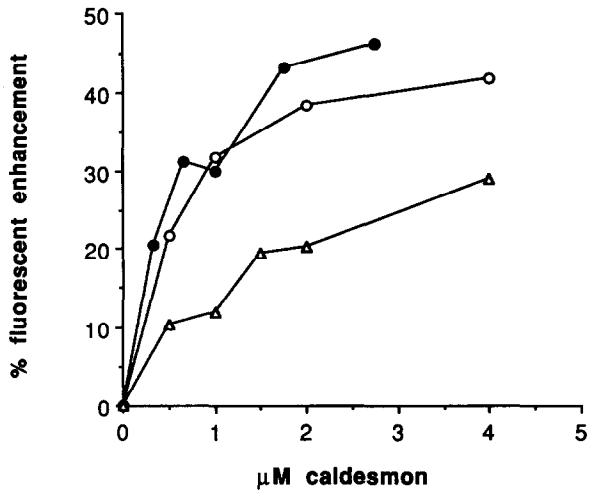

Fig. 4. Effect of caldesmon on pyrene-tropomyosin fluorescence. Legend as in Fig. 2. 0.5 μM tropomyosin labelled with *N*-(1-pyrenyl)-maleimide [19] was mixed with 0–4 μM caldesmon in 5 mM K_2 · PIPES, 40 mM KCl, 5 mM $MgCl_2$, 1 mM DTT pH 7.0. Fluorescence emission peaks at 377 nm were measured after excitation at 340 nm in a Perkin-Elmer fluorescence spectrophotometer (model LS-5). Data are expressed as % increase compared with pyrene-tropomyosin alone.

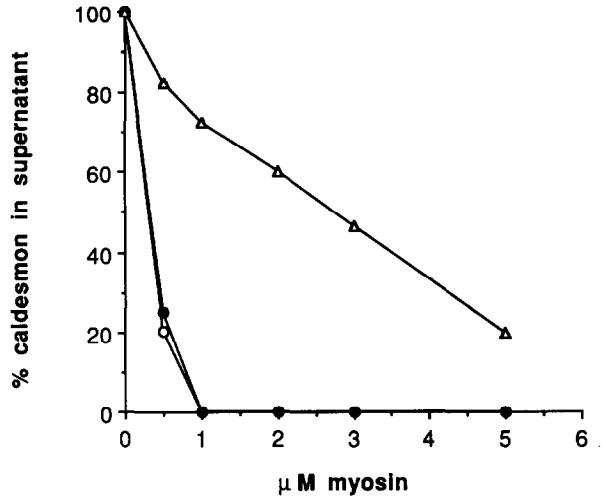

Fig. 5. Cosedimentation of caldesmon with aorta myosin. Legend as in Fig. 2. 1 μM caldesmon was mixed with 0–5 μM thiophosphorylated smooth muscle myosin in 5 mM K_2 · PIPES, 2.5 mM $MgCl_2$, 1 mM DTT pH 7.0. Myosin filaments were pelleted in an airfuge and the supernatants analysed by SDS-PAGE. The caldesmon bands were quantified by scanning densitometry.

3.3. *Functional properties of mutant caldesmon (amino acids 1–578)*

Measurements were made on 4 preparations. The mutant caldesmon did not alter actin-tropomyosin activation of myosin ATPase (Fig. 2), it did not bind to actin (Fig. 3) and exhibited weak binding to actin-tropomyosin (10% of 1 μM caldesmon cosedimented with 40 μM actin-tropomyosin). There was significant binding of the mutant to tropomyosin (Fig. 4) and to smooth muscle myosin (Fig. 5) but the affinities were significantly reduced compared with the wild type, 5-fold for tropomyosin binding and >10-fold for myosin binding.

4. DISCUSSION

4.1. *Expression of wild type and mutant caldesmon*

The level of expression of both the wild type and mutant caldesmon was at least one order of magnitude lower than that achieved with certain other proteins, e.g. gelsolin and myosin light chain (Dr. M. Way and T. Rowe respectively, LMB, Cambridge, personal communication) using the same expression system. The level is however higher than that achieved using the same construct in BL21(DE3) cells without pLysE and higher than that obtained using the pLcII/QY13 system of Nagai and Thogersen [13] (data not shown and [11]). Despite the poor expression the use of large volumes of culture and the development of a simple purification procedure which exploited the heat and acid stability of caldesmon yielded sufficient pure protein to make quantitative measurements of caldesmon function in biochemical assays. On SDS-PAGE the expressed wild type protein comigrated with native caldesmon with an

apparent molecular weight of 120 000 [5] while the mutant showed an apparent molecular weight of 100 000 (Fig. 1). Both apparent molecular weights were considerably higher than their molecular weights calculated from their sequence [11], 87 000 and 68 000 respectively.

4.2. *Expressed wild type caldesmon is fully functional*

Whilst the expression of small amounts of caldesmon has been reported [11,20] only a few properties have been examined in non-quantitative assays. We have now shown that wild type expressed caldesmon is essentially identical to native caldesmon in quantitative assays of inhibition of actin-tropomyosin activation of myosin ATPase and reversal by Ca^{2+}-calmodulin (Fig. 2, [3]), binding to actin and actin-tropomyosin (Fig. 3, [3]), smooth muscle myosin (Fig. 5), tropomyosin (Fig. 4, [19]) and calmodulin [5]. These results indicate that caldesmon expressed in a bacterial cell refolds into its native state and remains stable under the harsh conditions used for its subsequent purification (95°C [14], pH 3 [3]). Thus the ability of caldesmon to refold into a fully functional state allows one to express mutants and test their functional properties.

4.3. *Properties of mutant caldesmon (amino acids 1 to 578)*

The expressed mutant caldesmon containing amino acids 1 to 578 was found to be unable to bind to actin or Ca^{2+}-calmodulin or to inhibit actin-tropomyosin activated myosin ATPase; however, it did retain the ability to bind to tropomyosin although the affinity was diminished about 5-fold. Actin and calmodulin binding have been localised in a C-terminal 15 000 to 20 000 proteolytic fragment of caldesmon [8–11] which is absent from the mutant. The mutant does contain the sequence 508–565 which has been identified as a potential tropomyosin binding site on the basis of homology with the tropomyosin binding site of troponin T [11,20]. The weak tropomyosin binding indicates either that the mutant contains some but not all of the residues responsible for tropomyosin binding or that the deletion of the 178 residues affected the folding in the remainder of the protein, causing the reduced affinity for tropomyosin.

The mutant binds poorly to smooth muscle myosin; this contradicts previous results [10,21] obtained using myosin affinity columns which have indicated that there is a discrete myosin binding site within the first 200 amino acids from the N-terminus of caldesmon; however, both the results with tropomyosin and myosin binding are in agreement with Katayama et al. [22] who reported that full binding to these two proteins required the presence of the C-terminal 40K fragment.

Since the wild type expressed caldesmon is indistinguishable from native caldesmon, this expression system will be of great use in relating structure to function of caldesmon and is potentially much more versatile than current work using proteolytically or chemically fragmented native caldesmon. Although we have so far looked at only one mutant caldesmon we have already been able to confirm the gross predictions from previous work and show that the precise dependence of function on structure is likely to be rather more complex than previously suggested.

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