Biochem. J. (1991) 279, 1–16 (Printed in Great Britain)



# REVIEW ARTICLE
# The molecular anatomy of caldesmon

Steven B. MARSTON and Charles S. REDWOOD
Department of Cardiac Medicine, The National Heart and Lung Institute, Dovehouse Street, London SW3 6LY, U.K.

## INTRODUCTION

In 1980 Kakiuchi and his colleagues isolated a high-molecular-mass calmodulin-binding protein from chicken gizzard which they named caldesmon [1]. This protein was subsequently found to be ubiquitous in smooth muscle and non-muscle tissue and was eventually identified as the major regulatory component of smooth muscle thin filaments [2]. Because of its potential role in regulating contractility it has been the subject of intense scrutiny during the last 10 years and has proved to be a unique and interesting protein.

Smooth muscle caldesmon is a large protein (87 kDa) with an extended structure which is exceptionally stable to heat and acid denaturation [3]. It is a multifunctional protein which binds tightly and specifically to actin, calmodulin, tropomyosin and myosin [4–6], besides being a substrate for many protein kinases. *In vivo*, caldesmon is an integral component of the thin filaments of the contractile apparatus of smooth muscles [7,8] and the binding interactions between the proteins of the filament have important regulatory functions. Caldesmon is an essential component of the $Ca^{2+}$-regulatory mechanism of the thin filament: it is a potent inhibitor of actin–tropomyosin activation of myosin MgATPase activity and of contractility and the inhibition is regulated in response to $Ca^{2+}$ via its interaction with $Ca^{2+}$-binding proteins such as calmodulin [5,6]. It also crosslinks thick and thin filaments via its ability to bind both actin and myosin and this property is of potential importance during maintained contractions [9]. Caldesmon exists as several isoforms which appear to be specific to smooth muscle and non-muscle contractile cells [10,11].

The objective of this Review is to describe the structure of the caldesmon molecule and to try and identify the domains which are responsible for its multiple functional properties. Since a number of caldesmons have been sequenced and since the functional domains have been thoroughly studied by a variety of techniques, it is possible to build up a detailed picture of the mechanism by which caldesmon works.

## CALDESMON STRUCTURE

In Fig. 1 we have tabulated all the primary sequences of caldesmon which are known. We have followed Sobue [55] in using the notation *h* for the high-molecular-mass isoforms (756–769 residues) and *l* for the low-molecular-mass isoforms. Chicken caldesmon has been particularly well studied with four isoforms published; we have introduced the terminology $\alpha$ and $\beta$ to distinguish between the two CD*l* and two CD*h* sequences. At the time of writing no mammalian CD*h* sequence has been determined but the complete sequence of rat low-molecular-mass caldesmon (CD*l*) has been published and two sequences of human CD*l* are known.

The first sequence to be published in full was chicken gizzard CD*h* $\beta$, determined in Bryan's laboratory [13]. In common with the majority of recent publications, we have adopted his numbering of amino acids for the purposes of this Review. The other isoforms differ due to insertions (CD*h* $\alpha$) or deletions (CD*l*) (Fig. 1).

On SDS-gel electrophoresis smooth muscle caldesmon (CD*h*) usually runs as two closely spaced bands of roughly equal intensity. This pattern, though first recognized in chicken gizzard [1,3], is universal and has been reported in the smooth muscles of turkey gizzard [18,37], sheep and rat aorta (K. Pinter, unpublished work; [40]), human umbilical artery (K. Pritchard, unpublished work) and rabbit uterus [41], stomach and trachea [7]. The migration of this doublet has been variously reported as corresponding to 120 [7,19], 125 [20], 140 [21] and 165 [22] kDa. The two chicken CD*h* sequences that have been derived from cDNA clones give significantly lower calculated molecular masses; CD*h* $\beta$ has 756 amino acids which give it a calculated molecular mass of 86954 Da whereas CD*h* $\alpha$ has an additional 15 amino acids inserted at 380 and a calculated mass of 88743 Da (Fig. 1). The expressed protein from both clones comigrates with caldesmon purified from chicken gizzard [13,53,95] and we believe the two clones correspond to the two bands of the doublet. Inspection of the sequences shows that smooth muscle caldesmon is composed of approximately 50% charged amino acids and with a large proportion of glutamate (23%), lysine (15%), alanine (11%) and arginine (10%) residues. The reason for the anomalous migration on gels appears to be the high content (26%) of glutamic and aspartic acid residues [23]; neutralization of these by EDC changes the apparent molecular mass on SDS gels to 93 kDa [24,30].

The smaller isoform of caldesmon, termed CD*l*, has been located in many non-muscle avian and mammalian tissues on the basis of its heat stability and crossreactivity with anti-*h*-caldesmon antibodies [42,43]. These tissues include platelets [44,45], fibroblasts [59], foetal smooth muscle [11,46], adrenal medulla [47], liver [48], erythrocytes [49], thyroid glands [50], absorptive epithelial cells [43], atherosclerotic plaques [10] and many cultured cell lines [46,51,63]. In addition caldesmon-like proteins have been reported in molluscan catch and striated muscles [137,148]. CD*l* also migrates anomalously on SDS/polyacrylamide gels with an apparent mass between 76 and 80 kDa; the two cloned chicken CD*l* isoforms [54,57] have calculated molecular masses of 60 and 58 kDa and the cloned human and rat non-muscle isoforms have masses of 63 and 65 kDa respectively [59,61]. The two chicken small isoforms, CD*l* $\alpha$ and $\beta$, have 524 and 517 amino acids respectively and differ only at the extreme *N*-terminus as shown in Fig. 1. CD*l* $\alpha$ is identical to the smooth muscle CD*h* isoforms except for a deletion of 234 amino acids between residues 197 and 432. The pattern of sequence similarities and differences suggests that the isoforms may be derived from a single gene by alternative splicing of the RNA.

The cloned mammalian CD*l* molecules show extensive similarities to the chicken isoforms; the regions that are highly conserved are shown in boxes in Fig. 1. There is 85% identity in the region amino acids 496–729 with only two amino acid changes being

Abbreviations used: CD, caldesmon; TN, troponin; HMM, LMM, heavy and light meromyosin; p[NH]ppA, adenosine 5′-[$\beta\gamma$-imido]triphosphate; EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodi-imide; NTCB, 2-nitro-5-thiocyanobenzoic acid.


**Fig. 1. Complete amino acid sequences of caldesmon from chicken, rat and human cDNA libraries**

The sequence of chicken gizzard CD*h* $\alpha$ and CD*l* $\beta$ was determined by Hayashi *et al.* [53,57] using a chick embryo library. The chicken CD*h* $\beta$ and CD*l* $\alpha$ were determined by Bryan *et al.* [13,54] from a chicken gizzard $\lambda$ NM1149 cDNA library. Peptide sequencing of the '40 K' fragment (see Fig. 4) by Leszyk *et al.* [37] and of two long peptides within the '40 K' fragment by Tagaki [73] are in agreement with the sequences derived from cDNA. Small sequence differences have been reported in gizzard caldesmon from turkey (Val-566→met) and duck (Cys-580 is absent). The rat CD*l* sequence was determined by Matsumura [59] from a rat liver (Clontech) cDNA library and the human CD*l* sequence was determined by Lin *et al.* [61] from WI-38 human foetal lung fibroblast library. A partial sequence of human caldesmon from a foetal liver library which codes from

negatively related (611 Thr→gln and 630 Val→glu) plus a single additional amino acid in human (Ser between 603 and 604). The mammalian caldesmons lack the *C*-terminal 20 amino acids of the chicken. There is also considerable conservation of sequence at the *N*-terminus. Amino acids 1–171 of human CD*l* have a 77% similarity to gizzard caldesmon. Within this region there is virtual identity between 1 and 53 and between 85 and 120, whilst both mammalian sequences have an insertion of seven amino acids after 84. Surprisingly the rat *N*-terminal sequence up to 27 is longer and entirely different from both human and chicken [59].

### Size and shape

Most physical measurements of caldesmon size in solution have indicated a much larger molecule. For instance, on gel filtration columns smooth muscle caldesmon elutes at a position equivalent to a globular protein with a mass of 600 kDa [3,14,15]. Whilst it has been suggested that caldesmon could be a multimeric protein under certain conditions [1,15,16], the prime reason for these observations is that caldesmon is a very extended molecule.

Caldesmon molecules have been observed directly by electron microscopy using negative staining [25] and rotary shadowing [8,26,27]. Generally they appeared as very thin threads with lengths up to 150 nm which may represent single molecules or oligomers. In a recent study using an improved technique of rotary shadowing and very low caldesmon concentrations [27], single caldesmon molecules were observed with lengths up to 80 nm (Fig. 2*a*). They seemed to be made up from a rather rigid rod section, 30–40 nm long, in the middle of the molecule and more flexible conformations, about 20 nm long, at each end of the molecule which were sometimes not visible. Markers cross-linked to cysteines 153 and 580 (see Fig. 2*b*) were about 60 nm apart.

These dimensions have been confirmed in solution by equilibrium sedimentation measurements [17,25,168] and by $^1$H-n.m.r. measurements [38].

### Secondary structure

Predictions of secondary structure based on the Robson and Chou–Fasman algorithms indicate that there may be up to 80% $\alpha$-helix but little $\beta$ structure (Fig 3*c*). The predicted regions of $\alpha$-helix are almost entirely confined to the first 600 amino acids. The regions of highest $\alpha$-helical probability, amino acids 239–397 and 510–570, coincide with regions containing repeat sequences as identified using a self-comparison Diagon plot (Fig. 3*b*). The region 239–397 is composed almost entirely of lysine, arginine, glutamate and alanine and reveals a repeating motif of 15 amino acids, based on the sequence EEE(R/K)KAAEERERAKA. The motif is repeated nine times in CD*h* $\beta$ and ten times in CD*h* $\alpha$ [13,53]; the 15-amino-acid difference between the two isoforms corresponds to one repeating unit. This region is entirely absent from the CD*l* sequences. Calculation of the positions which the residues in this repeating region of the sequence would occupy in an $\alpha$-helix shows that the acidic groups would be found above and below each basic group and similarly basic groups would be above and/or below every acidic group, indicating that the helix may be stabilized in solution by salt bridge interactions of the side chains [31,74]. Analogous structures have been identified in the central helix of troponin C and residues 81–145 of skeletal muscle troponin I [29], but the repeating region (239–397) on CD*h* is considerably longer, constituting 55 turns of $\alpha$-helix. The proximity of acidic and basic side groups in this region of the molecule has been demonstrated by chemical crosslinking [30]. There is no evidence of a heptad repeat which would be required for the formation of an $\alpha$-helical coiled coil structure in this region or elsewhere in caldesmon.

A second smaller and weaker pattern on the diagon plot begins near residue 510 and ends at 570 [13]; this region does not possess the same repeating motif but has regular, repeating stretches of three or four acidic and three or four basic residues (− and + in Fig. 1). This region in both the gizzard and the mammalian sequences [13,61,64,65] has been shown to have significant similarity to residues 90–146 of skeletal muscle troponin T. A further segment present in the mammalian sequences which seems to be an insert at the *N*-terminal end of this region also shows high similarity to skeletal muscle troponin T (120–134) and even better similarity to the equivalent sequence of cardiac troponin T [61]. These regions of similarity are located in the shaded areas on Fig. 1. The overall identity is 43% in gizzard CD*h* and 41% in human CD*l*. More striking is the similarity of the distribution of acidic and basic residues, which is conserved in all the sequenced caldesmons.

The similarity derived from the sequence data is also apparent at the protein level. It was noted some time ago that smooth muscle contains protein crossreacting with anti-troponin T monoclonal antibodies [66] and this crossreactivity was found to be with caldesmon [67]. Recently the reverse experiment has been reported; anti-caldesmon polyclonal antibodies crossreact with skeletal muscle troponin T and with a CNBr peptide CB2 (residues 71–151) [68]. This similarity is of considerable interest since this region of troponin T has been identified as a calcium-insensitive skeletal muscle $\alpha$-tropomyosin binding site [69,70]. The troponin T CB2 peptide was analysed in detail by Parry [71]. It is predicted to be 80% $\alpha$-helical and up to 10 nm long. It is made up of the repeating motif of alternating groups of four basic and four acidic amino acids. The pattern does not match the tropomyosin sequence very well except in the region 121–173 of skeletal muscle tropomyosin (see Fig. 7). Since the structural motif is well conserved in caldesmon it has been proposed by a number of investigators as the tropomyosin binding site of caldesmon [13,64,65]. Biochemical studies (see later) have tended to confirm this, with reservations.

Circular dichroism measurements have attempted to define the amount of $\alpha$-helix actually present in caldesmon in solution. In one study Lynch *et al.* [26] reported 10% $\alpha$-helix but recently Graceffa & Jancso [32] have found 40% $\alpha$-helix at 20 °C in solution for whole caldesmon; the *N*-terminal fragment 1–482 was 50% $\alpha$-helix and the *C*-terminus (483–756) only 20% $\alpha$-helix which is in accord with the prediction that the *C*-terminus has little $\alpha$-helix (Fig. 3). The fragment 166/171–450, which includes the proposed $\alpha$-helical region, was found to be 60% $\alpha$-helix in solution [31].

amino acid 467 to the *C*-terminus was determined by Avent *et al.* [60]. It was identical to the sequence shown here except for one base change resulting in Met-275→Val (474 in CD*h* $\beta$ numbering). The sequences are aligned in the regions of similarity but no attempt has been made in this Figure to align the sequences between 190 and 495. Regions of high similarity are boxed with a solid line and regions of partial similarity with dotted lines. The regions of human caldesmon which are similar to troponin T are shaded and the sequence of rabbit skeletal muscle troponin T is given underneath. The sequence motif which is repeated nine or ten times between 251 and 377 is indicated by the bars with the 'helix' pattern.
**Note added in proof**: The sequencing of a human aorta caldesmon cDNA has recently been completed (J. Bryan, unpublished work). It codes for a protein of 793 residues with a calculated molecular mass of 93262 Da. The sequence is identical to the human CD*l* shown above except for a central 255-residue insertion, which includes domain 2, at residue 206. There is considerable homology with the chicken sequence in this region and in domain 2 the predicted salt-bridge-stabilized helical structure is conserved although the 15mer repeat motif is much less apparent.

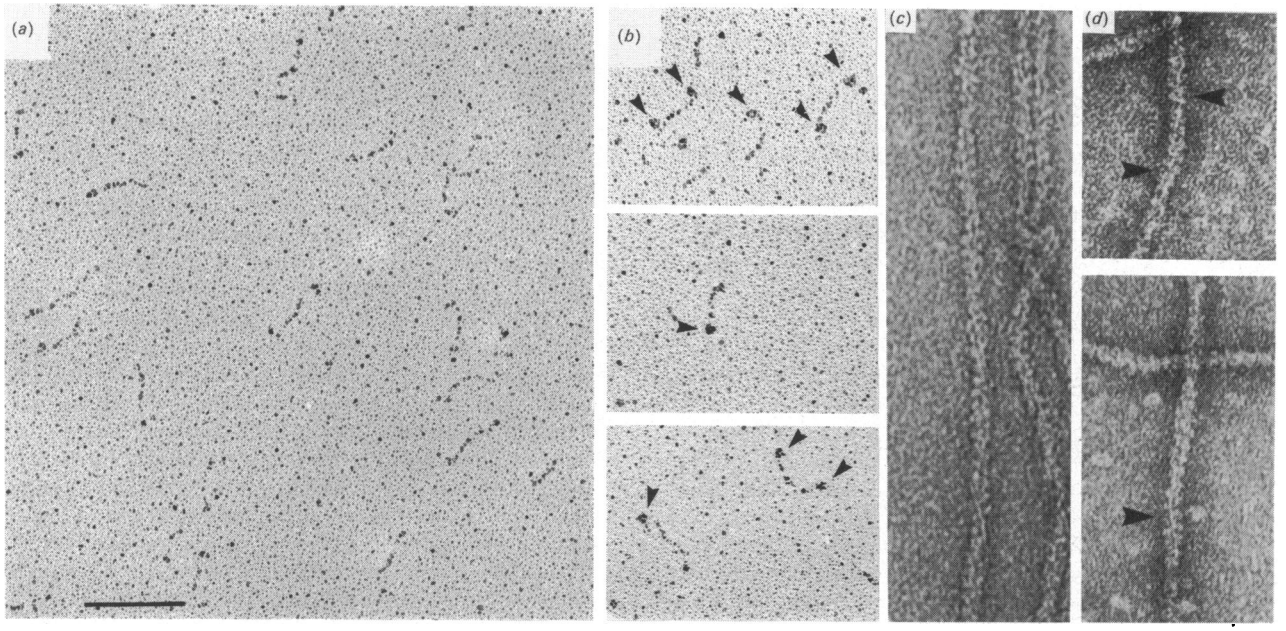


**Fig. 2. Electron microscope images of caldesmon and caldesmon–actin complex**

(*a*) Rotary shadowed single chicken gizzard CD*h* molecules are shown. The bar is 100 nm long. (*b*) Caldesmon molecules with caldmodulin crosslinked to cysteines 153 and 580. Calmodulin appears as a 10 nm globule (arrowed). These pictures are from the work of Mabuchi & Wang [27]. (*c*) and (*d*) Native thin filaments from chicken gizzard (*c*) and skeletal actin/chicken gizzard caldesmon complex (*d*) were negatively stained on thin carbon film and photographed using minimum dose electron microscopy, revealing actin substructure. Magnification 320000×. Reproduced from the work of Moody *et al.* [25].

## Domain structure

Proteolytic digestion of caldesmon under physiological conditions by α-chymotrypsin has suggested the presence of four domains which are relatively resistant to proteolysis separated by three sites where proteolysis is rapid (Fig. 4). Chicken gizzard caldesmon is first split into two portions of apparent mass 80 kDa and 40 kDa [19,33–36] which has been shown to be due to cleavage at Trp-450 [13]. Thrombin digestion cleaves at 480 [37]. Examination of the full sequence indicates a total of 16 potential sites for α-chymotryptic cleavage of which nine are within the '40 K' *C*-terminal fragment. Despite this, the '40 K' fragment is quite stable to further digestion and may readily be isolated [33,36]. This part of caldesmon seems therefore to represent a stable domain.

Further digestion by chymotrypsin causes it to be split into two parts by cleavage around 578–625 [33,35]. The *N*-terminal fragment ('15 K', Fig. 4) contains the sequence which has similarity to troponin T and is probably α-helical (490–580; Fig. 3) whilst the *C*-terminal '20 K' fragment (596–756) is quite different from the rest of the molecule. It has little predicted secondary structure, is more basic and more hydrophobic than the rest of caldesmon and contains most of the tryptophan, phenylalanine, tyrosine, histidine and proline residues. The '20 K' fragment also retains the actin and calmodulin binding properties of caldesmon [33] and therefore, presumably has a discrete tertiary structure.

The '80 K' *C*-terminal product of chymotryptic digestion (1–450) is further cleaved at Phe-165 or Trp-170 to yield two distinct fragments: one of 60 kDa which contains the long α-helix and the 15-mer repeats and one of 27 kDa, which includes the *N*-terminus [31,35,38]. Rather little is known about the '27 K' *N*-terminal domain although it has been shown that the myosin interacts with this domain. Secondary structure predictions suggest that it may contain some stretches of α-helix between 20 and 120 but that a single stable helix similar to the 230–430 region is unlikely. The region contains unusually long stretches of acidic and basic residues as seen by inspection of the primary sequence (Fig. 1) and acid–base plot (Fig. 3*a*).

Caldesmon is remarkable for such a large protein in being resistant to high temperatures [3,39] and to low pH [5,14]. The functional and structural properties are quite unaffected by these treatments once caldesmon has been returned to physiological pH and temperature, a property which has proven most useful for the isolation of caldesmon. This behaviour suggests that all the domains have a relatively simple tertiary structure which can spontaneously refold. Graceffa & Jancso [32] observed that thermal melting of the α-helical structure of caldesmon occurs reversibly and in a gradual linear fashion without any steep co-operative transitions. This might account for the high thermal stability.

In Fig. 4 we have produced a model of the large caldesmon isoform based on a consensus of the physical measurements, proteolytic degradation, predicted secondary structure and the regions which are highly conserved between species. We propose that there are four domains which are separated by mobile sections of the peptide chain. The *N*-terminal domain (1–170) is extended and may contain some α-helix. The second domain (170–480) is the central helix, responsible for the 30–40 nm rodlike structure in electron micrographs (Fig. 2). This domain has been measured at 43 nm by equilibrium sedimentation and at 35 nm by electron microscopy [31]. The third domain is, again, primarily a single α-helix stabilized by intrahelix salt bridges containing the troponin-T-like sequence (residues 509–565). The length of domain 2 and 3 is 60 nm [27] giving a length for domain 3 of around 20 nm. The fourth domain (*C*-terminal residues 590–756) is an extended stably folded region which contains little regular secondary structure. In the smaller isoform domain 2 is absent and in the mammalian sequences the linking peptide between domains 1 and 3 is significantly longer: 97 residues in rat, 96 in human and 69 in chicken.

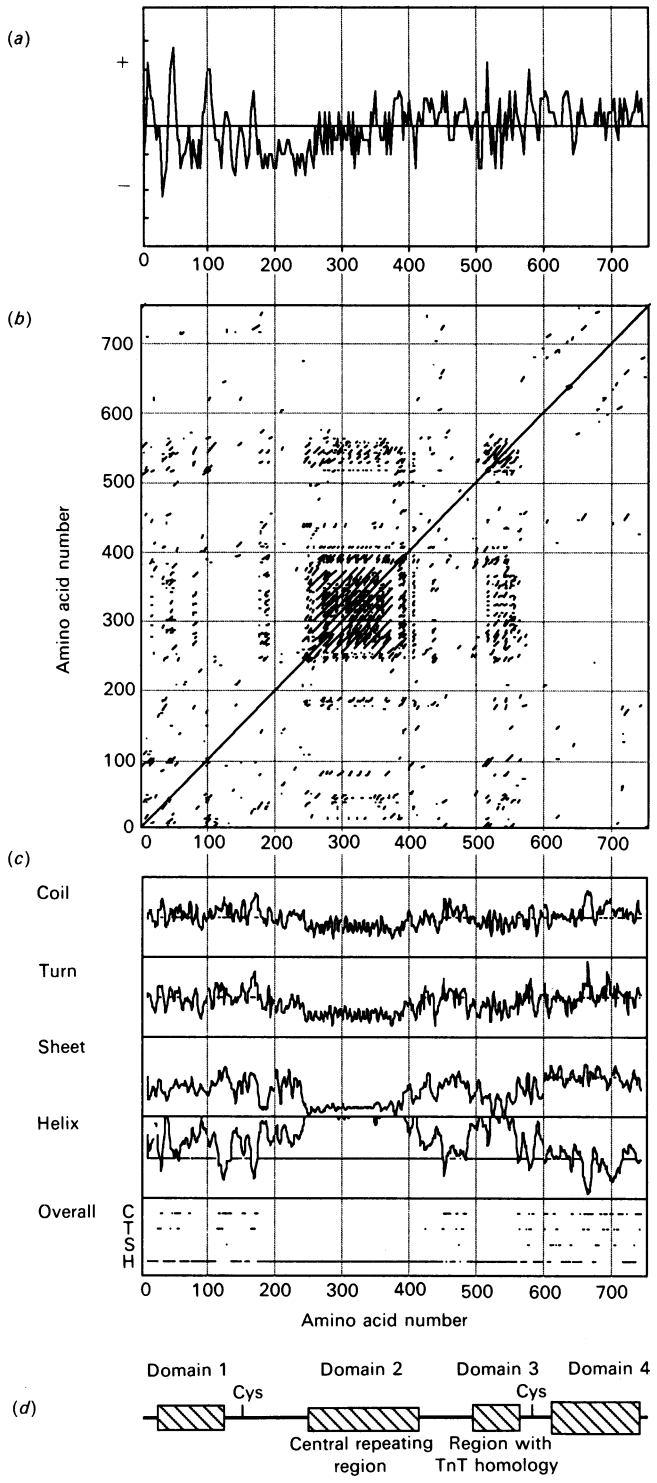


**Fig. 3. Structural features of chicken gizzard CD*h* caldesmon sequence**

(*a*) Plot showing the distribution of acidic (−) and basic (+) residues calculated using a computer program written by R. Staden (Cambridge, U.K.) with window size = 7. (*b*) Self-comparison (Diagon) plot calculated using MacVector (IBI) with window size = 8, used to identify regions of self-similarity. The markings indicate regions of the sequence similar to other regions of the same sequence. (*c*) Secondary structure prediction plot calculated using a computer program written by R. Staden (Cambridge, U.K.). The probabilities of the sequence adopting coil, turn, $\beta$-sheet or $\alpha$-helical conformations are plotted individually and an overall prediction of conformation is shown. The sequence is predicted to be up to 80% $\alpha$-helix (H), the balance comprising coil (C) and turn (T) with very little predicted $\beta$-sheet. (*d*) Simple model of caldesmon structure showing the main areas of structural interest.

## CALDESMON IN THIN FILAMENTS

*In vivo*, caldesmon is found associated with actin filaments rather than free in solution and its configuration when associated may be different from that considered up to now. Furst *et al.* [8] showed that caldesmon was located along with actin in the contractile domain of smooth muscles whilst in non-muscle cells it is found associated with actin and tropomyosin in stress fibres and membrane ruffles [45,52,63]. Anti-caldesmon antibodies have been used to select for caldesmon-containing structures. In smooth muscle tissue, native thin filaments were obtained made up of actin, tropomyosin and caldesmon at a ratio of one caldesmon molecule to 17 actin molecules [75] whilst in non-muscle cells microfilaments were extracted which were also composed of actin, tropomyosin and caldesmon [12,76]. Native thin filaments composed primarily of actin, tropomyosin and caldesmon have been purified from many types of smooth muscles [2,7,41]. The molar ratios of the thin filament proteins have been found to be quite consistent, being one tropomyosin to seven actins [7,67,77] and one caldesmon to 16 actins [78]. Clearly the thin filament is a stable multiprotein complex and caldesmon is part of it.

Electron microscopy combined with antibodies has shown the likely configuration of caldesmon in the thin filament. The backbone of the filament is the actin double helix with tropomyosin located in the groove between the helices (Figs. 2*c*, 2*d* and 5). The length of the tropomyosin molecule, which is equivalent to the length of seven actin monomers in the double helix, accounts for its stoichiometry. This structure has been analysed in considerable detail in skeletal muscle [79,80] and is known to be the same in smooth and non-muscle filaments [81].

No particles of appropriate size are observed attached to negatively stained thin filaments and polyclonal antibodies to caldesmon label the entire surface of the filament [75,82]. It is thus likely that caldesmon is bound to thin filaments in an extended configuration. In high resolution images of native thin filaments (Fig. 2) elongated molecules were seen following the long-pitch helices with no evidence of lateral projections. These were suggested to represent the side-by-side association of caldesmon and tropomyosin [25]. In synthetic filaments of caldesmon bound to actin long slender molecules were sometimes seen running along the filament axis like tropomyosin (Fig. 2).

Antibodies specific to a small region of caldesmon promote bundling of the filaments due to crosslinking through the IgG (or IgM) molecule. Unlike antibody to whole caldesmon, these antibodies produce bundles with a banded pattern reflecting a regular spacing of caldesmon along the filament. Antibody specific to '40 K' fragment of caldesmon (Fig. 4) or the *N*-terminal NTCB fragment (1–150) gave striations repeating every 38 nm with chicken gizzard thin filaments [25,75], whilst a monoclonal antibody produced bundles with $36 \pm 4$ nm repeat in microfilaments extracted from gerbil fibroma cells [12]. The interpretation of these results is that caldesmon binds specifically to tropomyosin and is therefore located in register with it since tropomyosin molecules repeat every 38 nm [83,84].

A completely self-consistent model of caldesmon location in the thin filament is possible using a few reasonable assumptions. Firstly it is assumed that caldesmon is in the same extended configuration as it is thought to be in solution with a length of about 80 nm (Figs. 2 and 5). Secondly the stoichiometry is assumed to be one caldesmon per two tropomyosins or 14 actins. As already mentioned, direct measurement of caldesmon content of thin filaments has given numbers of one per 16 and one per 17 in conditions where the loss of a caldesmon has been minimized. Binding stoichiometries are in the range one per 8 to one per 18 (Fig. 6). Lower caldesmon contents have been reported and may

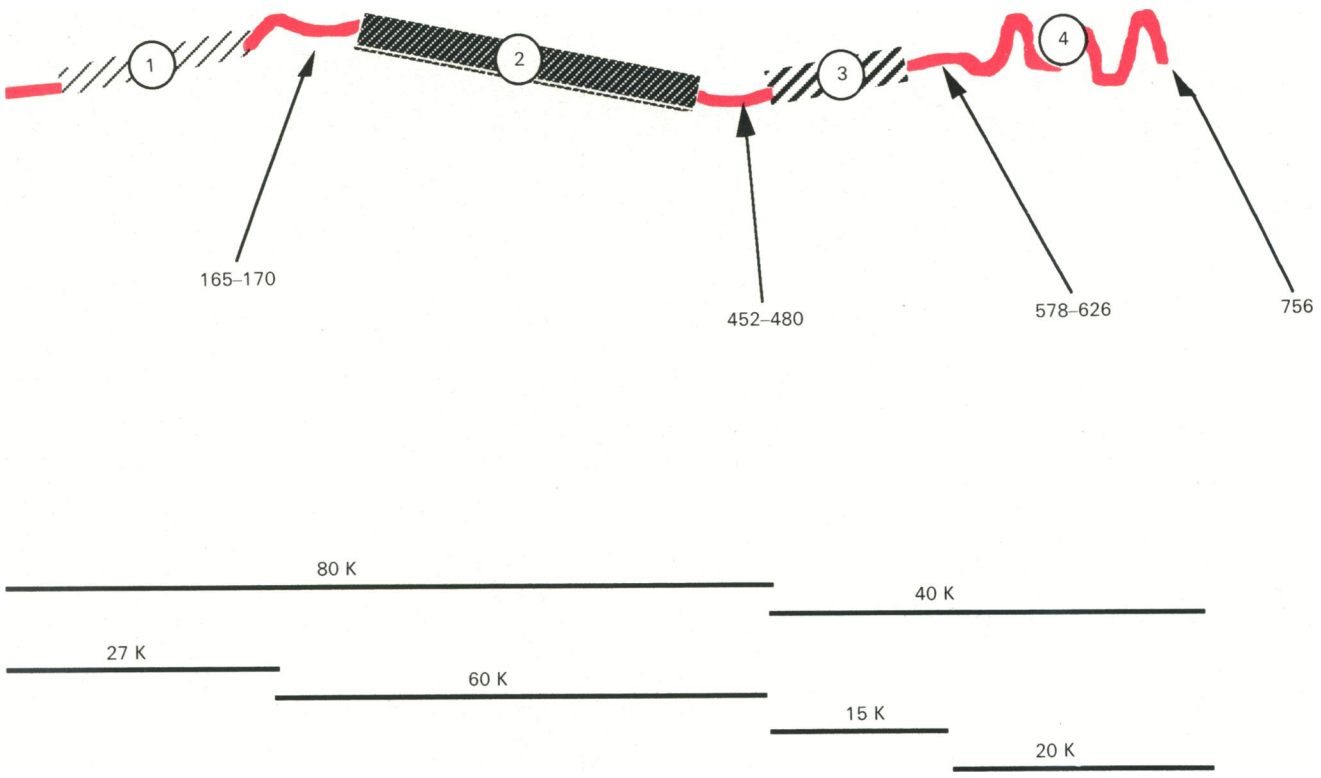


**Fig. 4. Structural domains of CD*h***

The four domains defined by structure analysis (Fig. 3), proteolytic digestion and conserved sequences are drawn to scale. The points where α-chymotrypsin cleaves gizzard CD*h* are indicated on the model. Below, the chymotryptic peptides and their origins are depicted. The names of these peptides derive from their apparent molecular masses on SDS gels. We use these names to describe the peptides defined here throughout the text; however, a variety of different apparent molecular masses may be found in the literature. For instance the '40 K' peptide is also known as a 35 K peptide [36,37].

be ascribed to mixed populations of filament types or caldesmon dissociation [85,86], but caldesmon contents in excess of one to 14 actins have never been observed. Given the ratio of one caldesmon to 14 actins, an 80 nm long caldesmon molecule can cover the entire length of an actin filament without gaps or significant overlap. There are two tropomyosins to every caldesmon and each caldesmon is the length of two tropomyosins. The final assumptions are that caldesmon is bound in register with tropomyosin (considered above) and that the caldesmon molecules in the two grooves are staggered by the length of one tropomyosin with respect to one another. Fig. 5 shows the proposed structure.

Whatever the exact details of the structure it is evident that caldesmon is in potential contact with actin and tropomyosin throughout its length and that caldesmon molecules might also form end-to-end contacts. In addition caldesmon has binding contacts with calcium-binding proteins such as calmodulin and with myosin. The next sections consider the molecular structure of these contacts and their functions.

## INTERACTION WITH ACTIN AND TROPOMYOSIN

Caldesmon's binding interaction with actin filaments is its most characteristic function [1,67,87]. Careful and quantitative binding measurements have been made by Smith *et al.* [5,14,88] with sheep aorta CD*h* and by Velaz *et al.* with chicken gizzard CD*h* [89]. Fig. 6 shows Scatchard plots of binding from this work. At room temperature and ionic strength 0.1 M the binding constant is around $5 \times 10^6$ $M^{-1}$ caldesmon. The affinity is essentially the same with skeletal muscle or smooth muscle actin but the additional presence of tropomyosin bound to the actin filaments increases the affinity by 2–3-fold [5,89]. Affinity also depends on temperature ($Q_{10} = 1.35$; $\Delta H = -30\ J \cdot K^{-1} \cdot mol^{-1}$ [88]) and salt concentration (3.5-fold decrease in affinity when ionic strength was raised from 0.1 to 0.16 M [5]).

The stoichiometry of the binding makes it clear that one caldesmon binds to several actins. Velaz *et al.* [89] analysed binding in terms of a linear array of potential binding sites [90]. An interaction factor, $\omega$, defines the extent to which there is a 'parking problem' or co-operativity. $\omega$ was a small positive number, not altered when tropomyosin was present, which indicates that there was no interference between caldesmons and slight positive co-operativity of binding to the actin filament [89]. The stoichiometry obtained with this analysis, adjusted for a molecular mass of 87.5 kDa, was a mean 7.8 actins per caldesmon and was independent of the presence of tropomyosin (Fig. 6*a*). Smith *et al.* [5] analysed the data on the basis of simple binding, which given the low value of $\omega$ is a reasonable approximation. However they observed further low-affinity binding at high caldesmon concentrations and therefore fitted the data to two sets of binding sites (Fig. 6*b*). The tighter sites had a stoichiometry of one caldesmon per $18.9 \pm 5.3$ ($n = 28$) actins (adjusted to 87.5 kDa) independent of tropomyosin. The non-muscle isoform, CD*l*, has been reported to bind with a similar affinity and at a stoichiometry of one per 9 actins (adjusted to the correct molecular mass) [12].

A number of less systematic estimations of stoichiometry have been made, mostly at high actin concentrations. Stoichiometries in the range one caldesmon per three to eight actins were obtained, but these may represent binding to both high ($K > 10^6$ $M^{-1}$) and low-affinity ($K < 10^5$ $M^{-1}$) sites [5,34,36,45,87,91,92].

The stoichiometry of caldesmon incorporated into carefully

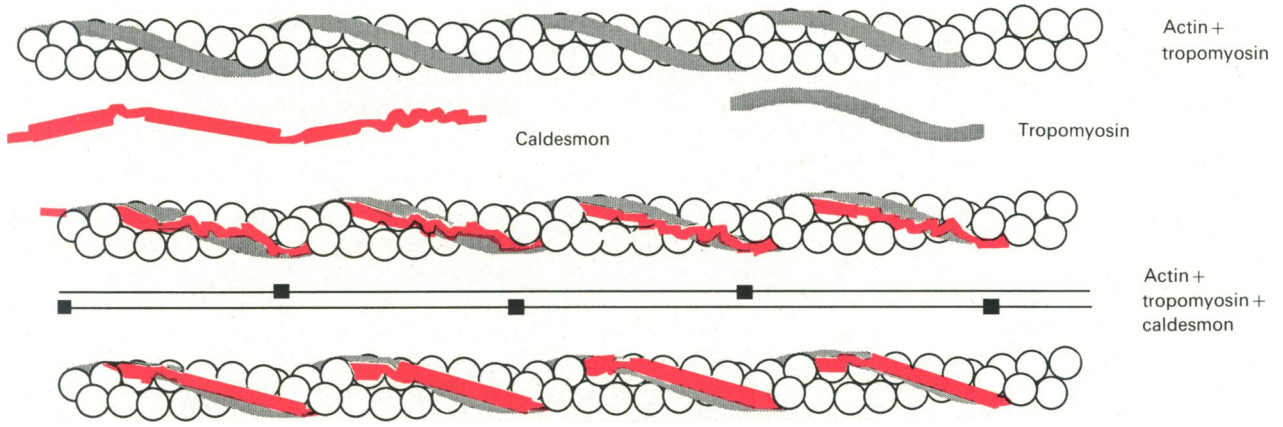


**Fig. 5. A model for the incorporation of an extended caldesmon molecule into the thin filaments of smooth muscle**

**Top**. The structure of F-actin complexed with tropomyosin is shown with actin monomers represented as circles. Actin is formed into a double helix with a pitch of 74 nm. Tropomyosin is represented by a grey rod; each tropomyosin molecule is 40 nm long and is located within the grooves of the actin filaments. Two tropomyosin molecules span one complete turn of the actin helix. **Middle**. Individual tropomyosin and caldesmon molecules are shown. The caldesmon structure is the same as that shown in Fig. 4 derived from structural and domain analysis. **Bottom**. The caldesmon molecule incorporated into the thin filament. In order to produce a 38 nm repeat of structure each caldesmon is placed in register with a tropomyosin and extends for 76 nm, the length of two tropomyosins. The caldesmon molecules in the two grooves of the actin helix are staggered by the length of one tropomyosin relative to each other as is shown schematically by the pair of lines with blocks. A consequence of this arrangement, besides producing the 38 nm repeat, is that the filament has no radial symmetry and consequently any particular part of caldesmon is on the same side of the filament. We have therefore shown the filament in two views which are rotated by 180 degrees. One shows the domains 3 and 4 of one caldesmon and the beginning of domain 1 of the next; the other shows the rest of domain 1 and the central helix domain 2. The features of this model are essentially the same as those previously published by Marston *et al.* [82] (Model A), Lehman *et al.* [75,167] and Watson *et al.* [93].

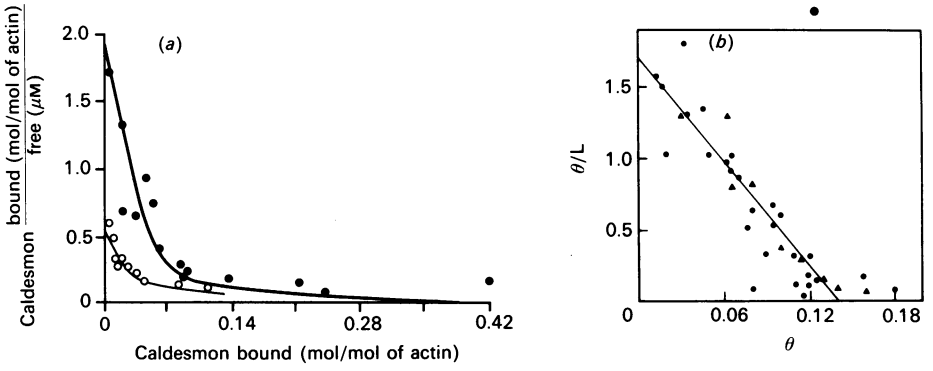


**Fig. 6. Caldesmon binding to actin**

Data are presented as Scatchard plots of bound caldesmon, $\theta$, expressed in mol/mole of actin, against bound caldesmon/free caldesmon ($\mu$M). (*a*) Sheep aorta caldesmon and actin in the presence (●) and absence (○) of sheep aorta tropomyosin, 25 °C, $I = 0.09$ M. Data are fitted to two classes of binding sites; the tight binding site $K_b$ was $4 \times 10^7$ M$^{-1}$ for actin–tropomyosin and $1 \times 10^7$ M$^{-1}$ for actin, stoichiometry one caldesmon per 19 actins. From Smith *et al.* [5]. (*b*) Binding of chicken gizzard caldesmon to skeletal (●) or chicken gizzard (▲) actin in the presence of smooth muscle tropomyosin, 25 °C, $I = 0.09$ M. Data are analysed according to McGee & von Hippel [90]. $K_1 = 1.7 \times 10^6$ M$^{-1}$, $\omega = 5$, $n = 7$ caldesmon per actin. From Velaz *et al.* [89].

isolated native thin filaments is optimally one per 16 to 17 actins [75,78] whilst the best model of filament structure predicts one caldesmon per 14 actins (Fig. 5) [75,93]. These values are in reasonable agreement with the binding studies; moreover, the apparent affinity of caldesmon for actin in native thin filaments is the same as purified caldesmon's affinity for synthetic actin–tropomyosin filaments [78] indicating that isolated caldesmon probably has the same binding properties as it has *in vivo*.

### What parts of caldesmon bind to actin?

Caldesmon has no significant sequence similarities to any currently known actin-binding protein. Since both CD*h* and CD*l* bind actin with high affinity [12], the 'central helix' domain 2 (190–480) cannot be involved. After cleavage around residues 450–480 with chymotrypsin or thrombin the *C*-terminal '40 K' fragment can bind to actin but the *N*-terminal fragment cannot (Fig. 4) [33,34,36,45]. Yazawa *et al.* reported that the binding affinity of the '40 K' fragment was almost as tight as that of intact caldesmon although it would appear that the stoichiometry had approximately halved [36].

Smaller fragments of the '40 K' still retain actin-binding ability. Szpacenko & Dabrowska were the first to show that the *C*-terminal '20 K' chymotryptic fragment (590–756, Fig. 4) bound to actin–tropomyosin and inhibited its activity as potently as did whole caldesmon [33]. The importance of the *C*-terminal region was independently confirmed by carboxypeptidase Y digestion, which abolished actin binding [94]. Leszyk *et al.* [65] found weak actin binding in the other half of the '40 K' fragment, i.e. the '15 K' fragment (amino acids 483–578) containing most of the third domain. On the other hand an expressed mutant of gizzard CD*h* containing amino acids 1–578 (i.e. including the first three domains) did not bind to actin in quantitative assays.

These set a limit to the affinity of binding in the third domain of less than $4 \times 10^3$ M$^{-1}$ [95]. The most important actin-binding region therefore is in the fourth, *C*-terminal, domain.

Recently an even smaller actin-binding fragment has been studied. This is a '10 K' peptide obtained by CNBr cleavage at the last methionine of turkey gizzard caldesmon (Fig. 1) containing the sequence from 659 to 756 [96]. Its affinity for actin was $0.7 \times 10^6$ M$^{-1}$ and for actin-tropomyosin $2 \times 10^6$ M$^{-1}$. This is within a factor of 5 of the affinities measured for whole caldesmon under similar circumstances. Perhaps even more remarkable is that despite this being only one-seventh of the caldesmon molecule its binding stoichiometry was 7 actins per molecule of peptide. Additional evidence for the role of this region comes from work with a monoclonal antibody specific for a region near the *N*-terminus of this '10 K' peptide which is able to displace caldesmon from actin [97].

Even closer definition of the actin-binding region could be obtained using genetically engineered caldesmon [74]. This work is very recent and not wholly consistent with previous work. Wang *et al.* [54,98] have detected actin binding in all their modified peptides of the *C*-terminal fourth domain except 629–710 and 666–756. Since the latter contains all but seven amino acids of the '10 K' fragment this is puzzling. The lack of binding of 629–710 might imply that the actin binding is restricted to 710–756; however, mammalian caldesmons are only homologous with gizzard as far as 727 (Fig. 1) which would indicate a conserved actin-binding site of only 17 amino acids (710–727). Sobue [57] has observed that a number of fragments starting at 451 but excluding the '10 K' peptide can bind to actin, albeit perhaps weakly (e.g. 451–620, 451–604, 451–563) whilst all the peptides which included the '10 K' bound well to actin. Without quantitative measurements [95] it is not possible to draw firm conclusions; however, there is a suggestion of an additional weak actin-binding site in the third domain as was proposed by Leszyk *et al.* [65].

### What part of actin is in contact with caldesmon?

Although caldesmon is not homologous with any actin-binding proteins it does bind to the same region near the *N*-terminus as many other proteins. Actin structure has been determined at atomic resolution [99,100] which permits the topography of contact to be closely defined. Actin is made up of four domains, two of which are on the outside of the filament and two of which are on the inside forming the backbone of the filament. The *N*-terminus and *C*-terminus are close together in one of the outer domains (domain I; see Fig. 7). When the actin–caldesmon complex was treated with zero-length crosslinking agents a complex was obtained made up of one caldesmon to 17.5 actins (molecular mass adjusted) in which one of the actins was covalently linked to caldesmon. The link was between domain 4 (*C*-terminus) of caldesmon and the extreme *N*-terminus of actin (1–12) [101] (see Figs. 4 and 7). Antibodies against residues 1–7 of actin strongly inhibited caldesmon binding, whilst antibodies against 18–24 showed only limited competition [102]. These sequences of actin have also been shown to interact with troponin I, myosin subfragment-1, cofilin, fragmin and $\alpha$-actinin [101].

$^1$H-n.m.r. measurements have defined this contact region even more precisely [28]. Caldesmon binding perturbed His-40, Phe-21 or -31, Thr-5 or -6 and the *N*-terminal acetyl group of a peptide containing the first 44 amino acids of skeletal muscle

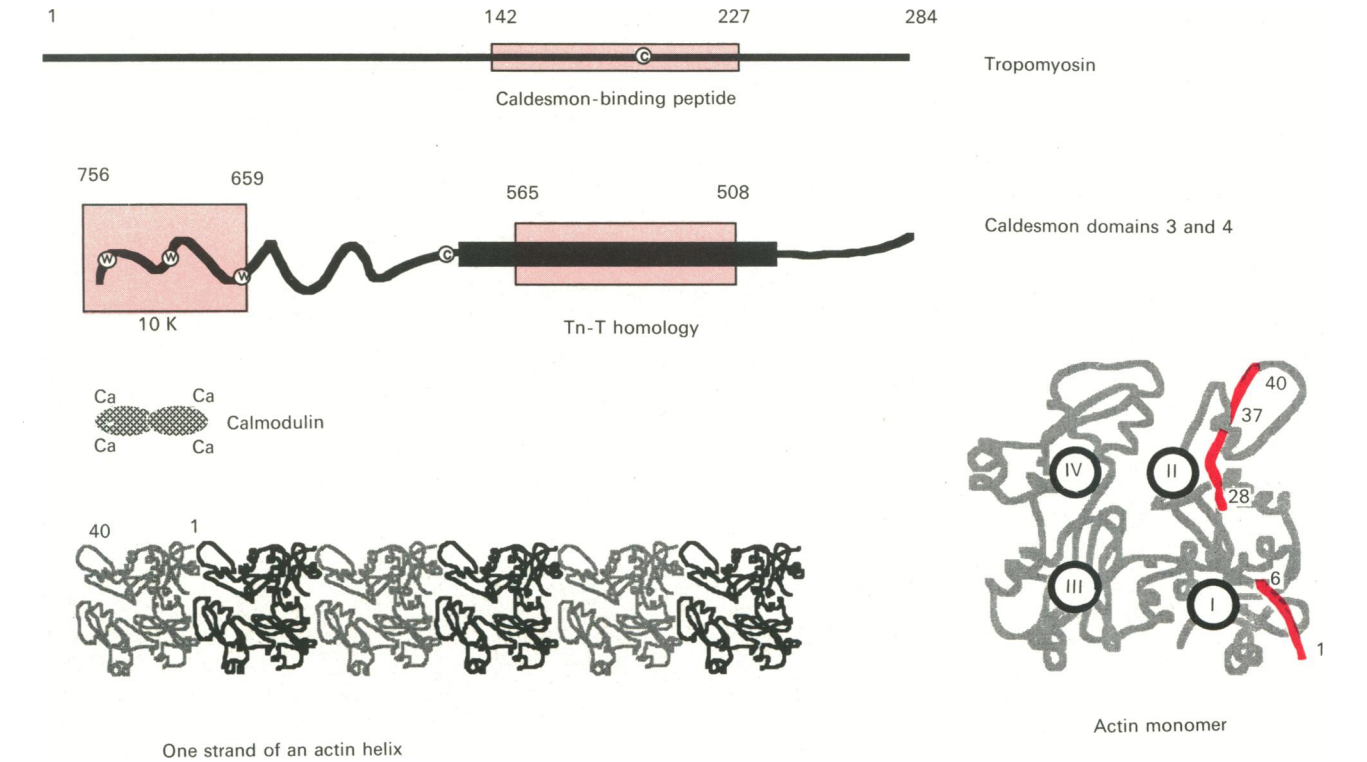


**Fig. 7. Location of caldesmon contact sites with actin, tropomyosin and calmodulin**

The actin structure (bottom right) is traced from Kabsch *et al.* [99]. The domains are numbered and the position of the peptide that interacts with caldesmon is highlighted in red. The strand of the actin polymer shows actin monomers attached to each other in the conformation proposed by Holmes *et al.* [100]. Above, tropomyosin, caldesmon and calmodulin molecules are drawn to the same scale. The caldesmon-binding peptide of tropomyosin and the '10 K' fragment and troponin T homology region of caldesmon are indicated. The location of tryptophans (W) and cysteines (C) are shown.

actin. In the three-dimensional structure of actin (Fig. 7) these residues stretch over 4 nm from the top to the bottom of the exposed small domain, suggesting they form an extended contact site or multiple sites. The '15 K' fragment (483–578) specifically perturbs just His-40, Phe-21/31, Gly-35 and Arg-38/37/39, indicating that it contacts actin in the region 28–40 and that the other contacts observed with whole caldesmon in the region 1–7 are a separate site, presumably in the *C*-terminal fourth domain of caldesmon. It should be noted that these experiments use extremely high protein concentrations [up to 200 $\mu$M-caldesmon, well in excess of physiological (10 $\mu$M)] so that it is possible for weak binding, like that of the '15 K', to be measured. It therefore has to be borne in mind that even specific contacts measured by n.m.r. may not be significant *in vivo*. The possibility of contact sites with the nearby *C*-terminus and other segments of the actin has not been investigated although work with labels attached to actin Cys-374 (the penultimate amino acid) suggests it could be involved [103].

The $^1$H-n.m.r. data is of some use in analysing the mechanism of caldesmon binding and function. Caldesmon binding is similar but not identical to troponin I binding, which contacts 1–7 and 23–26, and the caldesmon binding directly involves sites which are also recognized by myosin subfragment-1 rigor bonds. Caldesmon has been shown to promote polymerization of actin under conditions where it is normally monomeric [78,104–106]. This indicates that it probably binds preferentially to sites that are only present in polymeric actin. Given the extended structure of caldesmon it is not clear whether the two contact sites are on the same actin, on adjacent actins or even on actins separated by a few monomers (Figs. 5 and 7); nor is it clear how the stoichiometry of one caldesmon per 7–14 actins can be maintained when the binding is between a limited site on caldesmon and a closely defined site on a single actin.

**Caldesmon–tropomyosin interaction**

Measurements of smooth muscle tropomyosin binding to individual caldesmon molecules have been made using ligand-sensitive fluorescent labels attached to cysteine on caldesmon or tropomyosin (Cys-190) [95,107,108]. In low-ionic-strength buffers the binding constant is around $10^6$ M$^{-1}$ but affinity diminishes rapidly with increasing [KCl], reaching $10^5$ M$^{-1}$ at physiological salt concentrations, indicating largely ionic bonding. The interaction has also been demonstrated less directly by determining the effect of caldesmon on tropomyosin viscosity [109] and by binding to a caldesmon–Sepharose affinity column [5]. The stoichiometry appears to be 1:1.

The similarity of the segment of caldesmon in the third domain (508–565) to the tropomyosin binding peptide CB2 of troponin T (Fig. 1) pinpoints this as the most likely site for the interaction. This is supported by some experimental data. Firstly an expressed caldesmon fragment 1–578 (domains 1, 2 and 3) was found to bind tropomyosin with an affinity of the same order as whole caldesmon [95]. Secondly, a fraction of anti-caldesmon antibodies specific for the troponin T homology region was shown to displace tropomyosin from its binding site on caldesmon [171]. This may not be the only site, since some tropomyosin binding was reported in expressed mutants containing only sequences from the *C*-terminal fourth domain [57] and Katayama *et al.* [110] suggest that none of the domains in isolation can bind tropomyosin.

Using a series of tropomyosin fragments derived from proteolysis or site-directed mutagenesis, Watson *et al.* [93] have located a single binding region at 142–227 on tropomyosin (total 284 amino acids) with 220–227 being especially important (Fig. 7). The sequence includes Cys-190 to which the ligand-sensitive fluorescent labels were attached for caldesmon binding measurements [95,107] and 121–173 which was predicted to bind to troponin T [71]. Tropomyosin is, of course, a coiled-coil molecule; the two $\alpha$-helices are in parallel register so that one binding site would be contributed by two tropomyosin peptides [111,112].

The binding equilibria of caldesmon to actin and tropomyosin are coupled such that tropomyosin increases caldesmon affinity for actin (Fig. 6; [5,89]), actin increases tropomyosin affinity for caldesmon 5-fold [107] and caldesmon increases tropomyosin affinity for actin (this has been demonstrated with CD*l* and non-muscle tropomyosins which have a low affinity for actin [12]).

In the native smooth muscle thin filament tropomyosin is located in the grooves of the actin helix and it binds both to actin and to the next tropomyosin via an overlap region (reviewed in [67]). Tropomyosin is tightly bound and rarely dissociates until the filament concentrations are so low that the actin itself begins to depolymerize [78]. It is likely that caldesmon binds alongside tropomyosin in register (Figs. 5 and 7) [75]. This structure fits well with the observation of a specific binding site between caldesmon 508–565 and tropomyosin 142–227. The tropomyosin binding site sequence is significantly longer than the complementary gizzard caldesmon sequence, but in mammalian caldesmons there is an additional adjacent stretch with similarity to troponin T (Fig. 1). Since there are two tropomyosins to every caldesmon, alternate tropomyosins will not be able to bind to caldesmon 508–565; the binding site would instead be adjacent to the *N*-terminal caldesmon domain (Figs. 5 and 7; [93]). There is no direct experimental evidence for an interaction but sequence analysis could suggest possible sites. We have noted weak similarity to residues 70–150 of troponin T throughout the domain 2 helix and around residues 40–50 in domain 1. Certainly the *N*-terminus of caldesmon does appear to be attached to the thin filament backbone since caldesmon molecules are not seen peeling away from the filament (Figs. 2*c* and 2*d*) and the antibody to the *N*-terminus aggregates thin filaments into closely apposed bundles [25]. This may be due to binding of domain 1 to tropomyosin, to actin or to domain 4 of the adjacent caldesmon. There is some evidence for caldesmon end-to-end interaction since caldesmon molecules were often observed as 150 nm dimers in rotary shadowed micrographs [8] and a reversible dimerization (or polymerization) at physiological salt concentration has been demonstrated by gel filtration [16].

This type of interaction might account for the stoichiometry of caldesmon binding to actin since it now is clear that stoichiometry is not defined by the presence of tropomyosin (Fig. 6). There must be some reason why caldesmon does not bind to every actin as troponin I does in the absence of tropomyosin in skeletal muscle thin filaments. A simple steric explanation could be that it is blocked from doing so by the presence of domains 1, 2 and 3 of caldesmon in the groove of the actin helix; the observation of Smith *et al.* [5] that more caldesmon can bind at much higher concentrations (Fig. 6) suggests that it is possible eventually to displace the block. However this type of mechanism does not account for the binding of caldesmon fragments such as the '10 K'. This peptide could not cover more than three or four actins and yet it binds and inhibits with a stoichiometry of one per seven actins. We are therefore obliged to suggest that the formation of the tight bond between one actin and domain 4 of caldesmon can alter the structure of other actins, with which it is not in contact, via the actin so that they cannot bind caldesmon. The idea of propagation of effects along the actin filament is probably an essential feature of the mechanism by which caldesmon regulates actin activation of myosin ATPase.

**Caldesmon inhibition of actin activation**

The most important functional property of caldesmon is its inhibition of actin activation of myosin MgATPase. This prop-

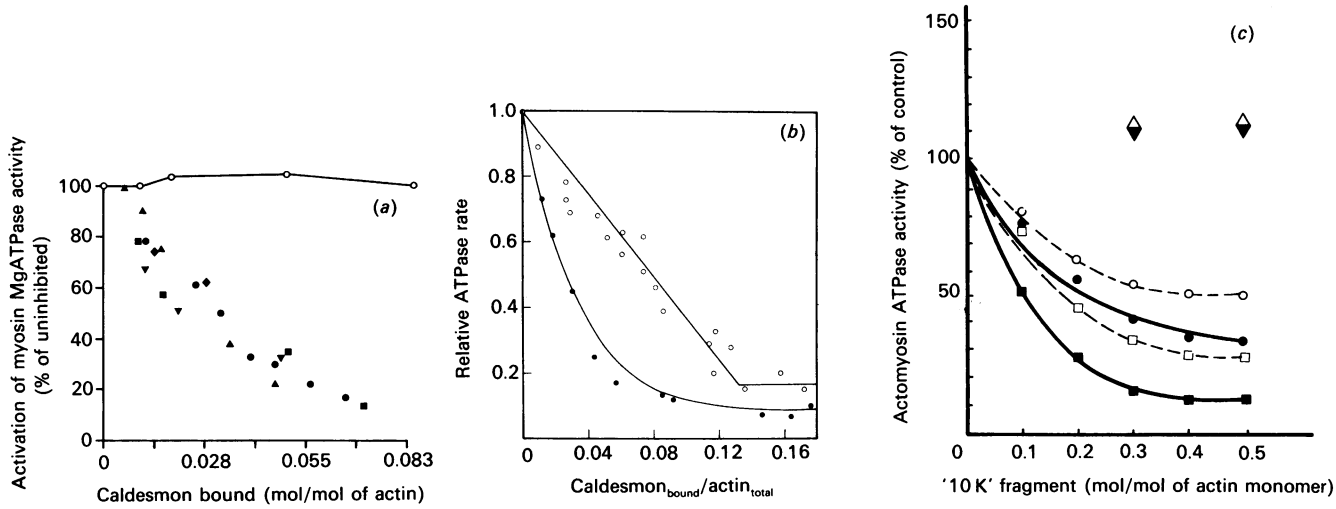


**Fig. 8. Relationship between caldesmon binding and inhibition of actin-activated myosin ATPase**

(*a*) Sheep aorta caldesmon, actin and tropomyosin, and skeletal muscle myosin. Open symbols, actin, 25 °C, 60 mM-KCl; closed symbols, actin–tropomyosin: (●) 4 °C, 50 mM-KCl; (■) 25 °C, 60 mM-KCl; (▲) 37 °C, 60 mM-KCl; (◆) 37 °C, 120 mM-KCl; (▼) skeletal muscle tropomyosin 25 °C, 60 mM-KCl. Reproduced from [5]. (*b*) Chicken gizzard caldesmon and skeletal muscle actin with 42 mM-NaCl (○) and skeletal actin-smooth muscle tropomyosin (●). Reproduced from [89]. (*c*) Chicken gizzard caldesmon and the '10 K' NTCB fragment of caldesmon. Skeletal muscle acto-S1 MgATPase was measured in 50 mM-Tris/HCl, 5 mM-ATP, 10 mM-KCl, 5 mM-$MgCl_2$, pH 7.5, in the presence (squares) or absence (circles) of smooth muscle tropomyosin. Added caldesmon (closed symbols) or '10 K' peptide (open symbols) inhibited acto-S1 ATPase. Reproduced from [96].

erty is central to the $Ca^{2+}$-dependent control of thin filament activity [82] and the effect of caldesmon on contractility [113]. Under optimal conditions caldesmon inhibits by over 90%, the inhibition is consequent upon caldesmon binding and all the actins are inhibited even though the strong binding interaction occurs with only one actin molecule. Smith *et al.* [5] found that maximal inhibition was correlated with the binding of one caldesmon to 14–15 actins. Velaz *et al.* [89] obtained a similar result (Fig. 6).

Optimal caldesmon inhibition is not dependent on temperature or ionic strength (Fig. 8; [5]) but it is strongly dependent on the proteins used in the assay. The additional presence of tropomyosin always increases the potency of inhibition [5,87,88,91,92,114]. Fig. 8(*a*) indicates an extreme position where actin alone is virtually uninhibited by caldesmon even though it is bound, whilst Fig. 8(*b*) illustrates a case where caldesmon strongly inhibits actin activation in the absence of tropomyosin. The important factor determining whether caldesmon can inhibit pure actin filaments is the state of the myosin. The experiment in Fig. 8(*a*) was done with filamentous myosin (whether from skeletal or smooth muscle is immaterial [67]) whilst the experiment in Fig. 8(*b*) was done with heavy meromyosin (HMM). When tested with any *soluble* myosin fragment (S-1 or HMM from smooth or striated muscle) caldesmon is a potent inhibitor of actin activation [74,115].

The data are not readily interpreted in terms of structure but it is useful to note that the experiments do indicate the existence of two states of actin–caldesmon complex; one in which activation is > 90% inhibited and one in which inhibition is small or absent. Inhibition will need to be propagated from the one actin which binds caldesmon (Fig. 8) to the other 7–14 actins which can be simultaneously inhibited but which do not bind strongly to caldesmon. It would appear that the propagation of inhibition is helped by tropomyosin [116] and hindered by the formation of myosin into filaments, but that neither are essential elements in the inhibitory mechanism.

The recent remarkable result obtained from the gizzard caldesmon '10 K' fragment (residues 659–756) sets limits on the possible mechanisms (Fig. 8*c* [96]). This fragment contains the principal actin–caldesmon binding site but it is not big enough to be in contact with more than three or four actin molecules at the most (Fig. 7). Nevertheless, it is a potent inhibitor of acto-HMM ATPase whether or not tropomyosin is present. When its 5-fold weaker binding affinity compared with whole caldesmon is taken into account [96] it seems likely that this fragment is as potent as whole caldesmon and can inhibit almost as many actins. This result would suggest that the propagation of caldesmon inhibition along the actin filament does not even require domains 1, 2 and 3. Other work using domains 2–3–4 [38], 3–4 and 4 [33] and 1–2–3 [95] likewise indicate that domains 1–3 do not play a vital role in inhibition.

The main switch thus resides in the contact between residues 659–756 and one actin molecule. Formation of this contact switches off not only the actin involved but also a number of others (up to 14). It was apparent from the topology of the contact that the caldesmon site on actin overlapped an S-1 binding site (Fig. 7); competition for the S-1 binding site would partly explain how caldesmon switches off actin activation of S-1 ATPase. There is little doubt that in the absence of ATP S-1 can displace caldesmon both from synthetic and native thin filaments [115,117–119] and interestingly the stoichiometry is one S-1 per actin for complete removal of caldesmon, indicating that S-1 is only competing for the one actin binding the *C*-terminal domain. Conversely caldesmon can displace S-1·p[NH]ppA and S-1·$PP_i$ from actin (115,118).

This aspect of caldesmon inhibition has been characterized by Velaz *et al.* using a modified caldesmon (domains 2–3–4, which lacks myosin binding ability which might interfere with the results) [38]. Large quantities of caldesmon displaced HMM·ADP·$P_i$ from actin during ATP hydrolysis. This was accompanied by inhibition of ATPase activity; however, it was a rather unphysiological situation and it was not clear whether low levels of caldesmon could inhibit without displacement of HMM.

We have investigated inhibition under conditions that resemble *in vivo* ratios of proteins with a whole caldesmon preparation

which was incapable of binding directly to HMM [120]. At a fixed ratio of one caldesmon added per eight actin molecules up to 95% inhibition of actin activation was obtained without any detectable change in the amount of HMM·ADP·$P_i$ bound to the actin. The conclusion was that caldesmon worked by reducing the $k_{cat.}$ of the acto-HMM ATPase.

These two sets of results are not contradictory since they represent different experimental designs. Indeed, they point the way to a detailed mechanism of inhibition. We propose that the main switch of caldesmon is a competitive reaction between residues 659–756 of caldesmon and S-1·ADP·$P_i$ for a common or overlapping site on actin. When caldesmon binds, displacing the S-1·ADP·$P_i$, it propagates an inhibitory signal along the actin filament, presumably via some conformational change. As a result the $k_{cat.}$ of the other actins controlled by the caldesmon is reduced by about 20-fold. The propagated inhibition would be the predominant effect when actin is well in excess of caldesmon and myosin as it is in the native thin filament [67,120]. The recent report from Horiuchi *et al.* [121] in which caldesmon '40 K' fragment was found to decrease both affinity and $k_{cat.}$ seems to represent conditions intermediate between those of Velaz and Marston.

## CALDESMON INTERACTION WITH CALMODULIN AND OTHER $Ca^{2+}$-BINDING PROTEINS

Caldesmon was first isolated on the basis of its ability to bind to a calmodulin affinity column [1] and was named caldesmon to indicate a calmodulin-binding protein; however, caldesmon does in fact bind to a variety of $Ca^{2+}$-binding proteins. Besides calmodulin it binds to troponin C [122,123], brain S-100 [123–125] and in native thin filaments it is associated with a functionally distinct $Ca^{2+}$-sensitizing protein [122,126]. In all these cases the binding of the $Ca^{2+}$-protein complex to caldesmon reverses caldesmon inhibition, although the mechanism is variable.

Calmodulin binding has been studied in some detail by a number of techniques including direct assays [127], changes in fluorescence [5,128] and fluorescence polarization [129] of covalently attached fluorophores as well as enhancement of caldesmon's intrinsic tryptophan fluorescence [18,21]. All the reports have produced similar results; $Ca^{2+}$–calmodulin has an affinity for caldesmon of $(2–4) \times 10^6$ M$^{-1}$ at $I = 0.1$ M and 25 °C and a stoichiometry of 1 $Ca^{2+}$–calmodulin per caldesmon. The affinity is virtually insensitive to temperature ($K = 2.2 \times 10^6$ at 48 °C [21]) but is reduced by high salt concentrations [21,129], suggesting that hydrophobic, electrostatic and hydrogen bonding all contribute to the stability of the complex. Calmodulin does not bind at all in the absence of $Ca^{2+}$.

The affinity of $Ca^{2+}$–calmodulin for caldesmon is at least two orders of magnitude less than typical calmodulin-regulated enzymes like myosin light chain kinase or phosphodiesterase [72,128]. It is therefore not surprising that there are no sequences in caldesmon homologous with known calmodulin binding sites. DeGrado has proposed that a positively charged amphiphilic $\alpha$-helix is a common structural feature of calmodulin-binding sites [72]. There is a weakly amphiphilic $\alpha$-helical sequence in the *C*-terminal domain of caldesmon from 596–643 [37]. However, the presence of proline residues at 608, 637 and 640 would destabilize an $\alpha$-helix and modification of His-610 has no effect on calmodulin binding [169]. Tagaki has suggested that there is homology between caldesmon 449–547 and 718–731 and the weak ($K = 2 \times 10^6$ M$^{-1}$) $Ca^{2+}$-independent calmodulin binding site of neuromodulin [73,130]. These weak homologies are of negligible predictive value.

The main $Ca^{2+}$–calmodulin binding region of caldesmon is colocalized with actin binding within the last 100 amino acids at the *C*-terminus since the '40 K' and '20 K' proteolytic fragments [33–36], the NTCB fragment 580–756 [34] and the '10 K' CNBr fragment 659–757 [73,96] all bind to calmodulin affinity columns and $Ca^{2+}$-calmodulin reverses actin inhibition by the fragment.

Calmodulin binding to the '10 K' fragment has been further refined by proteolytic digestion [131] with $\alpha$-chymotrypsin. Under optimal conditions the '10 K' fragment was split at Phe-665 and the three tryptophans, 659, 692 and 722. Binding of $Ca^{2+}$-calmodulin abolished the proteolytic susceptibility whilst actin and tropomyosin did not. The cleaved caldesmon could still bind to actin but did not bind to $Ca^{2+}$–calmodulin. It was proposed that actin and calmodulin sites lie on distinct portions of the '10 K' fragment and the integrity of at least one tryptophanyl peptide is required for calmodulin binding. The effect of $Ca^{2+}$–calmodulin on the fluorescence of tryptophan residues indicates that they are normally exposed in a polar environment which becomes less polar when $Ca^{2+}$–calmodulin binds [21]. A monoclonal antibody C21 [97] has been shown to displace both actin and calmodulin from caldesmon, indicating close or overlapping sites. The epitope of this is believed to be at the *N*-terminal end of the '10 K' fragment (around residue 660). Using this and additional evidence from mutations, Wang *et al.* [132] have made a case for the location of the binding site at 659–666. It is possible to crosslink calmodulin to both cysteines of gizzard caldesmon (residues 153 and 580) [133] but neither of these sites corresponds to any of the functional sites considered above.

### Reversal of caldesmon inhibition by $Ca^{2+}$–calmodulin

When $Ca^{2+}$–calmodulin binds to caldesmon attached to actin or actin-tropomyosin it reverses the inhibition of activation of myosin MgATPase. Since calmodulin does not bind to caldesmon in the absence of $Ca^{2+}$ this mechanism is a $Ca^{2+}$-dependent switch of the activity of the thin filament [6,134]. Early work on caldesmon led to a simple mechanism in which $Ca^{2+}$–calmodulin competed with actin for caldesmon binding and therefore reversed inhibition by displacing caldesmon from actin. This was termed a flip–flop mechanism [6,135]. However, it soon became apparent that in many cases, including experiments done under near-physiological conditions, $Ca^{2+}$–calmodulin did not displace caldesmon from actin although it did reverse inhibition (reviewed in [67]). We therefore need to consider two distinct modes of $Ca^{2+}$–calmodulin action.

We have investigated how the mode of regulation depends upon conditions. At room temperature and in KCl concentrations of 60 mM or less the 'flip–flop' mechanism operates with smooth muscle actin and tropomyosin and is not dependent on the myosin type [5,129]. Direct binding measurements confirm that $Ca^{2+}$–calmodulin does not bind to caldesmon–actin complex [129] and it is generally observed that under such conditions a high concentration of $Ca^{2+}$–calmodulin is required to reverse inhibition [67]. This is because caldesmon has a lower affinity for $Ca^{2+}$–calmodulin than for actin from which it must be displaced to reverse inhibition [5,129]. The flip–flop mechanism is readily explained by the structural data discussed above which suggested that $Ca^{2+}$–calmodulin and actin binding sites on caldesmon between residues 656 and 756 are close or overlapping (Fig. 7) and thus binding may be mutually exclusive.

'Flip–Flop' seems to be an extreme case and in general it has been found that $Ca^{2+}$–calmodulin can reverse inhibition by binding whilst caldesmon remains bound to actin [5,82,114,129,134]. In fact, there is a continuum of conditions in which $Ca^{2+}$–calmodulin binding to caldesmon weakens actin binding to caldesmon to a greater or lesser extent and it is not difficult to reach a condition where caldesmon affinity for actin is quite unaffected by $Ca^{2+}$–calmodulin binding [5,129]. Under

these conditions, of course, $Ca^{2+}$–calmodulin binding would be quite unaffected by actin binding to caldesmon and this has been demonstrated [129]. A consequence of the absence of a competition is that caldesmon inhibition may be switched off with relatively low $Ca^{2+}$–calmodulin concentrations. It is likely that this mode of $Ca^{2+}$ regulation occurs in native thin filaments, since these contain low levels of calcium-binding proteins and it is known that increasing $Ca^{2+}$ can activate the filaments without displacing the caldesmon [78,136].

Clearly the site where $Ca^{2+}$–calmodulin binds is altered in some way, even though the affinity of caldesmon for $Ca^{2+}$–calmodulin is not [129]. Non-competitive behaviour is favoured by increasing temperature and ionic strength [5,129], conditions which would favour hydrophobic at the expense of ionic and hydrogen bonding. It is also much more pronounced when skeletal actin is used rather than smooth muscle actin [5,129]. It is of interest to note that exactly the same changes in mechanism of reversal of caldesmon inhibition have been observed using another calcium binding protein of different structure, brain S-100 [124].

If $Ca^{2+}$–calmodulin does not displace caldesmon from actin, how can it control caldesmon inhibition? We have already considered how actin inhibition by caldesmon probably involves two processes: competition with S-1·ADP·$P_i$ for a site on one actin and propagation of an inhibitory effect to a number of other actins. If $Ca^{2+}$–calmodulin blocked propagation it would account for the non-competitive mode. For such a mechanism to work we need to propose that there are two $Ca^{2+}$–calmodulin contact sites (or two conformations of one contact site) on caldesmon, one of which is in competition with the actin-binding site and another which is not in competition and which blocks propagation of caldesmon's inhibitory signal. $Ca^{2+}$–calmodulin would bind to one site or the other depending on conditions.

In native smooth muscle thin filaments the regulation is by the non-competitive mode under all conditions of ionic strength and temperature and the $Ca^{2+}$-sensitizing factor has one additional property, namely that its binding to the thin filament is $Ca^{2+}$-independent [67,82,122,137]. Since calmodulin does not bind in the absence of $Ca^{2+}$ it is probable that native thin filaments have a different $Ca^{2+}$-binding protein or additional linking proteins. Crude extracts containing proteins with the expected properties have been obtained and a number of candidate proteins have been excluded [122,124].

## CALDESMON BINDING TO MYOSIN

Caldesmon is unique in being able to bind tightly to both actin and smooth muscle myosin at the same time. This property sets caldesmon apart from any other actin-binding protein, and there is every reason to believe myosin binding is physiologically significant since it can be observed not only with pure caldesmon but also in native thin filaments containing caldesmon.

The binding of caldesmon to myosin filaments has been measured under a variety of conditions. In direct assays skeletal myosin did not bind but both phosphorylated and unphosphorylated smooth muscle myosin did bind with affinities of at least $10^6$ $M^{-1}$ in 10 mM-KCl buffer [119,138,139]. The affinity was highly dependent on salt concentration, decreasing to around $10^4$ $M^{-1}$ in 75 mM-NaCl and $2 \times 10^3$ $M^{-1}$ in 100 mM-NaCl [138]. It has been reported that $Ca^{2+}$–calmodulin reduces the affinity [4,140]. The stoichiometry of binding appears to depend somewhat on conditions, and published values range from one to three caldesmon per myosin [119,138,139]. Both CD*h* and CD*l* isoforms can bind to myosin with equal affinity [119].

The main contact site on caldesmon is in domain 1, since the *N*-terminal NTCB fragment (residues 1–150) and the *N*-terminal '27 K' chymotryptic fragment (residues 1–165/170; Fig. 4) are retained on smooth muscle myosin affinity columns whilst the *C*-terminal fragments are not [38,141]. In direct assays the gizzard caldesmon '27 K' fragment [138] and the sequence 1–128 expressed from gizzard cDNA [74] bound myosin with an affinity similar to that of whole caldesmon. Comparisons of the caldesmon sequence with other myosin-binding sequences such as the putative consensus sequence present in titin and myosin light chain kinase has not revealed any similarity [142]. There is evidence that cysteine is involved in the myosin binding site in mammals since titration of sheep aorta caldesmon with iodoacetamide or *N*-ethylmaleimide resulted in complete inhibition of myosin binding when one cysteine per caldesmon was blocked [119]; however, in chicken gizzard, cysteine modification does not alter myosin binding [138].

The binding site for caldesmon on smooth muscle myosin was identified as being within the S-2 portion of the myosin rod by Ikebe [4]. Subsequent work has confirmed that myosin fragments containing the S-2 portion, such as rod, single-headed myosin and HMM, bind to caldesmon as tightly as whole myosin, whilst light meromyosin (LMM) does not [92,117,119,138,143]. It has been reported that S-1 from smooth or skeletal muscle and skeletal muscle HMM can bind very weakly to caldesmon [138] but so far there are no reports of isolated S-2 binding to caldesmon.

Caldesmon binding is specific to smooth muscle myosin (non-muscle myosins have not been tested); both skeletal and smooth muscle myosins have been sequenced but comparison has not yielded any insight. The S-2 portion stretches from residues 849 to 1279 and shows only 38% homology between the isoforms [144]. Under physiological conditions smooth muscle myosin and rod form characteristic 'side polar' filaments which are quite distinct from the bipolar structure of skeletal muscle [145,146]. Filament structure depends on the LMM portion of myosin (residues 1279–1979); the S-2 portion of the rod is not part of the filament shaft but lies on its surface with a certain degree of flexibility. Filaments made from myosin rods have the same backbone structure as myosin filaments and the binding of caldesmon does not alter its structure; however, one may see caldesmon coating the S-2 portions and causing them to splay out from the filament shaft [119,139].

Myosin also binds to caldesmon when it is bound to actin [92] or incorporated into thin filaments [143]. In fact, the myosin–caldesmon interaction was first detected as a tight binding between smooth muscle HMM and actin–caldesmon in the presence of MgATP [92]. The affinity was at least $10^6$ $M^{-1}$, some 40 times stronger than the 'weak' binding of HMM·ADP·$P_i$ to actin involved in actin-activated ATPase. This tight binding requires caldesmon and is specific for fragments of smooth muscle myosin containing S-2 [117,143] and is independent of the phosphorylation level of the myosin.

In other respects the myosin–caldesmon–actin interaction is qualitatively different from myosin–caldesmon binding. The stoichiometry is one HMM molecule per actin, equivalent to up to 14 HMM binding at high affinity per caldesmon [143] and quite the reverse of the one myosin to three caldesmon stoichiometry found in sedimentation assays using isolated caldesmon and filamentous myosin [119,138,139]. Tight binding of HMM to thin filaments is not greatly affected by salt concentrations (there is a 2-fold change in $K_b$ between 10 and 100 mM-KCl [143]) but it is destroyed by the uranyl acetate used to stain filaments on electron microscope grids, unlike the caldesmon–myosin interaction [119].

The ability of caldesmon to link myosin to actin is probably unique. It can account for a number of properties of smooth muscle thin filaments. Firstly, the 'tethering' of the enzyme

(myosin) to its activator (actin) stabilizes the thin filament proteins, which would otherwise tend to dissociate at low concentrations [78,117]. Under certain conditions this promotes the movement of actin–caldesmon filaments in motility assay [147] and it also accounts for the unusually high $Ca^{2+}$-sensitivity of thin filaments activated by smooth muscle myosin compared with activation by skeletal muscle myosin [78]. Secondly, caldesmon can crosslink thick and thin filaments. Individual thick and thin filaments become bundled into large parallel aggregates due to crosslinking *in vitro* [119,139]. This may be of physiological significance either in stabilizing and directing the assembly of actin and myosin into a contractile apparatus or in acting as a crosslink that can maintain tension without energy expenditure [9].

## CALDESMON PHOSPHORYLATION

Caldesmon is a substrate for a number of protein kinases *in vitro* and some recent reports have shown caldesmon phosphorylation *in vivo*.

### $Ca^{2+}$–calmodulin kinase II

Ngai & Walsh [149] were the first to report phosphorylation of caldesmon by a $Ca^{2+}$–caldmodulin-dependent kinase activity which co-purified with chicken gizzard caldesmon [76,114,149,150]. This was subsequently identified as calmodulin-dependent kinase II [151] and has recently been purified from chicken gizzard [152]. This kinase has a mass of 56 kDa on SDS/PAGE and substrate specificity and autophosphorylation similar to the well-characterized brain enzyme.

$Ca^{2+}$–calmodulin kinase II phosphorylates gizzard caldesmon rapidly at Ser-73, but prolonged incubation results in up to 8 mol of $P_i$ being incorporated at residues including Ser-26, -726 and -587 [152]. The pattern of phosphorylation of pig arterial caldesmon is different. Again a maximum of 8 phosphates are incorporated but the rapidly phosphorylated residues are Ser-726 (32%) and Ser-194 (22%), the latter being absent from the gizzard sequence. Mammalian caldesmons do possess Ser-73 (see Fig. 1) but the flanking amino acids are different to those found in the gizzard sequence.

The functional effects of phosphorylation with this kinase remain controversial. Early reports that phosphorylated caldesmon did not inhibit actomyosin ATPase [76,149] were not reproduced by others [91,92] using a similar caldesmon preparation. Phosphorylation of gizzard CD*h* by the endogenous kinase II was shown also to inhibit the binding of caldesmon to smooth muscle myosin-Sepharose [141] and to reduce its affinity for myosin in direct assays [140]. Ser-73 lies within the *N*-terminal domain 1 which can bind to myosin, thus providing a possible mechanism to modulate myosin binding [9,154].

### Protein kinase C

Phosphorylation of caldesmon by protein kinase C has been reported by a number of groups. There is a general consensus that two major serine residues are phosphorylated in the *C*-terminus (domain 4 and 3–4 connection) [48,58,155,156]. These have been identified as Ser-587 (50%) and -600 (35%) in porcine CD*h* with a small amount of phosphorylation also occurring at Ser-726 (15%) [153]. There is some evidence that CD*l* is phosphorylated by protein kinase C *in vivo*. The protein kinase C activator tetradecanoylphorbol acetate caused a 4-fold increase in the level of CD*l* phosphorylation in platelets and the sites of phosphorylation appeared to be the same as the sites phosphorylated by protein kinase C *in vitro* [48]. The situation is rather less clear in smooth muscles: Park & Rasmussen [140,157] reported slow phosphorylation of a 140 kDa protein along with other cytoskeletal proteins in bovine trachea following either carbachol or phorbol ester stimulation, but a positive identification of CD*h* has not been made.

Sobue reported that protein kinase C phosphorylation of chicken gizzard CD*h* weakened caldesmon binding to both actin and $Ca^{2+}$–calmodulin in proportion to the degree of phosphorylation. Consistent with the change in actin binding, the potency of caldesmon inhibition decreased with increasing amounts of phosphate incorporated [156]. However, no change in the interaction of caldesmon with calmodulin was found upon phosphorylation of duck gizzard CD*h* [58].

### $p34^{cdc2}$ kinase

The kinase $p34^{cdc2}$ is specifically active during mitosis and has been linked to the profound changes preceding mitosis such as chromosome condensation, cytoskeletal rearrangement and nuclear membrane breakdown [159,160]. Matsumura and co-workers found that CD*l* is phosphorylated in fibroblasts undergoing mitosis [59,158]. Two groups have now shown that $p34^{cdc2}$ kinase phosphorylates both CD*h* and CD*l* *in vitro* [158,170]. Both serine and threonine residues were phosphorylated but the principal site was located in the *C*-terminal 10 kDa CNBr fragment (residues 660–756) [170]. The $p34^{cdc2}$ kinase is known to phosphorylate (Ser/Thr)-Pro sequences [162] of which there are four in domain 4 of caldesmon, all of which are conserved between species (Fig. 1) [59]. The third residue is commonly a basic amino acid [162]. There is only one such site in caldesmon: residues 673–676 have the sequence TPNK, which is identical to a site in $pp60^{c\text{-}src}$ which is phosphorylated by $p34^{cdc2}$ kinase *in vivo* [163].

CD*l* was observed to be phosphorylated in mitotic cells at the same site phosphorylated by $p34^{cdc2}$ *in vitro*. The phosphorylated CD*l* was dissociated from the microfilaments and when isolated proved unable to bind to actin filaments [59,158]. This effect was also shown *in vitro*: $p34^{cdc2}$ phosphorylation of CD*l* or CD*h* reduced its affinity for actin [158,170]. It is interesting to note that the site for $p34^{cdc2}$ kinase phosphorylation is near the *N*-terminus of the '10 K' fragment, the region which also seems to be most important for actin and $Ca^{2+}$–calmodulin binding (Fig. 7). The phosphorylation of caldesmon by this kinase and its subsequent dissociation from actin filaments is of great interest since it implies that caldesmon may play a role in the alteration or disassembly of the actin cytoskeleton at mitosis.

### Phosphorylation of CD*h* *in vivo*

Although there is evidence of a role for $p34^{cdc2}$ kinase and protein kinase C phosphorylation modulating CD*l* function in non-muscle contractile systems there is much less certainty about smooth muscle caldesmon phosphorylation. It has been speculated [9,154] that CD*h* phosphorylation could regulate tension maintenance by controlling actin–myosin crosslinking, but no satisfactory mechanism has been proposed. Adam *et al.* [164,165] have carefully determined the level of caldesmon phosphorylation in porcine carotid and coronary arteries. A basal level of caldesmon phosphorylation was found of 0.27 mol of phosphate/mol of caldesmon and, in contrast to Park & Rasmussen [157], no increase in phosphate content was found after 60 min exposure to carbachol. However after 60–75 min stimulations by KCl, phorbol dibutyrate, histamine, ouabain and endothelin-1 an increase to approximately 0.6 mol of phosphate/mol was observed. Similar results have been reported by Barany *et al.* [166]. NTCB cleavage of the caldesmon indicated that the phosphate groups were located in both the *N*- (54%) and *C*- (46%) terminal fragments [153] but the pattern of phosphorylated peptides did not match the peptides phosphorylated by $Ca^{2+}$–calmodulin kinase II or protein kinase C. It is known that

caldesmon phosphorylation is considerably changed when it is incorporated into thin filaments and it is probable that other, as yet unidentified, kinases can phosphorylate caldesmon *in vivo*.

## CALDESMON FUNCTION IN THE CELL

Caldesmon is present in significant quantity in all smooth muscle and non-muscle cells; its concentration in smooth muscle has been estimated at 10 $\mu$M [154]. Inside the cell caldesmon is nearly always found bound to the thin filaments [8,12,26,67,75,86]. In smooth muscles it has further been shown that caldesmon is bound to the thin filaments in the contractile domain where thick and thin filaments are overlapped rather than near the dense bodies or 'cytoskeletal domain' [8,86]. Thus there is enough caldesmon in the right place within the contractile apparatus of the intact cell for it to interact with actin, tropomyosin, calcium-binding proteins and myosin in the same way as it does *in vitro*.

From its properties *in vitro* one would predict that caldesmon, with a calcium-binding protein, could provide a $Ca^{2+}$-dependent controller of actomyosin ATPase and hence contractility analogous to troponin in striated muscles [67,127,179]. $Ca^{2+}$-dependent regulation of the thin filaments may be detected in crude smooth muscle actomyosin [172,173] and the use of anti-caldesmon antibodies has confirmed that this regulation is due to caldesmon [40,174]. In addition Taggart & Marston have demonstrated by using a model muscle fibre system [62], that caldesmon is able to inhibit isometric contraction at low doses in the absence of $Ca^{2+}$. Apart from one brief report [175], there is no direct evidence that caldesmon does control contractility in smooth muscles; this is largely a consequence of the experimental difficulties involved in testing for caldesmon function *in vivo* [40].

Both smooth muscle and non-muscle actomyosin have a powerful $Ca^{2+}$-dependent control mechanism mediated by $Ca^{2+}$-dependent phosphorylation of the myosin light chain. The question then arises as to whether there is any need for caldesmon regulation as well, since in many intact tissues myosin phosphorylation fully explains tension development (reviewed in [176,177]). In defence of a role for caldesmon it should be noted that the majority of muscles in invertebrates have dual regulatory systems on thick and thin filaments and that it has been calculated that synergy between two regulators can greatly enhance response to a small change in $[Ca^{2+}]$ [178]. In smooth muscle, activation of contraction occurs over a surprisingly small range of $[Ca^{2+}]_i$ [176,189]. There is a good deal of evidence from work on skinned fibre or single smooth muscle cells using a $Ca^{2+}$-independent fragment of myosin light chain kinase that myosin phosphorylation is necessary and sufficient for force development [180–183] in those preparations. This work should be interpreted with some care since Kossman *et al.* [184] have shown that caldesmon is degraded in skinned smooth muscle fibre preparations.

In addition to tension development, caldesmon may play a role in tension maintenance through its ability to crosslink thick and thin filaments. These bonds were calculated to be capable of bearing substantial tension [143]. Many smooth muscles maintain high tension with low $Ca^{2+}$, low levels of myosin phosphorylation and little or no contractile activity (reviewed in [9,179]). Although this state has been explained on the basis of a modification of the myosin phosphorylation mechanism [176,185] there are plenty of instances where this explanation fails [40,186–189]. A number of laboratories have suggested caldesmon could be responsible for tension maintenance in smooth muscles [4,9,92,117,143,154] but direct evidence is still lacking.

Caldesmon's properties *in vitro* suggest a potential role in organizing the assembly of the contractile apparatus, which might be a particularly appropriate function in non-muscle cells where both the actin and myosin filaments are transient structures [181]. Caldesmon promotes actin polymerization [104–106] and the assembly of soluble myosin 10 S species into filaments [4]. Once formed, caldesmon links between thick and thin filaments could be necessary to orientate filament polarity and to maintain the filament lattice structure in the absence of Z lines and M lines. The phosphorylation of caldesmon by $p34^{cdc2}$ kinase [59,158] could be taken as an example of the need to switch off caldesmon's organizing function prior to mitosis.

## CONCLUSION

Present knowledge of the molecular anatomy of caldesmon shows that it is an interesting subject of study. Caldesmon has many unique structural features as well as an unmatched combination of interaction sites with other proteins of the contractile apparatus, and yet it has little in common with any protein having analogous functions. However, despite knowing the complete sequence and having mapped out the location of the interaction sites into four domains our knowledge of how caldesmon regulates actin filament activity is at best incomplete. We have proposed that a two-stage process of binding followed by propagation of inhibition could explain an apparently contradictory body of experimental data, but this raises many further questions as to how such a mechanism could work and whether it does. Similarly, we have described how caldesmon can crosslink thick and thin filaments yet we have little knowledge of what function this may have or whether it actually occurs *in vivo*.

Physiologically, caldesmon is probably also important in the contraction of every smooth muscle and non-muscle cell. If caldesmon manifests *in vivo* the functions it has *in vitro* then it must play a significant part in $Ca^{2+}$-regulated contraction and in the crosslinking of thick and thin filaments, and these functions could be additionally controlled by phosphorylation reactions. Conversely, if it does not manifest those functions *in vivo* it will be necessary to find out why and how.

We are grateful to our colleagues throughout the world who have provided us with copies of unpublished work, illustrations and useful criticism. We are particularly indebted to the British Heart Foundation, which has supported most of our work.

## REFERENCES

1. Sobue, K., Muramoto, Y., Fujita, M. & Kakiuchi, S. (1981) Proc. Natl. Acad. Sci. U.S.A. **78**, 5652–5655
2. Marston, S. B. & Smith, C. W. J. (1984) J. Muscle Res. Cell Motil. **5**, 559–575
3. Bretscher, A. (1984) J. Biol. Chem. **259**, 12873–12880
4. Ikebe, M. & Reardon, S. (1988) J. Biol. Chem. **263**, 3055–3058
5. Smith, C. W., Pritchard, K. & Marston, S. B. (1987) J. Biol. Chem. **262**, 116–122
6. Sobue, K., Morimoto, K., Inui, M., Kanda, K. & Kakiuchi, S. (1982) Biomed. Res. **3**, 188–196
7. Marston, S. B. & Lehman, W. (1985) Biochem. J. **231**, 517–522
8. Furst, D. O., Cross, R. A., De Mey, J. & Small, J. V. (1986) EMBO J. **5**, 251–257
9. Marston, S. B. (1989) J. Muscle Res. Cell Motil. **10**, 97–100
10. Glukhova, M. A., Kabakov, A. E., Frid, M. G., Ornatsky, O. I., Belkin, A. M., Mukhin, D. N., Orekhov, A. N., Koteliansky, V. E. & Smirnov, V. N. (1988) Proc. Natl. Acad. Sci. U.S.A. **85**, 9542–9546
11. Glukhova, M. A., Frid, M. G. & Koteliansky, V. E. (1990) J. Biol. Chem. **265**, 13042–13046
12. Yamashiro-Matsumura, S. & Matsumura, F. (1988) J. Cell Biol. **106**, 1973–1983
13. Bryan, J., Imai, M., Lee, R., Moore, P., Cook, R. G. & Lin, W. G. (1989) J. Biol. Chem. **264**, 13873–13879
14. Smith, C. W. J. (1985) Ph.D. Thesis, London University
15. Sobue, K., Takahashi, K. & Wakabayashi, I. (1985) Biochem. Biophys. Res. Commun. **132**, 645–651

16. Cross, R. A., Cross, K. E. & Small, J. V. (1987) FEBS Lett. **219**, 306–310
17. Graceffa, P., Wang, C. L. & Stafford, W. F. (1988) J. Biol. Chem. **263**, 14196–14202
18. Malencik, D. A., Ausio, J., Byles, C. E., Modrell, B. & Anderson, S. R. (1989) Biochemistry **28**, 8227–8233
19. Fujii, T., Imai, M., Rosenfeld, G. C. & Bryan, J. (1987) J. Biol. Chem. **262**, 2757–2763
20. Ball, E. H. & Kovala, T. (1988) Biochemistry **27**, 6093–6098
21. Shirinsky, V. P., Bushueva, T. L. & Frolova, S. I. (1988) Biochem. J. **255**, 203–208
22. Cavanni, P., Cavallini, P., Ratti, G. G. & Dalla Libera, L. (1989) Biochem. Biophys. Res. Commun. **160**, 174–180
23. Bryan, J. (1989) J. Muscle Res. Cell Motil. **10**, 95–96
24. Graceffa, P. & Jancso, A. (1990*a*) J. Muscle Res. Cell Motil. **11**, 440 (abstr.)
25. Moody, C., Lehman, W. & Craig, R. (1990) J. Muscle Res. Cell Motil. **11**, 176–185
26. Lynch, W. P., Riseman, V. M. & Bretscher, A. (1987) J. Biol. Chem. **262**, 7429–7437
27. Mabuchi, K. & Wang, C-L. A. (1991) J. Muscle Res. Cell Motil. **13**, 146–151
28. Levine, B. A., Moir, A. J. G., Audemard, E., Mornet, D., Patchell, V. B. & Perry, S. V. (1990) Eur. J. Biochem. **193**, 687–696
29. Subdaralingam, M., Drendel, W. & Greaser, M. (1985) Proc. Natl. Acad. Sci. U.S.A. **82**, 7944–7947
30. Martin, F., Harricane, M.-C., Audemard, E., Pons, F. & Mornet, D. (1991) Eur. J. Biochem. **195**, 343–350
31. Wang, C-L. A., Chalovich, J. M., Graceffa, P., Lu, R. C., Mabuchi, K. & Stafford, W. F. (1991), unpublished work
32. Graceffa, P. & Jansco, A. (1990*b*) J. Muscle Res. Cell Motil. **11**, 440 (abstr.)
33. Szpacenko, A. & Dabrowska, R. (1986) FEBS Lett. **202**, 182–186
34. Riseman, V. M., Lynch, W. P., Nefsky, B. & Bretscher, A. (1989) J. Biol. Chem. **264**, 2869–2875
35. Katayama, E. (1989) J. Biochem. (Tokyo) **106**, 988–993
36. Yazawa, M., Yagi, K. & Sobue, K. (1987) J. Biochem. (Tokyo) **102**, 1065–1073
37. Leszyk, J., Mornet, D., Augemard, E. & Collins, J. H. (1989) Biochem. Biophys. Res. Commun. **160**, 1371–1378
38. Velaz, L., Ingraham, R. H. & Chalovich, J. M. (1990) J. Biol. Chem. **265**, 2929–2934
39. Lynch, W. & Bretscher, A. (1986) Methods Enzymol. **134**, 37–42
40. Taggart, M. J. (1991) Ph.D. Thesis, London University
41. Marston, S. (1989) Am. J. Obstet. Gynecol. **160**, 252–257
42. Ban, T., Ishimura, K., Fujita, H., Sobue, K. & Kakiuchi, S. (1984) Acta Histochem. Cytochem. **17**, 331–338
43. Ueki, N., Sobue, K., Kanda, K., Hada, T. & Higashino, K. (1987) Proc. Natl. Acad. Sci. U.S.A. **84**, 9049–9053
44. Kakiuchi, R., Inui, M., Morimoto, K., Kanda, K., Sobue, K. & Kakiuchi, S. (1983) FEBS Lett. **154**, 351–356
45. Dingus, J., Hwo, S. & Bryan, J. (1986) J. Cell Biol. **102**, 1748–1757
46. Yamashiro-Matsumura, S., Ishikawa, R. & Matsumura, F. (1988) Protoplasma **2** (Suppl.), 9–21
47. Burgoyne, R. D., Cheek, T. R. & Norman, K. M. (1986) Nature (London) **319**, 68–70
48. Litchfield, D. W. & Ball, E. H. (1987) J. Biol. Chem. **262**, 8056–8060
49. der Terrossian, E., Deprette, C. & Cassoly, R. (1989) Biochem. Biophys. Res. Commun. **159**, 395–401
50. Fujita, H., Ishimura, K., Ban, T., Kurosumi, M. & Sobue, K. (1984) Cell Tissue Res. **237**, 375–378
51. Owada, M. K., Hakura, A., Iido, K., Yagara, I. & Sobue, K. (1984) Proc. Natl. Acad. Sci. U.S.A. **81**, 3133–3137
52. Bretscher, A. & Lynch, W. (1985) J. Cell Biol. **100**, 1656–1663
53. Hayashi, K., Kanda, K., Kimizuka, F., Kato, I. & Sobue, K. (1989) Biochem. Biophys. Res. Commun. **164**, 503–511
54. Bryan, J. & Lee, R. (1991) J. Muscle Res. Cell Motil. **12**, 372–375
55. Sobue, K. & Fujio, Y. (1989) Adv. Exp. Med. Biol. **255**, 325–335
56. Glukhova, M. A., Kabakov, A. E., Ornatsky, O. I., Vasilevskaya, T. D., Koteliansky, V. E. & Smirnov, V. N. (1987) FEBS Lett. **218**, 292–294
57. Hayashi, K., Fujio, Y., Kato, I. & Sobue, K. (1991) J. Biol. Chem. **266**, 355–361
58. Vorotnikov, A. V. & Gusev, N. B. (1991) Biochem. J. **273**, 161–163
59. Yamashiro, S., Yamakita, Y., Hosoya, H. & Matsumura, F. (1991) Nature (London) **349**, 169–172
60. Avent, N., Redwood, C., Marston, S. & Huber, P. (1991), unpublished work
61. Novy, R. M., Lin, J. L-C. & Lin, J. J-C. (1991) J. Biol. Chem. **266**, in the press
62. Taggart, M. & Marston, S. (1990) J. Muscle Res. Cell Motil. **11**, 39 (abstr.)
63. Lin, J. J-C., Lin, J. L-C., Davis-Nanthakumar, E. J. & Lourim, D. (1988) Hybridoma **7**, 273–287
64. Hayashi, K., Yamada, S., Kanda, K., Kimizuka, F., Kato, I. & Sobue, K. (1989) Biochem. Biophys. Res. Commun. **161**, 38–45
65. Leszyk, J., Mornet, D., Audemard, E. & Collins, J. H. (1989) Biochem. Biophys. Res. Commun. **160**, 210–216
66. Lim, S. S., Tu, Z. & Lemanski, L. F. (1984) J. Muscle Res. Cell Motil. **5**, 515–526
67. Marston, S. B. & Smith, C. W. (1985) J. Muscle Res. Cell Motil. **6**, 669–708
68. Shirinsky, V. P., Biryukov, K. G., Vorotnikov, A. V. & Gusev, N. B. (1989) FEBS Lett. **251**, 65–68
69. Pearlstone, J. R. & Smillie, L. B. (1982) J. Biol. Chem. **257**, 10587–10592
70. Mak, A. S. & Smillie, L. B. (1980) J. Mol. Biol. **149**, 541–550
71. Parry, D. A. D. (1981) J. Mol. Biol. **146**, 259–263
72. O'Niel, K. T. & DeGrado, W. F. (1990) Trends. Biochem. Sci. **15**, 59–64
73. Takagi, T., Yazawa, M., Ueno, T., Suzuki, S. & Yagi, K. (1989) J. Biochem. (Tokyo) **106**, 778–783
74. Redwood, C. S. (1991) Ph.D. Thesis, London University
75. Lehman, W., Craig, R., Lui, J. & Moody, C. (1989) J. Muscle Res. Cell Motil. **10**, 101–112
76. Ngai, P. K. & Walsh, M. P. (1987) Biochem. J. **244**, 417–425
77. Fatigati, V. & Murphy, R. A. (1984) J. Biol. Chem. **259**, 14383–14388
78. Marston, S. B. (1990) Biochem. J. **272**, 305–310
79. Egelman, E. H. (1985) J. Muscle Res. Cell Motil. **6**, 129–151
80. Hanson, J., Lednev, V., O'Brian, E. J. & Bennett, P. M. (1972) Cold Spring Harbor Symp. Quant. Biol. **37**, 311–318
81. Hanson, J. & Lowy, J. (1963) J. Mol. Biol. **6**, 46–60
82. Marston, S. B., Lehman, W., Moody, C. J., Pritchard, K. & Smith, C. W. (1988) in Calcium and Calcium Binding Proteins (Gerday, Ch., Gilles, R. & Bollis, L., eds.), pp. 69–81, Springer Verlag, Heidelberg
83. Matsumura, F. & Lin, J. J-C. (1982) J. Mol. Biol. **157**, 163–171
84. Cohen, C., Caspar, D. L. D., Johnson, J. P., Nauss, K., Margossian, S. S. & Parry, D. A. D. (1972) Cold Spring Harbor Symp. Quant. Biol. **37**, 287–298
85. Haeberle, J. & Hathaway, D. R. (1990) Biophys. J. **57**, 166a (abstr.)
86. Lehman, W., Sheldon, A. & Madonia, W. (1987) Biochim. Biophys. Acta **914**, 35–39
87. Dabrowska, R., Goch, A., Galazkiewicz, B. & Osinska, H. (1985) Biochim. Biophys. Acta **842**, 70–75
88. Moody, C. J., Marston, S. B. & Smith, C. W. (1985) FEBS Lett. **191**, 107–112
89. Velaz, L., Hemric, M. E., Benson, C. E. & Chalovich, J. M. (1989) J. Biol. Chem. **264**, 9602–9610
90. McGee, J. & von Hippel, P. (1974) J. Mol. Biol. **84**, 469–489
91. Horiuchi, K. Y., Miyata, H. & Chacko, S. (1986) Biochem. Biophys. Res. Commun. **136**, 962–968
92. Lash, J. A., Sellers, J. R. & Hathaway, D. R. (1986) J. Biol. Chem. **261**, 16155–16160
93. Watson, M. H., Kuhn, A. E., Novy, R. E., Lin, J. J-C. & Mak, A. S. (1990) J. Biol. Chem. **265**, 18860–18866
94. Makuch, R., Walsh, M. P. & Dabrowska, R. (1989) FEBS Lett. **247**, 411–414
95. Redwood, C. S., Marston, S. B., Bryan, J., Cross, R. A. & Kendrick-Jones, J. (1990) FEBS Lett. **270**, 53–56
96. Bartegi, A., Fattoum, A., Derancourt, J. & Kassab, R. (1990) J. Biol. Chem. **265**, 15231–15238
97. Lin, J J.-C., Davis-Nanthakumar, E. J., Jin, J-P., Lourim, D., Novy, R. E. & Lin, J. L-C. (1991) unpublished work
98. Wang, C-L. A., Wang, L-W. C., Lu, R. C., Saavedra-Alanis, V. & Bryan, J. (1991) Biophys. J. **59**, 219a (abstr.)
99. Kabsch, W., Mannherz, H. G., Suck, D., Pai, E. F. & Holmes, K. C. (1990) Nature (London) **347**, 37–44
100. Holmes, K. C., Popp, D., Gebhard, W. & Kabsch, W. (1990) Nature (London) **347**, 44–49
101. Bartegi, A., Fattoum, A. & Kassab, R. (1990) J. Biol. Chem. **265**, 2231–2237

102. Adams, S., DasGupta, G., Chalovich, J. M. & Reisler, E. (1990) J. Biol. Chem. **265**, 19652–19657
103. Crosbie, R., Adams, S. B., Velaz, L., Chalovich, J. M. & Reisler, E. (1991) Biophys. J. **59**, 56a (abstr.)
104. Galazkiewicz, B., Mossakowska, M., Osinska, H. & Dabrowska, R. (1985) FEBS Lett. **184**, 144–149
105. Galazkiweicz, B., Belagyi, J. & Dabrowska, R. (1989) Eur. J. Biochem. **181**, 607–614
106. Galazkiewicz, B., Buss, F., Jockusch, B. M. & Dabrowska, R. (1991) Eur. J. Biochem., in the press
107. Horiuchi, K. Y. & Chacko, S. (1988) Biochemistry **27**, 8388–8393
108. Fujii, T., Ozawa, J., Ogoma, Y. & Kondo, Y. (1988) J. Biochem. (Tokyo) **104**, 734–737
109. Graceffa, P. (1987) FEBS Lett. **218**, 139–142
110. Katayama, E., Horiuchi, K. Y. & Chacko, S. (1990) Biochem. Biophys. Res. Commun. **160**, 1316–1322
111. Cohen, C. & Parry, D. A. D. (1986) Trends. Biochem. Sci. **11**, 245–248
112. Hodges, R. S., Sodek, J., Smillie, L. B. & Jurasek, L. (1972) Cold Spring Harbor Symp. Quant. Biol. **37**, 299–310
113. Taggart, M. J. & Marston, S. B. (1988) FEBS Lett. **242**, 171–174
114. Ngai, P. K. & Walsh, M. P. (1985) Biochem. J. **230**, 695–707
115. Chalovich, J. M., Hemric, M. E. & Velaz, L. (1990) Ann. N.Y. Acad. Sci. **599**, 85–99
116. Horiuchi, K. Y. & Chacko, S. (1989) Biochemistry **28**, 9111–9116
117. Hemric, M. E. & Chalovich, J. M. (1988) J. Biol. Chem. **263**, 1878–1885
118. Chalovich, J. M., Cornelius, P. & Benson, C. E. (1987) J. Biol. Chem. **262**, 5711–5716
119. Marston, S. B., Bennett, P. M. & Pinter, K. (1991), unpublished work
120. Marston, S. (1988) FEBS Lett. **238**, 147–150
121. Horiuchi, K. Y., Samuel, M. & Chacko, S. (1991) Biochemistry **30**, 712–717
122. Pritchard, K. & Marston, S. B. (1988) Biochem. Soc. Trans. **16**, 355–356
123. Skripnikova, E. V. & Gusev, N. B. (1989) FEBS Lett. **257**, 380–382
124. Pritchard, K. & Marston, S. (1991) Biochem. J. **277**, 819–824
125. Fujii, T., Machino, K., Andoh, H., Satoh, T. & Kondo, Y. (1990) J. Biochem. (Tokyo) **107**, 133–137
126. Pritchard, K. & Marston, S. B. (1988) in Sarcomeric and Non-sarcomeric Muscles: Basic and Applied Research Prospects for the 90's (Carraro, U., ed.), pp. 649–654, Unipress, Padova
127. Marston, S., Lehman, W., Moody, C. & Smith, C. (1985) Adv. Protein Phosphatases **2**, 171–189
128. Mills, J. S., Walsh, M. P., Nemcek, K. & Johnson, J. D. (1988) Biochemistry **27**, 991–996
129. Pritchard, K. & Marston, S. B. (1989) Biochem. J. **257**, 839–843
130. Liu, Y. & Storm, D. R. (1990) Trends Pharmacol. Sci. **11**, 107–111
131. Bartegi, A., Fattoum, A., Derancourt, J. & Kassab, R. (1991) Biophys. J. **59**, 65a (abstr.)
132. Wang, C-L. A., Wang, L-W. C., Xu, S., Lu, R. C., Saavedra-Alanis, V. & Bryan, J. (1991) J. Biol. Chem. **266**, 9166–9172
133. Wang, C. L. (1988) Biochem. Biophys. Res. Commun. **156**, 1033–1038
134. Smith, C. W. J. & Marston, S. B. (1985) FEBS Lett. **184**, 115–119
135. Kakiuchi, S. & Sobue, K. (1983) Trends. Biochem. Sci. **8**, 59–62
136. Lehman, W. (1986) Biochim. Biophys. Acta **885**, 88–90
137. Bartegi, A., Fattoum, A., Dagorn, C., Gabrion, J. & Kassab, R. (1989) Eur. J. Biochem. **185**, 589–595
138. Hemric, M. E. & Chalovich, J. M. (1990) J. Biol. Chem. **265**, 19672–19678
139. Marston, S., Redwood, C. & Bennett, P. (1991) in Muscle and Motility Vol. 2: Proceedings of XIXth European Conference in Brussels (Marechal, G. & Carraro, U., eds.), pp. 351–357, Intercept, Andover
140. Rasmussen, H., Takuwa, Y. & Park, S. (1987) FASEB J. **1**, 177–185
141. Sutherland, C. & Walsh, M. P. (1989) J. Biol. Chem. **264**, 578–583
142. Labeit, S., Barlow, D. P., Gautrel, M., Gibson, T., Holt, J., Hsieh, C-L., Francke, U., Leonard, K., Wardale, J., Whiting, A. & Trinick, J. (1990) Nature (London) **345**, 273–276
143. Marston, S. B. (1989) Biochem. J. **259**, 303–306
144. Yanagisawa, M., Hamada, Y., Katsuragawa, Y., Imamura, M., Mikawa, T. & Masaki, T. (1987) J. Mol. Biol. **198**, 143–157
145. Cooke, P. H., Fay, F. S. & Craig, R. (1989) J. Muscle Res. Cell Motil. **10**, 206–220
146. Craig, R. & Megerman, J. (1977) J. Cell Biol. **75**, 990–996
147. Haeberle, J. R., Trybus, K. M. & Warshaw, D. M. (1991) Biophys. J. **59**, 58a (abstr.)
148. Bennett, P. M. & Marston, S. B. (1990) J. Muscle Res. Cell Motil. **11**, 302–312
149. Ngai, P. K. & Walsh, M. P. (1984) J. Biol. Chem. **259**, 13656–13659
150. Scott-Woo, G. C. & Walsh, M. P. (1988) Biochem. J. **252**, 463–472
151. Abougou, J. C., Hagiwara, M., Hachiya, T., Terasawa, M., Hidaka, H. & Hartshorne, D. J. (1989) FEBS Lett. **257**, 408–410
152. Ikebe, M., Reardon, S., Scott-Woo, G. C., Zhou, Z. & Koda, Y. (1990) Biochemistry **29**, 11242–11248
153. Hathaway, D. R. & Adam, L. P. (1990) J. Muscle Res. Cell Motil. **11**, 435 (abstr.)
154. Walsh, M. P. & Sutherland, C. (1989) Adv. Exp. Med. Biol. **255**, 337–346
155. Umekawa, H. & Hidaka, H. (1985) Biochem. Biophys. Res. Commun. **132**, 56–62
156. Tanaka, T., Ohta, H., Kanda, K., Hidaka, H. & Sobue, K. (1990) Eur. J. Biochem. **188**, 495–500
157. Park, S. & Rasmussen, H. (1986) J. Biol. Chem. **261**, 15734–15739
158. Yamashiro, S., Yamakita, Y., Ishikawa, R. & Matsumura, F. (1990) Nature (London) **344**, 675–678
159. Lewin, B. (1990) Cell **61**, 743–752
160. Draetta, G. (1990) Trends. Biochem. Sci. **15**, 378–382
161. Vorotnikov, A. V., Shirinsky, V. P. & Gusev, N. B. (1988) FEBS Lett. **236**, 321–324
162. Moreno, S. & Nurse, P. (1990) Cell **61**, 549–551
163. Shenoy, S., Choi, J-K., Bagrodia, S., Copeland, T. D., Maller, J. L. & Shalloway, D. (1989) Cell **57**, 763–774
164. Adam, L. P., Haeberle, J. R. & Hathaway, D. R. (1989) J. Biol. Chem. **264**, 7698–7703
165. Adam, L. P., Milio, L., Brengle, B. & Hathaway, D. R. (1990) J. Mol. Cell. Cardiol. **22**, 1017–1023
166. Barany, M., Rokolya, A. & Barany, K. (1991) FEBS Lett. **279**, 65–68
167. Lehman, W., Moody, C. & Craig, R. (1990) Ann. N.Y. Acad. Sci. **599**, 75–84
168. Stafford, W. F., Jancso, A. & Graceffa, P. (1990) Arch. Biochem. Biophys. **281**, 66–69
169. Bonet-Kerrache, A. & Walsh, M. P. (1991) FEBS Lett. **281**, 81–84
170. Mak, A. S., Watson, M. H., Litwin, C. M. & Wang, J. H. (1991) J. Biol. Chem. **266**, 6678–6681
171. Birukov, K. C., Shirinsky, V. P., Vorotnikov, A. V. & Gusev, N. B. (1990) FEBS Lett. **262**, 263–265
172. Marston, S. B., Trevett, R. M. & Walters, M. (1980) Biochem. J. **185**, 355–365
173. Bloomquist, E. L. & Yaney, G. C. (1979) J. Gen. Physiol. **74**, 5a
174. Marston, S. B., Redwood, C. S. & Lehman, W. (1988) Biochem. Biophys. Res. Commun. **155**, 197–202
175. Szpacenko, A., Wagner, J., Dabrowska, R. & Ruegg, J. C. (1985) FEBS Lett. **192**, 9–12
176. Hai, C. & Murphy, R. A. (1989) Annu. Rev. Physiol. **51**, 000–000
177. Kamm, K. E. & Stull, J. T. (1985) Annu. Rev. Pharmacol. Toxicol. **25**, 593–620
178. Lehman, W. & Szent-Gyorgyi, A. G. (1975) J. Gen. Physiol. **66**, 1–30
179. Kamm, K. E. & Stull, J. T. (1989) Annu. Rev. Physiol. **51**, 299–313
180. Walsh, M. P., Bridebraugh, R., Hartshorne, D. J. & Kerrick, W. G. L. (1982) J. Biol. Chem. **257**, 5987–5990
181. Cande, W. Z., Tooth, P. J. & Kendrick-Jones, J. (1983) J. Cell. Biol. **97**, 1062–1071
182. Itoh, T., Ikebe, M., Kargacin, G. J., Hartshorne, D. J., Kemp, B. E. & Fay, F. S. (1989) Nature (London) **338**, 164–167
183. Kargacin, G. J., Ikebe, M. & Fay, F. S. (1990) Am. J. Physiol. **259**, C315–C324
184. Kossman, T., Furst, D. & Small, J. V. (1987) J. Muscle Res. Cell. Motil. **8**, 135–144
185. Hai, C. & Murphy, R. A. (1988) Am. J. Physiol. **254**, C99–C106
186. Tansey, M. G., Hori, M., Karaki, H., Kamm, K. E. & Stull, J. T. (1990) FEBS Lett. **270**, 219–221
187. Kenney, R. E., Hoar, P. E. & Kerrick, W. G. L. (1990) J. Biol. Chem. **265**, 8642–8649
188. Paul, R. J., Gibbs, C. & Wendt, I. (1990) Biophys. J. **57**, 166a
189. Jiang, M. J. & Morgan, K. G (1989) Pflugers Arch. **413**, 637–643