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Localization and Characterization of a 7.3-kDa Region of Caldesmon Which Reversibly Inhibits Actomyosin ATPase Activity*

Joseph M. Chalovich‡,§, **Joseph Bryan**¶, **Caryl E. Benson**‡, and **Laly Velaz**‡

‡*From the Department of Biochemistry, East Carolina University School of Medicine, Greenville, North Carolina 27858-4354 and the*

¶*Department of Cell Biology, Baylor College of Medicine, Houston, Texas 77030*

Abstract

Cleavage of caldesmon with chymotrypsin yields a series of fragments which bind both calmodulin and actin and inhibit the binding of myosin subfragments to actin and the subsequent stimulation of ATPase activity. Several of these fragments have been purified by cation exchange chromatography and their amino-terminal sequences determined. The smallest fragment has a molecular mass of about 7.3 kDa and extends from Leu^{597} to Phe^{665}. This polypeptide inhibits the actin-activated ATPase of myosin S-1; this inhibition is augmented by smooth muscle tropomyosin and relieved by Ca^{2+}-calmodulin. The binding of the 7.3-kDa fragment to actin is competitive with the binding of S - 1 to actin. Thus, this polypeptide has several of the important features characteristic of intact caldesmon. However, although an intact caldesmon molecule covers between six and nine actin monomers, the 7.3-kDa fragment binds to actin in a 1:1 complex. Comparison of this fragment with others suggests that a small region of caldesmon is responsible for at least part of the interaction with both calmodulin and actin.

Caldesmon is an actin- and calmodulin-binding protein isolated from smooth muscle (1) and later shown to be a component of some nonmuscle cells (2–4). The role of caldesmon in these cells has not been firmly established, but it does interact with several contractile proteins and is a potent inhibitor of actin-activated ATP hydrolysis by myosin (5–8) and its subfragments (9,10).

Structurally, caldesmon consists of two globular regions joined, in smooth muscle caldesmon, by an elongated helical chain. The amino-terminal region of caldesmon binds to myosin (11, 12) forming a relatively strongly associated 1:1 complex in the presence of ATP (13). The isolated myosin- binding segment does not affect ATP hydrolysis. The binding of caldesmon to myosin is weakened both by phosphorylation of caldesmon (14,15) and by the binding of Ca^{2+}-calmodulin (12,15). Adjacent to the myosin binding region is an elongated helical structure (16) which probably serves a structural function since it has no known activity and is absent in the known nonmuscle caldesmon's (17–19). The carboxyl-terminal region of caldesmon was first isolated as a 40-kDa fragment from chymotryptic digests and was shown to inhibit ATP hydrolysis in a Ca^{2+}-calmodulin dependent manner (20–23). Further digestion of the 40-kDa fragment yielded a product of about 20 kDa with properties similar to the 40-kDa precursor (20,21,23). Both the 40-kDa (24) and the 20-kDa fragments (25) inhibit ATPase

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§ To whom correspondence should be addressed. Tel.: 919-551-2973; Fax: 919-551-2012..

activity while lacking the ability of cross-linking myosin with actin. The 20-kDa fragments also inhibit the binding of myosin S-1 to actin in the presence of ATP and can be displaced from actin by rigor crossbridges.[1] Furthermore, the 20-kDa fragments inhibit the attachment of weakly binding cross-bridges in intact muscle fibers blocking force production (26).

In the present study we show that 20-kDa chymotryptic fragments of caldesmon originate at either Lys^{579} or Leu^{597} and extend close to the carboxyl terminus of caldesmon. The population of fragments beginning at Lys^{597} is similar, if not identical, to the 20-kDa fragment prepared by Riseman *et al.* (23). We have shown that further digestion results in the production of a 7.3-kDa fragment which begins at Leu^{597} and terminates at Phe^{665}. This fragment binds to actin with a stoichiometry of 1:1, inhibits ATPase activity, and binds to calmodulin. By comparison with other known regions of caldesmon, these data further localize the location of major sites of interaction with actin and calmodulin.

MATERIALS AND METHODS

Skeletal actin was isolated from rabbit back and leg muscles by a modification of the method of Spudich and Watt (27,28). Smooth muscle tropomyosin was isolated from turkey gizzards (29). Calmodulin was purified from porcine brains by the method of Yazawa *et al.* (30). Skeletal muscle myosin was isolated from the back and leg muscles of rabbits (31), and chymotryptic S-1 was prepared by the method of Weeds and Taylor (32). Smooth muscle myosin and S-1 were prepared from chicken gizzards (11). Caldesmon was isolated from turkey gizzards by a modification of the method of Bretscher (29) described previously (33).

Protein concentrations were determined by absorbance at 280 nm except for caldesmon which was determined by the Lowry assay using bovine serum albumin as a standard. The molecular weights used for calculation of protein concentrations were S-1 (120,000), actin (42,000), tropomyosin (68,000), calmodulin (16,500), and caldesmon (87,000). The average molecular mass of the three actin-binding fragments of caldesmon *c*, *d*, and *e* was estimated to be 20 kDa on the basis of mobility on SDS[2] gels, size exclusion chromatography, and amino-terminal amino acid sequence determination.

Caldesmon was cleaved with either a 1:500 or 1:1,000 mg ratio of chymotrypsin for 5 min at 25 °C in a buffer composed of 0.1 M KC1, 20 mM Tris-HCl (pH 8.0), 1 mM EDTA, and 1 mM dithiothreitol. This procedure is similar to that used by Szpacenko and Dabrowska (21). To purify the actin-binding fragments of caldesmon, the digest was applied to a 1.5 × 95-cm column of Ultrogel AcA 54 (LKB) equilibrated with digestion buffer containing 1 M KCl. The second and third peaks, containing the low molecular weight actin-binding fragments, were pooled and applied to a 10-ml column of Affi-Gel Blue-Agarose (Bio-Rad) equilibrated with digestion buffer. After washing the column, actin-binding fragments of caldesmon were eluted with digestion buffer having a total KCl concentration of 0.5 M KCl. In some cases, the mixture of actin-binding fragments was separated into individual components by cation exchange HPLC using a Waters SP-R column. The column was equilibrated with 10 mM Tris-Mes (pH 7.0), 60 mM NaCl, 1 mM dithiothreitol, and the fragments were eluted with a NaCl gradient to 500 mM.

Binding Studies

The 7.3-kDa fragment of caldesmon was labeled with ^{125}I using the Iodogen reagent (Pierce) as described by the manufacturer. Labeling with [^{14}C] iodoacetamide, the preferred method,

[1] L. Velaz and J. M. Chalovich, unpublished data.
[2] The abbreviations used are: SDS, sodium dodecyl sulfate; Mes, 4-morpholineethanesulfonic acid EGTA, [ethylenebis (oxyethylenenitrilo)] tetraacetic acid.

was impossible since this fragment lacked Cys. The binding of ^{125}I-labeled caldesmon to actin was determined by the depletion of radioactivity from the supernatant following sedimentation of the reaction mixture at 160,000× g for 25 min in a 50-Ti rotor. The binding buffer contained 42 mM NaCl, 4.2 $MgC1_2$, 10 mM imidazole (pH 7.0), 0.25 mM EGTA, 1 mM dithiothreitol, 0.3 μM skeletal S-1, and 2 mM ATP. The binding data were corrected for 1) the fraction of caldesmon which did not form a sediment even at saturating actin concentrations; 2) the amount of caldesmon which was present in the sediment in the absence of actin; and (3) the fraction of actin (5%) which did not form a sediment.

ATPase Assays

The ATPase activity of myosin skeletal S-1 was measured by the liberation of ^{32}Pi from [^{32}P] ATP as described earlier (34).

Affinity Chromatography

Calmodulin was cross-linked to CNBr- activated Sepharose 4B according to the manufacture's protocol (Pharmacia LKB Biotechnology Inc.) as described earlier (25).

Electrophoresis and Autoradiography

Sodium dodecyl sulfate gels, with a 7–20% gradient of polyacrylamide, were run by the procedure of Laemmli (35) and stained with Coomassie Blue.

Molecular Weight Estimation

An estimate of the molecular weight of the 20-kDa fragments of caldesmon was made on the basis of retention time on a GF-250 (Du Pont) HPLC size exclusion column in a buffer containing 0.3 M Na_2HPO_4 (pH 7.0). The calibration curve was linear with eight standards having molecular weights between 94,000 and 12,400 (Sigma). Molecular weights of the 7.3- and 20-kDa fragments were also estimated from mobility on calibrated SDS gels.

Primary Structure Determination

For the determination of amino acid composition fragments were further purified by reversed phase HPLC on a μBondapak (Waters) C18 column equilibrated with 0.1% trifluoroacetic acid, 4.75% acetonitrile and eluted with a gradient of acetonitrile to 95%. Fractions containing the polypeptide of interest were dried under N_2 and hydrolyzed for amino acid analysis by the Pico-Tag method (Waters). The amino-terminal sequence of the peptides was determined following electrophoretic transfer from an SDS gel to an Immobilon (Millipore) polyvinylidene difluoride membrane in a LKB semi-dry transphore apparatus. The solvent was composed of 39 mM glycine, 48 mM Tris, 0.0373% (w/v) SDS, and 20% methanol. The transfer was run for 15 min at 50 mA, 15 min at 100 mA, 15 min at 150 mA, and finally 45 min at 190 mA. Following extensive washing with water and staining with Coomassie Blue, the bands of interest were excised and placed directly into the sequenator. Analyses were usually terminated after 15–20 residues. The carboxyl- terminal sequence of the 7.3-kDa polypeptide was determined by carboxypeptidase A (Worthington, 2 × crystallized) digestion at pH 8.5, 37 °C for 1 or 5 h. The reaction was stopped by the addition of HCl to pH 2, and released amino acids were determined.

RESULTS

Purification of Fragments and Localization in Caldesmon

Digestion of caldesmon with chymotrypsin has been shown earlier to produce a number of fragments which bind to actin (20,21) and one which binds to myosin (25). Fig. 1 shows the resolution of a chymotryptic digest of turkey gizzard caldesmon on cation exchange

chromatography. This is a more extensive digestion than reported earlier (25). With the exception of the first peak, all fractions inhibited the actin activation of ATP hydrolysis by skeletal S-1. Furthermore, most of the polypeptides with estimated molecular masses between 7 and 22 kDa were found to bind to actin in co-sedimentation assays. We had shown earlier that the three fragments migrating with a molecular mass of about 20-kDa (equivalent to those in fraction *j*) inhibited actin-activated ATP hydrolysis and binding of heavy meromyosin to actin (25). Furthermore, these fragments inhibit the interaction of myosin-ATP with actin in skinned muscle fibers (26). Because of the apparent importance of this region of caldesmon we determined the amino-terminal sequence of the 20-kDa fragments to localize them within caldesmon. We also isolated and characterized the lowest molecular weight polypeptide, ~7.3 kDa, in fraction *h* of Fig. 1. This polypeptide was of particular interest because it was the smallest polypeptide found to bind tightly to actin.

Purification of the 20-kDa fragments was best achieved from a digestion done with a 1:600 or 1:1,000 ratio of chymotrypsin to caldesmon. The digest was first chromatographed on a gel permeation column (see Fig. 3), and the fraction containing the 20-kDa fragments was resolved by chromatography on an SP HPLC cation exchange column (Waters). Fig. 2 is the elution profile of the fragments in the 20-kDa range. Four polypeptide fragments were identified in the peaks labeled *1, 2,* and *3;* each bound to actin in co-sedimentation experiments as shown in Fig. 2. Furthermore, each of these fractions inhibits the actin-activated ATPase activity of myosin (data not shown). The polypeptide fragments from a gel similar to that shown in Fig. 2 were transferred to a polyvinylidene difluoride membrane, and the individual bands were excised for sequence analysis. These four polypeptides are all derived from the same region of the caldesmon molecule. Two of the fragments began at Lys^{579}; the smaller fragment must be truncated at the carboxyl-terminal end. The other two fragments originate at Leu^{597}. The amino-terminal residues are given in Table I. The most likely carboxyl-terminal residue of these polypeptides was determined from estimates of their molecular weights. These results are also listed in Table I.

The smaller 7.3-kDa fragment was purified from a 1:500 digest with chymotrypsin following initial fractionation on the SP HPLC cation exchange column as described in Fig. 1. The peak enriched in the 7.3-kDa fragment (peak *h* in Fig. 1) was further purified by gel filtration as shown in Fig. 3. This two-step purification was usually sufficient to produce a single band of protein on heavily loaded SDS gels as shown in Fig. 3. The 7.3-kDa peptide was electrophoretically transferred to polyvinylidene difluoride membranes for sequence analysis and was shown to originate at Leu^{597} (Table I).

The carboxyl-terminal amino acid was determined by carboxypeptidase A digestion. Digestion at 37 °C for 1 h at a carboxypeptidase A to protein ratio of 1:220 yielded Phe (578 pmol) and a Val (206 pmol), whereas digestion for 5 h at a ratio of 1:20 yielded almost equal amounts of Phe and Val (594 and 685 pmol, respectively). Therefore, the carboxyl-terminal residues are Val-Phe. In the caldesmon sequence, there is only one Phe, Phe^{665}, within 10 kDa of Leu^{597}; the residue preceding Phe^{665} is Val^{664}. This is the only Val-Phe sequence between Leu^{597} and Pro^{756}. The presence of Phe at the carboxyl terminus is also consistent with a fragment produced by cleavage with chymotrypsin. Therefore, the fragment isolated extends from Leu^{597} to Phe^{665} and has a molecular mass of 7309 daltons (Table I).

Estimates of the carboxyl termini of the other fragments were made on the basis of their molecular weights determined on SDS- polyacrylamide gels. However, since intact caldesmon and other fragments display aberrant mobility on SDS gels (37) this result was confirmed by other methods. In the case of the actin-binding fragments, the molecular weights were also estimated by the retention time on an HPLC gel permeation column. The molecular mass and predicted location of all four fragments are given in Table I and Fig. 7. These data show that

the 7.3-kDa fragment is derived from the 20-kDa fragments, and both are derived from the 35–40- kDa fragment studied by others (20–23).

Interaction of the 7.3-kDa Fragment with Actin and Actin- Tropomyosin Filaments

The properties of the 7.3-kDa polypeptide were studied in more detail since this fragment binds to actin and is a derivative of larger fragments known to be important for the function of caldesmon. Fig. 4 shows the binding of highly purified 7.3-kDa fragment to actin and actin-tropomyosin filaments. Most of the data were obtained using ^{125}I-labeled peptide, but the three highest points were obtained with unlabeled caldesmon using quantitative gel electrophoresis. Binding saturated at a stoichiometry of 1.1 peptides/actin monomer with an association constant of $4.9 \times 10^4\ {}_{M}{}^{-1}$. There is no evidence of cooperativity in the binding. This binding is 1% as tight ($K_{l\omega}$) as intact caldesmon (33) and 5–10% as tight as the binding of the 20-kDa actin-binding fragments (Velaz *et al.*, Biophys. J. 1991). Although relatively weak, the interaction of the 7.3-kDa fragment is about 10- fold stronger than the binding of S-1-ATP to actin under identical conditions. Unlike intact caldesmon (one per seven to ten actin monomers) or the 20-kDa fragments (one per two actin monomers), the 7.3-kDa fragment binds to a single actin monomer and must contain a key determinant for actin binding.

The binding of intact caldesmon to actin is known to be enhanced by tropomyosin (33,38). However, there is little, if any, effect of smooth muscle tropomyosin on the binding of the 7.3-kDa fragment to actin. The *inset* to Fig. 4 shows the low concentration region of the binding curve in more detail. There is little difference between the binding in the presence (*solid circles*) and absence (*open circles*) of tropomyosin. In support of this observation, we have also observed a lack of tropomyosin effect on binding with the larger 20-kDa fragment.[1]

The binding of intact caldesmon to actin is inhibited by rigor S-1 binding (11) and by Ca^{2+}-calmodulin (33,38,39). The *inset* to Fig. 4 also shows that binding of the 7.3-kDa fragment is inhibited by both skeletal S-1 and Ca^{2+}-calmodulin. Using a simple competitive model the reversal of caldesmon-actin binding by calmodulin behaves as if the association constant of Ca^{2+}-calmodulin to caldesmon is about $2 \times 10^5\ {}_{M}{}^{-1}$. This is about 50% as tight as our estimate for binding to intact caldesmon by the same method (33).

The 7.3-kDa Fragment Binds to Ca^{2+}-Calmodulin

The ability of the 7.3-kDa fragment to bind directly to calmodulin was confirmed by affinity chromatography. Fig. 5 shows that the 7.3-kDa fragment binds to a calmodulin-Sepharose column in the presence of Ca^{2+} but is released when the free Ca^{2+} concentration is lowered by the addition of EGTA. This observation supports an earlier suggestion (40) that the sequence from TrP^{659} to Phe^{665} is essential and perhaps sufficient for binding to Ca^{2+}-calmodulin.

Inhibition of the Actin-activated ATPase of Myosin

Another characteristic property of caldesmon is the ability to inhibit the activation of myosin ATPase activity by actin. Fig. 6 demonstrates that the 7.3-kDa fragment of caldesmon inhibits the actin-activated ATPase activity in a concentration-dependent manner. The concentration of 7.3-kDa fragment required for inhibition is greater than for intact caldesmon in accord with the difference in affinity. However, the degree of inhibition by the fragment (about 90%) is similar to intact caldesmon. It is interesting that although tropomyosin has little effect on the binding of the 7.3-kDa fragment to actin, tropomyosin does facilitate inhibition of ATPase activity. Similar inhibition was observed on two additional preparations. Fig. 6 also shows that the 7.3-kDa fragment inhibits ATPase activity of smooth muscle S-1. Note that the effectiveness of this fragment for smooth and skeletal S-1 cannot be compared since different fragment preparations were used. The inset to Fig. 6 shows that inhibition of ATPase activity is reversed by Ca^{2+}-calmodulin.

DISCUSSION

Caldesmon has been shown to bind stoichiometrically to myosin (1:l in the presence of ATP) (13), actin (one per six to nine actin monomers in a filament) (9,33,41–44), calmodulin (one per two calmodulins) (40,45–47), and to tropomyosin (1:1) (48). The binding site for myosin (14,25) as well as one calmodulin binding site are in the amino-terminal region of caldesmon (45), whereas the actin binding site(s) and a second calmodulin binding site are located at the carboxyl- terminal region (20–22,49,50). These two regions of caldesmon are separated, in smooth muscle caldesmon, by a long helical region (16). It is unclear whether the carboxyl-terminal actin binding domain alone positions the long flexible caldesmon molecule on F-actin and may cooperatively regulate actomyosin or whether additional actin binding sites are necessary. One approach to understanding the function of caldesmon is to define the location of the various binding regions within the primary structure of caldesmon.

The actin binding site(s) of caldesmon is particularly interesting since binding to actin inhibits activation of ATP hydrolysis and could be of regulatory importance. Several recent studies have been directed toward defining the actin sequences involved in binding to caldesmon. There is agreement that caldesmon binds near the amino-terminal 7 residues of actin (51–53), a region that has been implicated in the binding of S-1-ATP (54,55). Caldesmon binding to actin is also perturbed slightly by antibodies directed against a peptide containing amino acid residues 18–28 of actin (51). This peptide may be involved in rigor myosin binding to actin (63). Recently, the carboxyl-terminal region of actin has been shown to affect the interaction with caldesmon (56–58), but this is probably an indirect effect resulting from the close proximity of the carboxyl- and amino-terminal regions of actin. The 20- kDa fragments, extending from Lys^{579} (or Leu^{597}) through the end of the molecule, are competitive with antibodies directed against the amino-terminal 7 residues of actin although the effect is not as dramatic as with intact caldesmon (51). Thus, the 20-kDa fragment contains part, but perhaps not all, of the actin binding site(s) of caldesmon.

We have defined the actin binding region of the 20-kDa fragment using more extensive chymotryptic digestion to produce smaller actin-binding fragments. A peptide, derived from the 20-kDa fragments, having an apparent molecular mass of about 7.3 kDa has been purified extensively. The 7.3- kDa peptide begins at Leu^{597} and ends at Phe^{665}. Fig. 7 summarizes the location of the fragments of caldesmon generated in this study along with several fragments produced in other laboratories. *Solid bars* indicate polypeptides with a demonstrated ability to block the actin activation of myosin ATPase. Note that not all of the fragments have been tested for inhibitory activity; an *open bar* does not necessarily imply a lack of inhibitory activity. The 20-kDa fragments originate either at Lys^{579} (*a*) or Leu^{597} (*b*). Both of these fragments have heterogeneity in their carboxyl-terminal regions and may end either at Phe^{752} or near Thr^{740}. These different carboxyl-terminal ends are not indicated in Fig. 7. The 7.3-kDa fragment derived from *a* and *b* and which contains their major activities is labeled *k.* All fragments which are known to inhibit ATPase activity overlap to some degree with fragment *k*. Thus it is highly likely that this region is important in the inhibition of ATP hydrolysis (it will be shown later that other regions may also be important for ATPase inhibition).

Our observation that the 7.3-kDa fragment binds to calmodulin is consistent with data from other laboratories. Fragment *g* (Wang's fragment 15) binds to calmodulin, whereas fragment *h* (Wang's fragment 15′) does not (40). This restricts the calmodulin binding sequence to residues 629–666. Comparison with fragment *i* (59) narrows the calmodulin binding region to residues 659–666, which is almost completely within the 7.3-kDa region of caldesmon (fragment *k*). Recently, a synthetic peptide including residues Gly^{651} through Ser^{667}, of caldesmon (Fig. 7, fragment *p*) was shown to bind to calmodulin and also weakly to actin (60).

It is also reasonable that our 7.3-kDa fragment binds to actin and inhibits actin-activated ATP hydrolysis. Consider fragments *l* and *m* (19), *n* (61), and *o* (36,62). Each has been reported to bind to actin to some degree, although only fragment *l*, which contains the entire 7.3-kDa sequence of fragment *k*, inhibits ATP hydrolysis. Comparison of the 7.3- kDa fragment, *k*, with fragments *l* and *m* (19) suggests that the peptide between 570 and 670 is critical for inhibition for ATPase activity. Hayashi *et al.* (19) have restricted this region further by arguing, from a comparison of fragments labeled *d* and *l*, that a smaller region, between 621 and 673, is sufficient for both inhibition of ATPase activity and reversal by Ca^{2+}- calmodulin.

Bartegi *et al.* (59) have reported that a CNBr fragment, *i*, inhibits ATPase activity and is reversed by Ca^{2+} in a manner similar to the 7.3-kDa fragment (fragment *k*). These fragments have only a 7-amino acid sequence overlap, between residues 659 and 665. It is unclear, however, whether the properties of fragment *i* (59) and fragment *k* are functionally the same since their reported stoichiometries of binding to actin are very different. The stoichiometry of the 7.3-kDa fragment to actin is 1 mol of fragment to 1 mol of actin. In contrast, the stoichiometry for fragment *i* reported by Bartegi *et al.* (59) is 1:7, more similar to intact caldesmon than to any of the shorter fragments. We consider the value for fragment *k* to be reasonable since we determined the stoichiometry of intact caldesmon to be between 1:7 and 1:10 and the 20-kDa fragment (fragment *a*) to be 1:2 using the same method. Furthermore, others have measured stoichiometries of binding for the 38-kDa actin binding region to be about 1:4 and that of the 20-kDa fragment (fragment *a*) to be about 1:2 (23). It is unclear how the shorter fragment *i* could either acquire additional binding sites and/or be able to sterically interfere with fragment binding to additional actin monomers. This issue needs to be resolved since it is crucial to understanding whether caldesmon has a single actin binding site centered around residues 659–665 or two sites flanking this peptide as suggested by Wang *et al.* (40). The available data are consistent either with two binding sites, one on fragment *k* and a second on fragment *i* or with a single site overlapping the small shared peptide.

It should be pointed out that Wang *et al.* observed that a deletion of the terminal 39 amino acid residues from fragment *a* resulted in a fragment *c* which was a poor inhibitor of actin- activated ATPase activity (40). They suggested that caldesmon binds to actin through residues 597–629 and at 711–756 and that the latter region, at the extreme carboxyl-terminal end of caldesmon, is important for maintenance of ATPase activity. The former region (597–629) is thought to have the higher actin affinity since fragments *f* and *c* (Wang's fragments 19a and CaD 579–717) bind to actin more tightly than fragment *e* (Wang's fragment 19b) (40). Note that fragment c (Wang's CaD 579–717) was shown to inhibit ATPase activity. The involvement of the carboxyl-terminal region in ATP-ase inhibition may be supported by the analysis of peptide *i* by Bartegi *et al.* (59). However, as stated earlier, peptide *i* overlaps residues 659–665 of peptide *k*, and it is possible that these residues, and not the extreme carboxyl-terminal region, are critical for inhibition. Wang *et al.* also observed that neither peptide *h* (their peptide 15′) (40) nor *j* (61) binds to actin, although both contain the carboxyl-terminal region of caldesmon. While this could be taken as evidence that the carboxyl-terminal region is not critical, Wang *et al.* suggested that the lack of binding by peptide *h* is because of the presence of a sequence which blocks binding to actin. However, we also note that peptide *h* is missing the 659–665 sequence which we suggest is important for inhibition of ATPase activity. Lack of binding of fragment *j* (61), equivalent to fragment *i* which does bind to actin (59), has been attributed to the harsh isolation conditions (59). We have also prepared a carboxyl-terminal caldesmon fragment using CNBr digestion (Table I). In our own preliminary experiments, this fragment did bind to actin in agreement with Bartegi *et al.* (53). Wang *et al.* (40) have isolated an additional fragment, *g* (their fragment 15), which has the peculiar property of binding calmodulin but not actin, although it contains the 659–665 overlap peptide. The lack of actin binding of this peptide has been attributed to an inhibitory sequence (40). The possibility of an additional inhibitory site at the carboxyl terminus of caldesmon must be considered.

Hayashi *et al.* (19) and Mornet *et al.* (36,62) have argued for an additional actin binding site on fragments *m*, *n*, and *o* in the sequence between 446 and 578. These fragments do not inhibit ATP hydrolysis, It is unclear whether all these putative actin binding sequences are in close proximity with each other in space forming a single actin binding site or whether they are separated and able to associate with different actin monomers. The presence of several actin binding regions on caldesmon which interact with different actin monomers is an attractive prospect since the stoichiometry of binding of caldesmon to actin varies with the size of the caldesmon fragment. However, only two separate, nonoverlapping sets of peptides have been shown to bind to actin, those containing sequence 446–578 and those containing sequence 659–665. Alternatively, binding of the carboxyl-terminal region of caldesmon to actin may position the rest of the caldesmon molecule so that the binding of additional caldesmon fragments is sterically blocked from binding. The longer the polypeptide attached to the carboxyl-terminal end, the greater is the apparent stoichiometry of binding. Determination of the region(s) of both actin and caldesmon involved in binding should differentiate between these models.

Caldesmon is able to virtually eliminate the binding of S-1-ATP to actin (10), whereas nucleotide-free S-1 is able to displace virtually all bound caldesmon from actin (11). Therefore any proposal for the binding of caldesmon to actin must be able to explain the ability of one caldesmon molecule to compete with about seven molecules of S-1. These competition data have been successfully modeled by assuming that 1) caldesmon binds to one or two actin monomers through the amino-terminal region of actin totally blocking the binding of S-1 to these residues, and 2) the rest of the caldesmon molecule only partially inhibits the binding of S-1 to actin[3]. Thus, the function of caldesmon does not require that it has a series of identical binding sites. Rather, a single strong binding site, such as that described in this report, as well as weaker interactions with other actin monomers (at undefined regions of the actin monomer) are sufficient to explain available binding data.

References

1. Sobue K, Muramoto Y, Fujita M, Kakiuchi S. Proc Natl Acad Sci U S A 1981;78:5652–5655. [PubMed: 6946503]
2. Owada MK, Hakura A, Iida K, Yahara I, Sobue K, Kakiuchi S. Proc Natl Acad Sci U S A 1984;81:3133–3137. [PubMed: 6328499]
3. Sobue K, Tanaka T, Kanda K, Ashino N, Kakiuchi S. Proc Natl Acad Sci U S A 1985;82:5025–5029. [PubMed: 2991905]
4. Sobue K, Fujio Y. Adv Exp Med Biol 1990;255:325–336. [PubMed: 2533456]
5. Dabrowska R, Goch A, Galazkiewicz B, Osinska H. Biochim Biophys Acta 1985;842:70–75. [PubMed: 2931121]
6. Smith CWJ, Marston SB. FEBS Lett 1985;184:115–119. [PubMed: 3987897]
7. Ngai PK, Walsh MP. Biochem J 1985;230:695–707. [PubMed: 2998332]
8. Sobue K, Takahashi K, Wakabayashi I. Biochem Biophys Res Commun 1985;132:645–651. [PubMed: 4062943]
9. Lash JA, Sellers JR, Hathaway DR. J Biol Chem 1986;261:16155–16160. [PubMed: 2946681]
10. Chalovich JM, Cornelius P, Benson CE. J Biol Chem 1987;262:5711–5716. [PubMed: 2952642]
11. Hemric ME, Chalovich JM. J Biol Chem 1988;263:1878–1885. [PubMed: 2962997]
12. Ikebe M, Reardon S. J Biol Chem 1988;263:3055–3058. [PubMed: 3257755]
13. Hemric ME, Chalovich JM. J Biol Chem 1990;265:19672–19678. [PubMed: 2246251]
14. Sutherland C, Walsh MP. J Biol Chem 1989;264:578–583. [PubMed: 2909541]
15. Hemric ME, Carey JO, Lu F, Chalovich JM. Biophys. J 1991;59:57.(abstr.)

[3]Y. -d. Chen and J. M. Chalovich, submitted for publication.

16. Wang CLA, Chalovich JM, Graceffa P, Lu RC, Mabuchi K, Stafford WF. J Biol Chem 1991;266:13958–13963. [PubMed: 1856225]
17. Ball EH, Kovala T. Biochemistry 1988;27:6093–6098. [PubMed: 3191109]
18. Bryan J, Lee R. J Muscle Res Cell Motil 1991;12:372–375. [PubMed: 1939602]
19. Hayashi K, Fujio Y, Kato I, Sobue K. J Biol Chem 1991;266:355–361. [PubMed: 1824698]
20. Fujii T, Imai M, Rosenfeld GC, Bryan J. J Biol Chem 1987;262:2757–2763. [PubMed: 2434491]
21. Szpacenko A, Dabrowska R. FEBS Lett 1986;202:182–186. [PubMed: 2941315]
22. Yazawa M, Yagi K, Sobue K. J Biochem (Tokyo) 1987;102:1065–1073. [PubMed: 3436963]
23. Riseman VM, Lynch WP, Nefsky B, Bretscher A. J Biol Chem 1989;264:2869–2875. [PubMed: 2914935]
24. Horiuchi KY, Chacko S. Biochemistry 1989;28:9111–9116. [PubMed: 2532547]
25. Velaz L, Ingraham RH, Chalovich JM. J Biol Chem 1990;265:2929–2934. [PubMed: 2137453]
26. Brenner B, Yu LC, Chalovich JM. Proc Natl Acad Sci U S A 1991;88:5739–5743. [PubMed: 2062853]
27. Spudich JA, Watt S. J Biol Chem 1971;246:4866–4871. [PubMed: 4254541]
28. Eisenberg E, Kielley WW. Cold Spring Harbor Symp Quant Biol 1972;37:145–152.
29. Bretscher A. J Biol Chem 1984;259:12873–12880. [PubMed: 6092349]
30. Yazawa M, Sakuma M, Yagi K. J Biochem 1980;87:1313–1320. [PubMed: 6104658]
31. Kielley WW, Harrington WF. Biochim Biophys Acta 1960;41:401–421. [PubMed: 14408979]
32. Weeds AG, Taylor RS. Nature 1975;257:54–56. [PubMed: 125854]
33. Velaz L, Hemric ME, Benson CE, Chalovich JM. J Biol Chem 1989;264:9602–9610. [PubMed: 2524487]
34. Chalovich JM, Eisenberg E. J Biol Chem 1982;257:2432–2437. [PubMed: 6460759]
35. Laemmli UK. Nature 1970;227:680–685. [PubMed: 5432063]
36. Leszyk J, Mornet D, Audemard E, Collins JH. Biochem Biophys Res Commun 1989;160:564–571.
37. Bryan J. J Muscle Res Cell Motil 1989;10:95–96. [PubMed: 2668328]
38. Smith CWJ, Pritchard K, Marston SB. J Biol Chem 1987;262:116–122. [PubMed: 2947901]
39. Sobue K, Morimoto K, Inui M, Kanda K, Kakiuchi S. Biomed Res 1982;3:188–196.
40. Wang CLA, Wang LWC, Xu S, Lu RC, Saavedra-Alanis V, Bryan J. J Biol Chem 1991;266:9166–9172. [PubMed: 2026616]
41. Yamazaki K, Itoh K, Sobue K, Mori T, Shibata N. J Biochem 1987;101:1–9. [PubMed: 3553171]
42. Ngai PK, Walsh MP. Biochem J 1987;244:417–425. [PubMed: 2822003]
43. Horiuchi KY, Miyata H, Chacko S. Biochem Biophys Res Commun 1986;136:962–968. [PubMed: 2941015]
44. Clark T, Ngai PK, Sutherland C, Groschel-Stewart U, Walsh MP. J Biol Chem 1986;261:8028–8035. [PubMed: 2940249]
45. Wang CLA. Biochem Biophys Res Commun 1988;156:1033–1038. [PubMed: 3190671]
46. Mabuchi K, Wang CLA. J Muscle Res, Cell Motil 1991;12:145–151. [PubMed: 2061408]
47. Wang CLA, Wang LWC, Lu RC. Biochem Biophys Res Commun 1989;162:746–752. [PubMed: 2757638]
48. Graceffa P. FEBS Lett 1987;218:139–142. [PubMed: 3595858]
49. Mornet D, Harricane MC, Audemard E. Biochem Biophys Res Commun 1988;155:808–815. [PubMed: 3421967]
50. Bryan J, Imai M, Lee R, Moore P, Cook RG, Lin WG. J Biol Chem 1989;264:13873–13879. [PubMed: 2760048]
51. Adams S, DasGupta G, Chalovich JM, Reisler E. J Biol Chem 1990;265:19652–19657. [PubMed: 2246250]
52. Levine BA, Moir AJG, Audemard E, Mornet D, Patchell VB, Perry SV. Eur J Biochem 1990;193:687–696. [PubMed: 2147415]
53. Bartegi A, Fattoum A, Kassab R. J Biol Chem 1990;265:2231–2237. [PubMed: 2298747]

54. Bertrand R, Chaussepied P, Audemard E, Kassab R. Eur J Biochem 1989;181:747–754. [PubMed: 2525090]
55. DasGupta G, Reisler E. J Mot Biol 1989;207:833–836.
56. Crosbie R, Adams S, Chalovich JM, Reisler E. J Biol Chem 1991;266:20001–20006. [PubMed: 1939062]
57. Graceffa P, Jancso' A. J Biol Chem 1991;266:20305–20310. [PubMed: 1834643]
58. Makuch R, Kolakowski J, Dabrowska R. FEBS Lett 1992;297:237–240. [PubMed: 1531959]
59. Bartegi A, Fattoum A, Derancourt J, Kassab R. J Biol Chem 1990;265:15231–15238. [PubMed: 2394719]
60. Zhan Q, Wong SS, Wang CLA. J Biol Chem 1991;266:21810–21814. [PubMed: 1939204]
61. Takagi T, Yazawa M, Ueno T, Suzuki S, Yagi K. J Biochem (Tokyo) 1989;106:778–783. [PubMed: 2613684]
62. Mornet D, Audemard E, Derancourt J. Biochem Biophys Res Commun 1988;154:564–571. [PubMed: 3401222]
63. Mejean C, Boyer M, Labbe JP, Derancourt J, Benyamin Y, Roustan C. Biosci Rep 1986;6:493–499. [PubMed: 3742019]

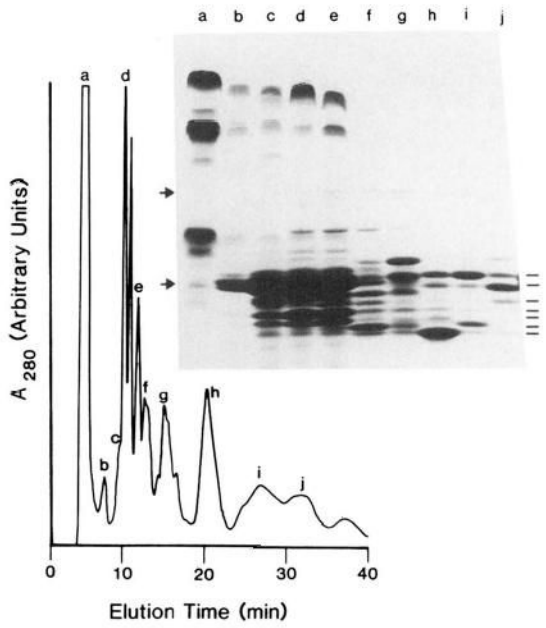

Fig. 1. HPLC cation exchange chromatography of a chymotryptic digest.

Caldesmon was digested with a 1:500 (w:w) ratio of chymotrypsin to caldesmon for 5 min at 25 °C. The digest was loaded onto a Waters SP R column equilibrated with 10 mM Tris- Mes (pH 7.0), 60 mM NaCl, 1 mM dithiothreitol. The column was washed with 15 ml of the same buffer followed by a gradient to 10 mM Tris-Mes, 500 mM NaCl, 1 mM dithiothreitol. Fraction *h* is enriched in the 7.3-kDa fragment, and fractions *i* and *j* were used as the source of the 20-kDa actin-binding fragments. The *arrows* denote the position of actin (42,000) and soybean trypsin inhibitor (21,500) standards.

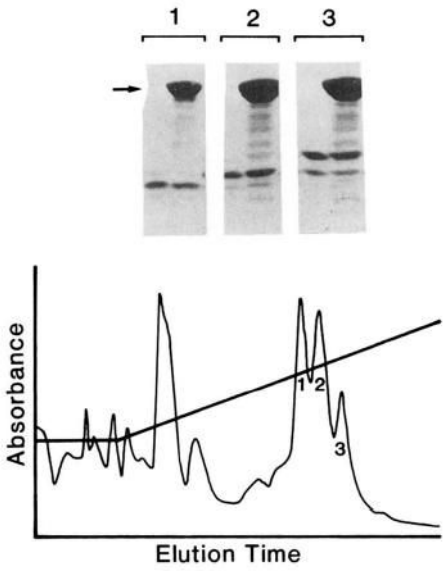

Fig. 2. HPLC cation exchange purification of the 20-kDA fragments and demonstration of binding to actin.

Crude 20-kDa fragments were first prepared by AcA 54 gel filtration of a 1:1,000 (*chymotrypsin:caldesmon*) digest of caldesmon. Fractions containing the 20-kDa fragments were applied to a Waters SP R column as described in Fig. 1, and the elution pattern is shown here. The peaks labeled *1*,*2*, and *3* were enriched in the polypeptides of interest. *Inset*, the contents of the three peaks were tested for actin binding in co-sedimentation assays; a Coomassie Blue-stained electrophoretic transfer of an SDS gel of both supernatants and pellets of peaks *1*,*2*, and *3* is shown. The *arrow* shows the position of actin which *is* present only in the pellets. Each peak is enriched in a single polypeptide which binds to actin. The bands were subsequently excised from the blots shown and used for sequence analysis.

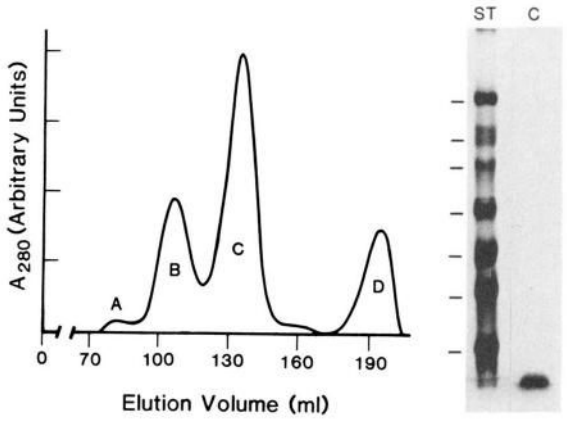

Fig. 3. Purification of the 7.3-kDa fragment of caldesmon.

Fraction *h* from Fig. 1 was applied to a 1.5 × 90-cm column of AcA 54 equilibrated with 500 mM KCl, 20 mM Tris (pH 8), 1 mM EDTA, 1 mM EGTA, and 1 mM dithiothreitol and washed with the same buffer at a flow rate of 15 ml/h. Traces of polypeptides greater than *35* kDa were eluted at 88 *ml* (peak *A*). 20-kDa fragments were eluted at 105 ml (peak *B*). Pure 7.3-kDa fragment was eluted at 134 ml (peak *C*), and peak *D* was unidentified. The *inset* shows the pure 7.3- kDa fragment (*C*) and molecular weight standards (*ST*). The pure 7.3-kDa fragment was subsequently transferred to a polyvinylidene difluoride membrane for amino-terminal analysis.

Fig. 4. Binding of the 7.3-kDa fragment of caldesmon to actin and actin-tropomyosin.

Either caldesmon or ^{125}I-caldesmon was co-sedimented with 5 μM actin (○) or 5 μM actin with 1.4 μM smooth muscle tropomyosin (•) at 25 °C in a buffer containing 42 mM NaCl, 4.3 mM $MgCl_2$, 11 mM imidazole-HCl (pH 7.0), 0.25 mM EGTA, and 1 mM dithiothreitol. In experiments with calmodulin, the EGTA was replaced with 0.5 mM $CaCl_2$. The *inset* is an expanded view of the data at low 7.3-kDa fragment concentration. Also included are data obtained in the presence of 6 μM S-1 (+) or 20 μM calcium-calmodulin (×). All data were obtained with a single preparation of the 7.3-kDA fragment.

Fig. 5. Binding of the 7.3-kDa fragment to calmodulin-Sepharose.

0.25 mg of 7.3-kDa fragment of caldesmon in 150 mM NaCl, 10 mM imidazole-HCl (pH 7.0), 1 mM $CaCl_2$, and 1 mM NaN_3 was loaded onto a 1-ml column of calmodulin-Sepharose (10 mg of calmodulin/ml of Sepharose) equilibrated with the same buffer. At the position indicated, the buffer was replaced with a similar buffer with the $CaCl_2$ replaced by 2 mM EGTA. The column was run at 5 °C at a flow rate of 5 ml/h, and the absorbance was monitored at 280 nm. Electrophoretic analysis confirmed the presence of the 7.3-kDa fragment in both the breakthrough peak and the major peak.

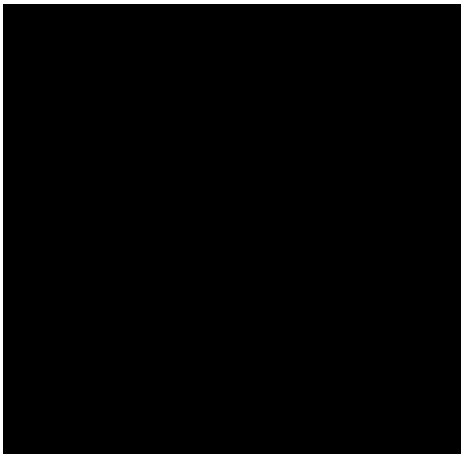

Fig. 6. Inhibition of the ATPase activity of acto-S-1 and reversal by calcium-calmodulin.

ATPase rates were measured at 25 °C in a buffer containing 1 mM ATP, 4.4 mM $MgCl_2$, 10.4 mM imidazole-HCl (pH 7.0), 0.25 mM EGTA, and 1 mM dithiothreitol. The protein concentrations were: 0.2 μM skeletal myosin S-1, and 10 μM actin without (○) or with (•) 2.8 μM smooth muscle tropomyosin. Also shown is the effect of a different preparation of the 7.3-kDa fragment on smooth muscle S-1 in the absence of tropomyosin (×). *Inset*, reversal of ATPase activity of skeletal S-1, in the presence of tropomyosin, with 10 and 20 μM total calmodulin. For this experiment 0.25 mM $CaCl_2$ was added rather than EGTA.

Fig. 7. Summary of polypeptide fragments from the region of caldesmon extending from residue 450 through the carboxyl terminus (residue 756).

The primary structure derived from Bryan *et al.* (50) is used as a reference; residue numbers from Hayashi *et al.* (19) have been reduced by 15 to compare with the other data shown. *Solid bars* represent fragments with a demonstrated ability to inhibit actin-activated ATPase activity. *Upper case* letters are used to indicate fragments which bind to actin *A* or calmodulin *C*. Fragments *a*, *b*, and *k* are from the present paper. Fragments *a* and *b* were also discussed by Velaz *et al.* (25), and fragment *a* was discussed by Hayashi *et al.* (19), and Riseman *et al.* (23). Fragments *c*, *e*, *f*, *g*, and *h* are from Wang *et al.* (40); *d*, *1*, and *m* are from Hayashi *et al.* (19); fragment *i* is from Bartegi *et al.* (59); *j* and *n* are from Takagi *et al.* (61); *o* is from Mornet *et al.* (36,62); and *p* is from Zhan *et al.* (60).

Table I

Primary structure and molecular weight summary of chymotryptic fragments

Molecular mass estimates				
SDS-PAGE	**Gel filtration**	**Amino acid composition**	**N terminus**	**C terminus**
		kDa		
22.9	22.9		Lys^{579}	Pro^{756}*a*
20.1	19.9	18.8	Lys^{579}	Thr^{740}-Phe^{752}*a*
20.1			Lys^{597}	Pro^{756}*a*
18.2	15	14.9	Leu^{597}	Thr^{740}-Thr^{746}*a*
10		7.8	Leu^{597}	Phe^{665}*b*
10*c*			Tyr^{659}-Ser^{666}	Pro^{756}*a*

a Estimated from molecular weight.

b Determined from carboxypeptidase *a* digestion.

c Prepared from a CNBr digestion of caldesmon.