Characterisation of the effects of mutation of the caldesmon sequence ^{691}glu-trp-leu-thr-lys-thr^{696} to pro-gly-his-tyr-asn-asn on caldesmon-calmodulin interaction

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Abstract We have investigated the functional properties of a mutant (Cg1) derived from the C-terminal 99 amino acids of chicken caldesmon, 658–756 (658C) where the sequence ^{691}glu-trp-leu-thr-lys-thr^{696} is changed to pro-gly-his-tyr-asn-asn. Cg1 bound Ca^{2+}-calmodulin with (1/7)th of the affinity as compared to 658C or whole caldesmon. NMR titrations indicate that the contacts of Ca^{2+}-calmodulin with the Trp-722 region of the peptide are retained but that those at the mutated site are lost. Most importantly Ca^{2+}-calmodulin is not able to reverse the Cg1-induced inhibition. We conclude that the interaction of calmodulin with this caldesmon sequence is crucial for the reversal of caldesmon inhibition of actin-tropomyosin activation of myosin ATPase. The results are interpreted in terms of multi-site attachment of actin and Ca^{2+}-calmodulin to overlapping sequences in caldesmon domain 4b.

Key words: Caldesmon; Calmodulin; Smooth muscle; NMR; Tryptophan fluorescence; Actin; Tropomyosin; Regulation

1. Introduction

Caldesmon is a component of the thin filaments of smooth muscles [1]. In vitro caldesmon binds to actin and inhibits actin-tropomyosin filament activity in a manner analogous to troponin-I [2–4]. The inhibition is released by EF hand Ca^{2+} binding proteins such as calmodulin, troponin-C and S100 [5–7]. The mechanism of this switch is based on the binding of caldesmon with both actin and Ca^{2+}-calmodulin [5,8]. Binding sites for both proteins are contained within the C-terminal 170 residues of caldesmon [9,10]. Analysis of various peptides, substitution mutants and deletion mutants of the C-terminus of caldesmon has shown that binding involves several discrete and separate sequences for actin as well as for calmodulin (reviewed in [11]). The C-terminal 99 residues of caldesmon (chicken sequence 658–756) have received particular attention since this peptide contains multiple actin and Ca^{2+}-calmodulin binding regions and retains the inhibitory and regulatory properties of the whole molecule [10,12,13].

There is an emerging consensus that Ca^{2+}-calmodulin binds to caldesmon through three short, tryptophan-containing, sequences which we have designated as sites A, B and B′ (^{658}MWEKGNVFS^{666}, ^{687}SRINEWLTK^{695} and ^{717}GKRNLWEKQ^{725} respectively). Various studies have aimed to determine the role of each of the three tryptophan-containing sequences of caldesmon in Ca^{2+}-calmodulin binding and the consequent release of ATPase but the data are not conclusive and sometimes contradictory [13–17].

In previous work we have analysed Ca^{2+}-calmodulin binding to caldesmon in combination with structural analysis using NMR spectroscopy and measurements of caldesmon regulatory function with a range of caldesmon and calmodulin mutant peptides [13,14,18]. We have interpreted these results in terms of multiple contacts between Ca^{2+}-calmodulin and the caldesmon C-terminus involving the interaction of site B with the C-terminal domain of calmodulin and site A and B′ with the N-terminal part of calmodulin. We also proposed that at least two contact sites are needed for Ca^{2+}-calmodulin binding to be able to reverse inhibition. In order to test this model we have constructed and characterised a mutant of the C-terminal 99 amino acids of caldesmon (658C) in which only one of the three putative Ca^{2+}-calmodulin contact sites remains intact (B′). In this mutant (which we call Cg1) we changed the sequence ^{691}glu-trp-leu-thr-lys-thr^{696} (site B) to pro-gly-his-tyr-asn-asn. We found that the mutation in Cg1 reduced the affinity for Ca^{2+}-calmodulin 7-fold and completely abolished the ability of Ca^{2+}-calmodulin to reverse the inhibition of the actin-tropomyosin activation of myosin ATPase, whereas interactions with actin were not greatly affected. This demonstrates the importance of site B in the release of 658C-induced inhibition and confirms the necessity for multi-point attachment between Ca^{2+}-calmodulin and caldesmon for reversal of inhibition.

2. Materials and methods

2.1. Proteins

Bovine brain calmodulin, rabbit skeletal muscle myosin subfragment-1 and actin and sheep or gizzard smooth muscle caldesmon and tropomyosin were prepared by our established methods [14,19]. Wheat germ calmodulin was prepared, purified and spin-labelled at Cys-27 as previously reported [20]. The C-terminal caldesmon mutant peptide 658C (chicken caldesmon *h* residues 658–756) was obtained by bacterial expression in the pMW172 plasmid/BL21(DE3) cell system and purified as described by Redwood and Marston [10]. The mutant plasmid for Cg1 was produced by the inverse PCR method [21] using the expression plasmid pMW172 encoding 658C [22]. Two back-to-

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Abbreviations: S-1, chymotryptic myosin subfragment-1; EGTA, ethylene glycol-*O*,*O*′-bis(2-aminoethyl)-*N*,*N*,*N*′,*N*′-tetraacetic acid; DTT, 1,4-dithio-DL-threitol; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulphate

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back primers, 5′-CCTGCTCCAAAACCTTCTGAT-3′ and 5′-CGATTTGTTACCCTCTGGGTTGTTGTAATGCCCTGGGTTGATACGACTGGAGAC-3′ (altered nucleotides underlined) were used to change ^{691}glu-trp-leu-thr-lys-thr^{696} to pro-gly-his-tyr-asn-asn. The yield of 658C and Cg1 was 15–20 mg pure protein per litre expression medium. On SDS-PAGE they migrated with identical apparent molecular weights of 10 500 Da. Protein concentrations were determined by the method of Lowry et al. [23].

2.2. Assays

Binding of 658C and Cg1 to Ca^{2+}-calmodulin was measured directly by cosedimentation of caldesmon peptides, 658C and Cg1, with Ca^{2+}-calmodulin coupled to Sepharose (Pharmacia) as described in [14,19]. Fluorescence spectra of 658C and Cg1 were recorded on a Perkin Elmer luminescence spectrometer (LS-5) with excitation at 295 nm; titrations with calmodulin were performed according to the procedure described by Huber et al. [14]. The effect of 658C and Cg1 on actin-tropomyosin activated myosin MgATPase activity was measured by our usual protocols [14,19].

2.3. Nuclear magnetic resonance (NMR) measurements

One-dimensional proton NMR spectra were obtained at 500 mHz on a Bruker AMX spectrometer using quadrature detection at a sample temperature of 25°C in D_2O solution. The binding titrations were carried out by addition of small aliquots of a concentrated stock solution of spin-labelled calmodulin as previously described [14] keeping dilution effects below 5%.

3. Results and discussion

The regulatory function of caldesmon in smooth muscle thin filaments consists of its tropomyosin-dependent inhibitory activity and a release mechanism, mediated by a Ca^{2+} binding protein such as calmodulin, by which caldesmon's interaction with actin-tropomyosin is altered to allow for myosin ATPase activation [11].

The 658C recombinant peptide of chicken gizzard caldesmon (residues 658–756), including a point mutation Trp-659→Gly (Ca^{2+}-calmodulin binding site A) retains binding to actin and to Ca^{2+}-calmodulin. Its tropomyosin-dependent inhibitory activity can be fully released upon interaction with Ca^{2+}-calmodulin like that of native caldesmon [4,10]. Nuclear magnetic resonance spectra have demonstrated that the interaction of this peptide with Ca^{2+}-calmodulin involves both tryptophan residues at positions 692 and 722 [14]. The Cg1 mutation (^{691}glu-trp-leu-thr-lys-thr^{696} to pro-gly-his-tyr-asn-asn) included the replacement of the hydrophobic residue tryptophan 692 by glycine, charge changes (lys > asn), the introduction of two unique residues, histidine and tyrosine, to aid NMR characterisation and a proline residue aiming to affect binding of Ca^{2+}-calmodulin to this site by affecting the ability of this sequence to adopt a helical conformation.

3.1. Binding of the caldesmon mutants to Ca^{2+}-calmodulin

Binding of 658C and Cg1 to calmodulin was measured by the established techniques of cosedimentation with calmodulin-Sepharose and tryptophan fluorescence titrations [13,14,16]. Both 658C and Cg1 cosedimented with calmodulin bound to Sepharose in the presence of Ca^{2+} (Fig. 1A). Binding was specific since control caldesmon peptides (e.g. N128, H10, [10,24]) not containing the Ca^{2+}-calmodulin binding sequences did not cosediment in the presence of Ca^{2+} and none of the peptides cosedimented in EGTA buffer (pCa9) (data not shown). The dissociation constants derived from five separate assays were for 658C: $K_d = 1.9 \pm 1.1$ μM ($n = 5$) and for Cg1: $K_d = 14.1 \pm 6.9$ μM ($n = 5$), thus the affinity of Cg1 is approximately 1/7 of the affinity of 658C. In previous work the affinity of 658C for Ca^{2+}-calmodulin was found to be indistinguishable from the whole caldesmon molecule [13] when measured by three different methods, thus the mutation in Cg1 induces a specific reduction in Ca^{2+}-calmodulin affinity. Graether et al. noted a similar, 3-fold, decrease in affinity for Ca^{2+}-calmodulin due to mutation of Trp-692 [16].

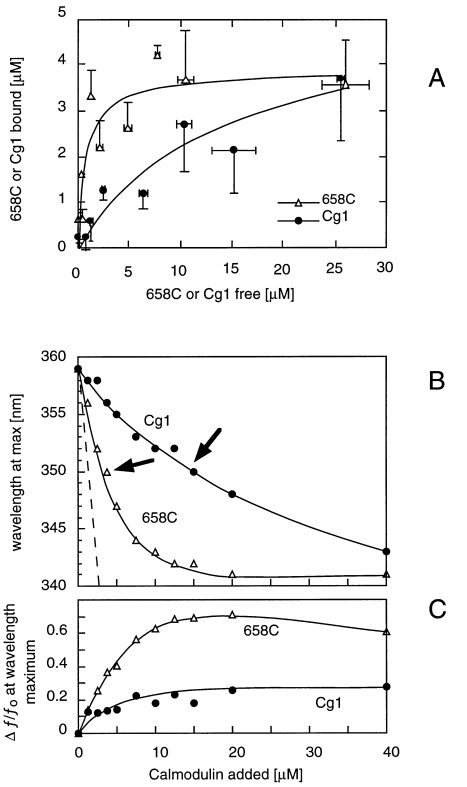

Fig. 1. Binding of Cg1 or 658C to Ca^{2+}-calmodulin. A: Increasing concentrations of Cg1 or 658C were cosedimented with calmodulin coupled to Sepharose (Pharmacia). Conditions: 10 mM KCl, 5 mM PIPES adjusted to pH 7.2 with KOH solution at 22°C, 2.5 mM $MgCl_2$, 0.5 mM DTT and 0.1 mM $CaCl_2$ or 0.5 mM EGTA, 22°C. A simple binding curve was fitted through the data and dissociation constants calculated for a single binding site: 658C: $K_d = 1.9 \pm 1.1$ μM ($n = 5$) and for Cg1: $K_d = 14.1 \pm 6.9$ μM ($n = 5$). Symbols: (●) Cg1; (△) 658C. Bars represent standard errors of data pooled from three experiments. B, C: Effect of Ca^{2+}-calmodulin on the tryptophan fluorescence emission of the caldesmon peptides Cg1 and 658C. Conditions were: 50 mM NaCl, 10 mM Tris-HCl, pH 7.2, 2.5 mM $MgCl_2$ and 0.1 mM $CaCl_2$ or 0.5 mM EGTA, 5 μM 658C or Cg1. B: The blue shift of the spectrum maximum. Dissociation constants were calculated on the basis of the equation K_d = concentration of free calmodulin at 50% change = (calmodulin added) $-0.5\times$(658C or Cg1). 50% changes are indicated by arrows. C: The emission intensity of the tryptophan fluorescence measured at the maximum of the spectrum. Data are expressed as the ratio of the change in fluorescence intensities of the caldesmon peptide induced by Ca^{2+}-calmodulin to the fluorescence intensities of Cg1 or 658C alone. Smooth lines were drawn through the data points. Symbols are used as in A.

The reduced affinity of Cg1 compared with 658C was confirmed in fluorescence titrations with Ca^{2+}-calmodulin. The fluorescence measurements suggested that both tryptophan residues in 658C, Trp-692 and Trp-722, contribute equally to the fluorescence intensity since the fluorescence of Cg1 was half of that measured for an equal concentration of

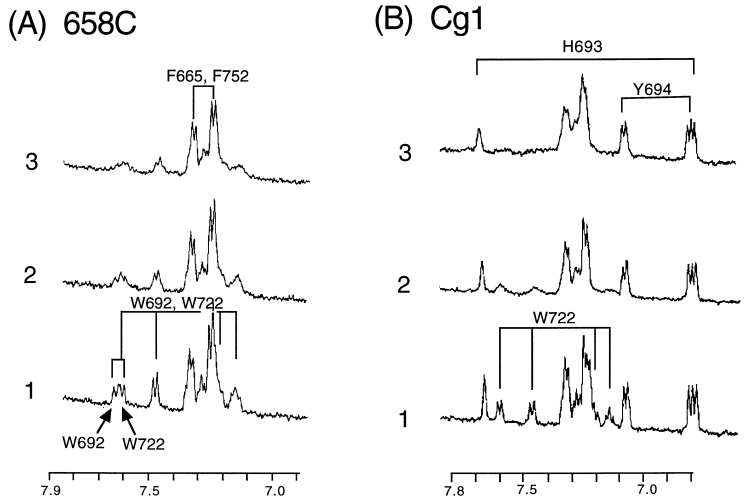

Fig. 2. Binding of 658C and Cg1 to calmodulin monitored by NMR spectroscopy. The aromatic spectral regions are shown: A: 658C: 1, 120 μM 658C; 2, 120 μM 658C+3 μM Ca^{2+}-calmodulin; 3, 120 μM 658C+7 μM Ca^{2+}-calmodulin. B: Cg1: 1, 160 μM Cg1; 2, 160 μM Cg1+7 μM Ca^{2+}-calmodulin; 3, 160 μM Cg1+27 μM Ca^{2+}-calmodulin.

658C. Upon complex formation with Ca^{2+}-calmodulin the intrinsic tryptophan fluorescence of both caldesmon peptides increased and the spectral maximum showed a distinct blue shift (Fig. 1B, C). The change in maximum fluorescence ($\Delta F/F_0$) with Cg1 was very much less than with 658C, indicating that the major change in fluorescence upon Ca^{2+}-calmodulin binding to 658C arises from Trp-692 [14]. The maximal blue shift caused by Ca^{2+}-calmodulin in Cg1, 16 nm, was similar to the maximal shift with 658C, 18 nm. The blue shift was sufficiently large to estimate a K_d of 2 μM for 658C and 13.5 μM for Cg1 assuming a linear relationship between binding and blue shift. This result is thus very similar to that obtained by cosedimentation.

3.2. NMR titration of 658C and Cg1 with Ca^{2+}-calmodulin

Precise information about caldesmon-calmodulin contacts can be obtained by nuclear magnetic resonance spectroscopy once individual resonances have been correlated with specific groups [25]. 1-D NMR spectra were recorded of 120 μM 658C and Cg1 titrated with Ca^{2+}-calmodulin. In the aromatic region of the spectrum, presented in Fig. 2, peaks arising from tryptophan, phenylalanine, tyrosine, and histidine are distinct. The doublet signals at 7.64 and 7.60 in the spectrum of 658C have been assigned to the C4H signal resonances of tryptophans 692 and 722 respectively [14,25] and it is clear that only the signal at 7.60 is present in Cg1, thus confirming its assignment to Trp-722. Upon titration with spin-labelled Ca^{2+}-calmodulin distinct broadening of the signals of both tryptophan residues of 658C indicated their combined involvement in the interaction with Ca^{2+}-calmodulin. Only the tryptophan signals showed this change; the phenylalanine signals (from Phe-665 and Phe-752, near the N- and C-termini of the peptide) remained unaffected by Ca^{2+}-calmodulin. The line broadening occurred progressively during titration and is indicative of fast-intermediate chemical exchange between free and bound forms of the peptide on the NMR timescale. The spectral effect of complex formation at any fraction bound is a sensitive function of the correlation time in the bound complex [26]. Hence the marked effect of substoichiometric Ca^{2+}-calmodulin on the tryptophan signals is a direct indication of close contact with between 658C and Ca^{2+}-calmodulin involving residues in the region of 692 and 722.

Titration of Cg1 with Ca^{2+}-calmodulin again showed specific broadening in the signals at 7.6 ppm (4H) and 7.45 ppm (7H) originating from the sole tryptophan at position 722 but these spectral changes required the addition of higher molar ratios of Ca^{2+}-calmodulin to caldesmon peptide indicating weaker interaction of Cg1 in accord with the direct binding experiments (Fig. 1). Hardly any change was detectable for the signals of the aromatic residues His-693 (7.72 ppm) or Tyr-694 (6.8 ppm) which had been introduced into Cg1 in place of the native site B. The data therefore indicate that the altered site B did not participate in the interaction of Cg1 with Ca^{2+}-calmodulin, while binding still occurred, albeit somewhat weaker, in the region of Trp-722 corresponding to site B′.

3.3. Reversal of ATPase inhibition

The mutation in Cg1 caused a 3-fold decreased ability to inhibit the actin-tropomyosin activated myosin MgATPase as demonstrated by higher concentrations of Cg1, compared to 658C, which were needed to achieve comparable levels of inhibition (Fig. 3; [27]). Ca^{2+}-calmodulin reversed the inhibition caused by 658C as expected but no reversal of the inhibition due to Cg1 was observed, even at very high Ca^{2+}-calmodulin concentrations (Fig. 3). Sedimentation analysis indicated that under these conditions Ca^{2+}-calmodulin displaced most of the 658C from actin-tropomyosin when bound. In contrast Ca^{2+}-calmodulin did not displace Cg1 from actin-tropomyosin. The lack of release of Cg1 inhibition by Ca^{2+}-calmodulin is thus largely due to its inability to compete with actin for binding to Cg1. This correlates with the complete loss of interaction between Cg1 and Ca^{2+}-calmodulin at site B whilst interaction with actin is retained.

Data from this and other laboratories have shown that deletion (or replacement) of tryptophan at any one of the three Ca^{2+}-calmodulin binding sequences had very little effect on the ability of Ca^{2+}-calmodulin to regulate the caldesmon-actin interaction [13,14,18]. Cg1 is the first inhibitory caldesmon peptide retaining only one intact Ca^{2+}-calmodulin bind-

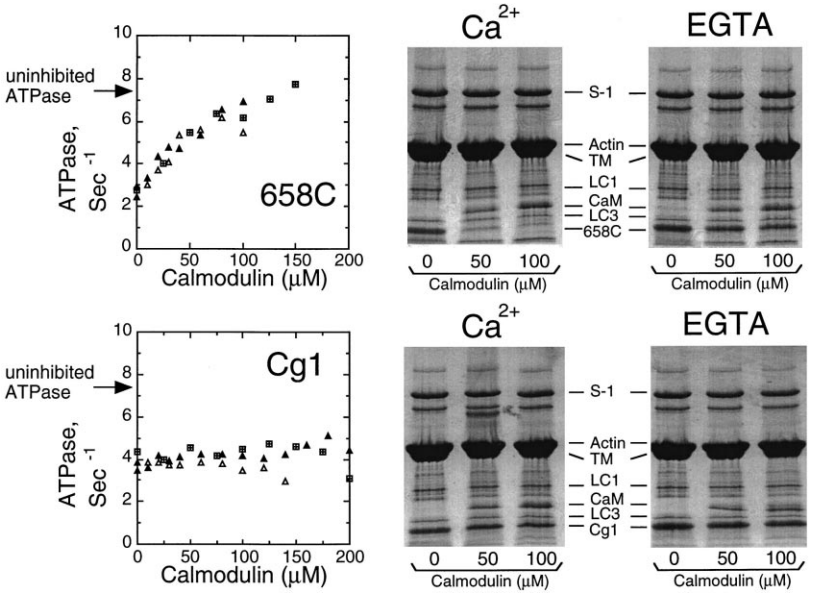

Fig. 3. Effect of calmodulin on the ATPase inhibitory activity and the binding to actin-tropomyosin of 658C and Cg1. Buffer conditions 20 mM KCl, 5 mM PIPES adjusted to pH 7.0 with KOH solution at 22°C, 2.5 mM $MgCl_2$, 0.1 mM DTT and 0.2 mM $CaCl_2$ or 5 mM EGTA. Mixtures of 1 μM skeletal muscle S-1, 12 μM skeletal muscle F-actin, 5 μM smooth muscle tropomyosin, 20 μM 658C (upper panel and gels) or 60 μM Cg1 (lower panel and gels) and 0 to 200 μM calmodulin were divided in two portions. One portion was sedimented and the pellets were analysed by gel electrophoresis and one portion was used for measurement of MgATPase activity. Uninhibited ATPase was 7.5 s^{-1}. The results of three separate experiments are presented. The gels of the pellets show that increasing amount of calmodulin (from 0 to 100 μM as indicated below the lanes) dissociated 658C from actin at pCa5 (Ca^{2+}) but not at pCa9 (EGTA). Cg1 is not dissociated from actin at both pCa5 and pCa9. A small amount of calmodulin (less than 5% of the total) sedimented. If the corresponding lanes are compared the same amount of calmodulin is observed in the pellets in Ca^{2+} or EGTA, with 658C or Cg1, indicating that it is probably trapped in the pellets and not bound.

ing site (site B′) which has been tested for functional interaction with Ca^{2+}-calmodulin. The absence of functional interaction indicates that multi-point attachment is likely to be a prerequisite for Ca^{2+}-calmodulin reversal of caldesmon inhibition.

3.4. Structural basis of Ca^{2+}-calmodulin control of caldesmon inhibition

In our recent work we have discussed the evidence that caldesmon inhibition of actin-tropomyosin filament activity is due to simultaneous binding of several discrete segments of caldesmon to actin and that the segments connecting these sequences contribute to positioning the actin binding sequences to form an inhibitory complex [11,19]. In 658C there are two actin binding sequences which overlap sites B and B′ respectively. The binding of Ca^{2+}-calmodulin at these two sites seems to be a prerequisite for inhibition reversal in this C-terminal peptide. Mabuchi et al. [28] have reported an extended structure of calmodulin in its interaction with caldesmon similar to the complex of troponin-I with troponin-C [29,30]. Our data suggest that an extended Ca^{2+}-calmodulin makes contacts on sites B and B′ of 658C inducing alteration of the caldesmon peptide-actin conformation accompanied by a weakening of actin-caldesmon binding which may result in dissociation and which results in release of the inhibition.

The change of sequence at site B in Cg1 reduces the affinity of this site for actin slightly but has a much greater effect upon the affinity of site B for Ca^{2+}-calmodulin. This may be related to the observations that, at least in short peptides, site B has an α-helical conformation when bound to Ca^{2+}-calmodulin [17]. This conformation would be greatly destabilised by the proline inserted at position 691 in Cg1. Both actin and Ca^{2+}-calmodulin contacts with site B′ are retained in the mutant Cg1. As a consequence inhibitory actin binding is essentially retained in Cg1 whilst the reversal of inhibition due to Ca^{2+}-calmodulin binding is lost.

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