Expressing Functional Domains of Mouse Calponin: Involvement of the Region Around Alanine 145 in the Actomyosin ATPase Inhibitory Activity of Calponin†

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ABSTRACT: Previously, we attributed the binding of F-actin to the 38-residue stretch of gizzard calponin encompassing the sequence A145−Y182 and postulated the hexapeptide motif VKYAEK, representing residues 142−147, as a putative actin-binding site [Mezgueldi, M., Fattoum, A., Derancourt, J. & Kassab, R. (1992) *J. Biol. Chem. 267,* 15943−15951]. Herein, the nature of the ATPase inhibitory amino acids of calponin and their relative position within the actin binding domain was investigated by expressing the following fragments of mouse calponin with or without substitution or deletion of the hexapeptide V142−K147: amino acids 1−228 (CaP1−228), 45−228 (CaP45−228), 131−228 (CaP131−228), and CaP1−228 with substitution of A145 with S (CaP1−228A145S) or deletion of V142−K147 (CaP1−228del142−147). All the recombinant fragments displayed most of the biochemical properties of the smooth muscle purified calponin including (a) expected electrophoretic mobility, (b) heat stability, (c) binding to actin, tropomyosin and calmodulin, and (d) zero-length cross-linking to actin switched by calmodulin in a calcium-dependent fashion. However, while the wild-type recombinant fragments inhibit the acto-S-1 ATPase activity to the same extent as do the parent calponin, modulation of the hexapeptide by either substitution or deletion strongly affect the inhibitory activity with only slightly decreasing actin binding capacity. The data indicate that the stretch VKYAEK is crucial for ATPase inhibition by calponin but represents only part of the actin-binding domain. These results are discussed in terms of multiple contact sites between actin and calponin.

Smooth muscle contraction was first considered to be regulated solely by a myosin linked system acting via the specific phosphorylation of the regulatory light chains of myosin by myosin light chain kinase (Adelstein & Eisenberg, 1980). Work over the last few years, however, strongly supports the existence of a functional thin filament-associated regulatory system, based on the proteins caldesmon or calponin or both (Marston & Redwood, 1991; Takahashi et al., 1986, 1988; Abe et al., 1990; North et al., 1994). Calponin is a 34 kDa actin-, calmodulin-, and tropomyosin-binding protein that inhibits the actomyosin ATPase activity (Takahashi et al., 1988; Abe et al., 1990) as well as the movement of actin filaments over coated myosin in *in vitro* enzyme and motility assays, respectively (Shirinsky et al., 1992; Haeberle, 1994). This inhibitory activity was abolished by the *in vitro* phosphorylation of the protein (Winder et al., 1993) or by its interaction with Ca^{2+}-calmodulin which has been first implicated in the modulation of calponin function (Takahashi et al., 1986). More recent studies have proposed other Ca^{2+}-binding proteins, such as caltropin or S100b protein (Wills et al., 1993, 1994a), as more likely candidates for the calcium regulation of calponin function.

We have localized the inhibitory activity as well as the actin-, calmodulin-, and tropomyosin-binding sites of gizzard calponin in the 22 kDa N-terminal domain corresponding to the sequence N7−Y182 (Mezgueldi et al., 1992) and postulated that the segment A145−Y182 contains the major structural determinants for F-actin binding. These conclusions were confirmed by Nakamura et al. (1993) and were in agreement with the data of Winder and Walsh (1990a) indicating that a chemical 21 kDa fragment, most probably corresponding to the sequence C61−R237, exhibits all calponin functions. The former study (Mezgueldi et al., 1992) also suggested the six-residue stretch VKYAEK, at position 142−147, as an actin-interactive sequence. More recently, using synthetic peptides we defined the sequence A145−I163 as the minimum sequence necessary for actin binding and inhibitory activity (Mezgueldi et al., 1995). In the latter study we also described the sequence Q153−I163 as a new calmodulin, and caltropin-binding site and proposed the coupling of this site to the reversal of calponin inhibition by Ca^{2+}-binding proteins. While the location of the critical F-actin recognition site within the 145−182 region was closely established from those studies, the nature of the actin-binding and inhibitory amino acids as well as their positioning relative to the regulatory calmodulin-binding site remain to be delineated. This would help to gain a better under-

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standing of the calponin's regulatory mechanism. In this regard, we (Mezgueldi et al., 1992) reported the interaction of calponin on the COOH-terminal region of actin at or near E334 and emphasized the involvement of this actin region in the inhibitory property of the protein on the actomyosin ATPase. Recently, the actin segment P332−E334 has been presented as a major hydrophobic contact site in the actin−myosin-S-1 interface (Rayment et al., 1993).

In this work, we have produced various recombinant fragments of mouse h1-calponin. We have characterized their functions using cosedimentation with F-actin and actomyosin S1-ATPase assays, cross-linking reactions, and affinity chromatography over immobilized calmodulin or tropomyosin. Collectively, the data show, for the first time, that the polypeptide A131−I228 missing the large N-terminal and C-terminal domains displays the actin binding and the inhibitory activity in a concentration range comparable to the parent calponin. This segment also interacts with tropomyosin and Ca^{2+}-calmodulin. The binding of the latter protein is coupled to the reversal of ATPase inhibition. Furthermore, alterations of the VKYAEK at position 142−147 motif by site-directed mutagenesis strongly affect the inhibitory activity of calponin with only a slight decrease of actin binding, further indicating that this region is essential for calponin inhibitory capacity but does not represent the entire actin binding site. This allows one to distinguish, for the first time, the actin binding function of calponin from its actomyosin ATPase inhibitory activity. These findings emphasize the use of recombinant technology and mutagenesis to study calponin structure−function relationships as has been largely done for the other regulatory protein, caldesmon (Wang et al., 1991; Redwood & Marston, 1993; Marston et al., 1994) and briefly reported for calponin (Gong et al., 1993).

MATERIALS AND METHODS

Chemicals. 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) and *N*-hydroxysuccinimide (NHS) were from Sigma, Heidelberg.

Molecular Cloning, Expression, and Purification of the Recombinant Calponin Fragments. All DNA manipulations were performed according to standard protocols (Maniatis et al., 1982). Mouse h1-calponin cDNA fragments were cloned into the bacterial expression vector pMW172 and expressed in *Escherichia coli* BL21(DE3) as described (Strasser et al., 1993). For the construction of N- and C-terminal deletion mutants, the cDNA was digested with suitable restriction enzymes, and the fragments were subcloned either directly into pMW172 or, when necessary, after polishing of the DNA ends by Klenow polymerase or Mung bean nuclease. In several cases linkers had to be introduced to create compatible restriction sites for subcloning into the expressing vector. Internal deletion or base substitutions were generated by the polymerase chain reaction (PCR) using primers carrying the desired mutations. The DNA sequences of all constructs were confirmed prior to expression in *E. coli*.

The recombinant proteins were purified as recently described (Wills et al., 1994b). Prior to use, the mutant CaP1−228A145S and CaP1−228del142−147 were further purified by affinity chromatography over tropomyosin-Sepharose using 10 mM Tris-HCl, 10 mM KCl, 1 mM $MgCl_2$, 0.1 mM dithiothreitol, and 1 mM NaN_3, pH 7.0, for loading and the same buffer containing 200 mM KCl for elution.

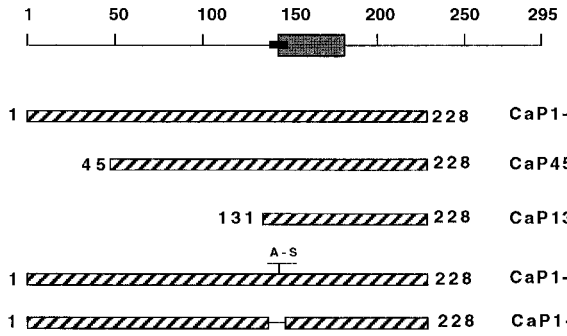
Muscle Proteins. Calponin from fresh turkey gizzards, F-actin, and chymotryptic myosin subfragment-1 (S-1) from rabbit skeletal muscle were prepared as previously described (Mezgueldi et al., 1992, 1994, 1995). Bovine brain calmodulin was purified by published procedures (Gopalakrishna & Anderson, 1982; Dedman & Kaetzel, 1983). The CH22 chymotryptic fragment of calponin was produced as previously described (Mezgueldi et al., 1992, 1995).

Protein Concentrations. The concentrations of S-1, F-actin, calmodulin, and calponin were measured spectrophotometrically using $A^{1\%}_{280nm}$ = 7.5, 11.7, 2.0, and 6.8 (Mezgueldi et al., 1992, 1995), respectively. The concentration of the recombinants peptides was determined assuming $A^{1\%}_{280nm}$ = 7.2 for CaP1−228 and CaP1−228A145-S calculated from the knowledge of the presence of two tryptophan residues and six tyrosine residues/mol peptide, 6.8 for CaP1−228del142−147 calculated from the knowledge of the presence of two tryptophan residues and five tyrosine residues/mol peptide, 4.9 for CaP45−228 calculated from the knowledge of the presence of one tryptophan residue and four tyrosine residues/mol peptide, and 3.1 for CaP131−228 calculated from the knowledge of the presence of three tyrosine residues/mol peptide (Gill & Von Hippel, 1989). Calculations were based on molecular mass values of 115 kDa for S-1, 42 kDa for actin, 17 kDa for calmodulin, and 32kDa for calponin. The molecular masses of recombinant peptides were 25.3 kDa for CaP1−228 and CaP1−228A145S, 24.7 kDa for CaP1−228del142−147, 20.5 kDa for CaP45−228, 10.8 kDa for CaP131−228, and 19.7 kDa for the chymotryptic peptide CH22, as calculated from the corresponding sequences.

Electrophoresis. SDS−polyacrylamide gel electrophoresis was carried out in 5−18% gradient acrylamide gels or 8−18% thin layer SDS gel electrophoresis (Pharmacia LKB Biotechnology Inc., ExcelGel) (Laemmli, 1970). The running buffer was 50 mM Tris and 100 mM boric acid, pH 8.0. The gels were stained with Coomassie Blue R-250 and destained with 7% acetic acid. Densitometric scanning of the protein bands was performed with a Howtek Scanmaster 3 scanner and Software PDI version 2.2. The immunoblotting was performed as previously reported (Strasser et al., 1993).

F-Actin-, Calmodulin-, and Tropomyosin-Binding Assays. The interaction of F-actin and recombinant fragments was analyzed by cosedimentation in 10 mM imidazole-HCl, 30 mM NaCl, 2 mM $MgCl_2$, and 1 mM NaN_3, pH 7.0, using a Beckman Airfuge at 140000*g*, for 30 min at 25 °C.

Peptides were dialyzed twice for 12 h at 4 °C in 3.5-kDa cut-off tubing against the 10 mM imidazole-HCl buffer, pH 7.0. After cosedimentation with F-actin, the pellet was dissolved in the Laemmli buffer, separated by gel electrophoresis, and analyzed by scanning densitometry. The amount of bound CaP, CaP1−228, and CaP1−228del142−147 was determined from a calibration curve generated by scanning known quantities of calponin or calponin mutants loaded on the same gel. All the points were within the linear range. The binding curves were obtained by cosedimenting 4 μM calponin or calponin mutants and 0−50 μM actin (Figure 3B) or 10 μM actin and 0−40 μM calponin or calponin mutants (data not shown).



	Molecular weight (kDa)	$A^{1\%}_{280nm}$
CaP1-228	25.3	7.2
CaP45-228	20.5	4.9
CaP131-228	10.8	3.1
CaP1-228A145S	25.3	7.2
CaP1-228del142-147	24.7	6.8

FIGURE 1: Schematic representation of the various calponin recombinant fragments used. The recombinant peptides are drawn to scale compared to the native mouse h1 molecule (top). The shaded area over the calponin sequence refers to the putative actin binding segment A145−Y182 and the overlapping solid bar to the motif VKYAEK at position 142−147 (Mezgueldi et al., 1992). The molecular weights and $A^{1\%}_{280nm}$, calculated from the corresponding sequences and on the basis of their tryptophan and tyrosine contents as described in Materials and Methods, are indicated.

The binding of the recombinant proteins to calmodulin and tropomyosin were assayed by affinity chromatography as previously reported (Mezgueldi et al., 1992; Vancompernolle et al., 1990).

Cross-Linking Reactions. The recombinant fragments (0.2−0.4 mg/mL) in 10 mM imidazole HCl, 30 mM NaCl, 2 mM $MgCl_2$, and 1 mM NaN_3, pH 7.0, were mixed with F-actin (fragments/actin molar ratio = 1:3) in the presence of 2 mM EDC and 5 mM NHS. The reaction was carried out at 25 °C for 0−30 min in the absence or presence of Ca^{2+}-calmodulin (peptides/Ca^{2+}-calmodulin molar ratio = 1:6) and was monitored by SDS gel electrophoresis.

ATPase Measurements. The Mg^{2+}-ATPase of the skeletal muscle acto-S-1 was determined in a medium (1 mL) containing 50 mM Tris-HCl, 10 mM KCl, and 2.5 mM $MgCl_2$, pH 7.5, using 0.42 mg/mL F-actin and 0.050 mg/mL S-1. The reaction was started by adding 5 mM Mg-ATP and terminated after 10 min by trichloroacetic acid. P_i was determined colorimetrically as described previously (Mezgueldi et al., 1992, 1995).

RESULTS

Expression and Purification of Calponin Mutants. We expressed a set of recombinant calponin fragments comprising the presumed actin binding region A145−Y182 and focused on the six-residue stretch corresponding to the putative actin-binding motif V142−K147. Figure 1 lists the five mutants produced and used in this study: CaP1−228, CaP45−228, and CaP131−228 are the fragments encompassing residues 1−228, 45−228, and 131−228, respectively, of the h_1 isoform of mouse calponin (Strasser et al., 1993). On the other hand, residue 145 in the mutant CaP1−228 was changed from A to S, generating mutant CaP1−228A145S. In addition, the sequence V142−K147 in the same mutant CaP1−228 was deleted to produce mutant CaP1−228del142−147. All the fragments and proteins have been expressed and obtained in high yield and purity (Figure 2A). The mobility of the recombinant fragments on SDS−PAGE gels matched the expected molecular mass as estimated from the corresponding sequences. The expressed fragments were also positively identified by immunoblotting using a polyclonal anti-calponin antibody (Figure 2 B). They were all soluble and retained the heat stability observed with native calponin. Even the shortest fragment, CaP131−228, was stable after boiling for 2 min at 95 °C in a water bath.

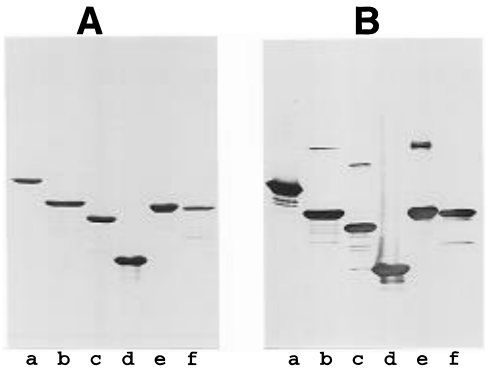


FIGURE 2: Coomassie Blue stained SDS−PAGE gel (A) and immunostaining with calponin antibodies (B) of the recombinant calponin mutants. The various fragments are indicated as follows: (a) CaP, (b) CaP1−228, (c) CaP45−228, (d) CaP131−228, (e) CaP1−228A145S, and (f) CaP1−228del 142−147.

Gong et al. (1993) have previously reported that full-length recombinant chicken calponin obtained by bacterial expression is indistinguishable from native calponin. Therefore, calponin's properties are not dependent upon posttranslational modifications. As detailed below, the wild-type recombinant fragments display the same biological activities as native smooth muscle calponin indicating that the procedures we employed for their purification from bacterial cells did not affect their functional conformation.

Interaction of F-Actin with the Calponin Mutants and Its Regulation by Ca^{2+}-Calmodulin. The binding of skeletal F-actin to each of the five recombinant fragments was analyzed by high speed cosedimentation and EDC cross-linking. Following centrifugation of each recombinant fragment in the absence or presence of F-actin (actin/recombinant fragment molar ratio = 3:1), the corresponding supernatants and pellets were analyzed by electrophoresis on a 5−18% gradient polyacrylamide gel (Figure 3A). The C-terminally truncated recombinant fragment lacking the last 67 amino acids of mouse calponin (CaP1−228) as well as the doubly truncated mutant additionally lacking the first 44 amino acids (CaP45−228) or the first 130 amino acids (CaP 131−228) bound to F-actin to the same extent as native purified calponin (Figure 3A, lanes c and d, and Figure 3B). Figure 3A also shows that CaP1−228A145S and CaP1−228del142−147 bound to F-actin, but the extent of this binding was slightly lower when compared to the native purified calponin or to the wild-type recombinant fragments. In the absence of F-actin all the five recombinant fragments remained in the supernatant (Figure 3A, lanes a and b).

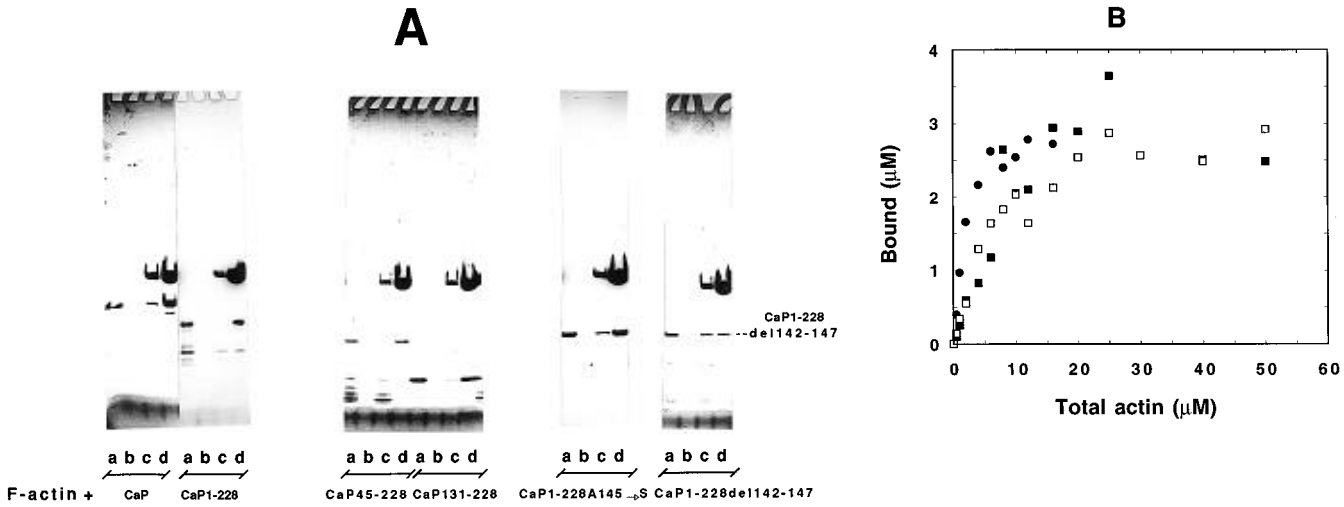


FIGURE 3: Interaction between F-actin and calponin mutants. (Panel A) The binding of the recombinant fragments, CaP1−228, CaP45−228, CaP131−228, CaP1−228 A145S, and CaP1−228del142−147, to F-actin (actin/fragment molar ratio = 3:1) was determined by cosedimentation followed by gel electrophoresis of the supernatant and pellet. The binding of calponin (CaP) to F-actin was assayed in parallel as a control. (Lanes a and b) Supernatant and pellet, respectively, of each polypeptide centrifuged alone. (Lanes c and d) Supernatant and pellet, respectively, of each polypeptide pelleted with F-actin. (Panel B) The binding of CaP (●), CaP1−228 (■), or CaP1−228del142−147 (□) to F-actin was determined by cosedimentation as described in Materials and Methods. Calponin or calponin recombinant fragments (4 μM) were incubated with a range of actin concentrations, as given in the abscissa. The molar amount of calponin or calponin fragments bound to actin is given in the ordinate.

More complete binding experiments of calponin mutants to actin support these observations. The binding of CaP, CaP1−228, and CaP1−228del142−147 to actin was determined under different conditions. Figure 3B shows that increasing concentrations of actin from 0 to 50 μM lead to an increase in the amount of bound calponin or calponin mutants to actin. The estimated K_d values were $<10^{-6}$ M for calponin, $1-3 \times 10^{-6}$ M for CaP1−228, and $2-5 \times 10^{-6}$ M for CaP1−228del142−147. A maximum of 75% of calponin or the recombinant fragments (CaP1−228 and CaP1−228del142−147) bound to actin. A similar value of 80% for maximum binding was recently reported for calponin binding to actin (Lu et al., 1995). We obtained similar results by cosedimenting fixed actin concentration (10 μM) and increasing concentration of calponin, CaP1−228, and CaP1−228del142-147 (0−40 μM) (data not shown). The deduced stoichiometry of binding of calponin or calponin recombinant fragments to actin is approximately 1:1. The results obtained shows that deletion of the hexapeptide 142−147 has only a slight effect on the actin binding property of calponin to actin.

These results provide the first direct experimental evidence that the hexapeptide motif of residues 142−147 contributes to the actin-binding activity of calponin, although weakly, and clearly suggest that the smallest recombinant fragment spanning residues 131−228 owns the major if not all the structural elements of the calponin actin-binding activity. We further tested the validity of this suggestion by investigating the ability of CaP131−228 to compete with native gizzard calponin for binding to F-actin. Figure 4 shows the potency of CaP131−228 to displace the parent protein molecule from the actin filament in a concentration-dependent manner. Displacement was only observed when the total calponin material (calponin + CaP131−228) exceeded the actin concentration (10 μM). This finding indicates that CaP131−228 contains the major structural determinants necessary for F-actin recognition and interacts with the same region of F-actin as the parent calponin.

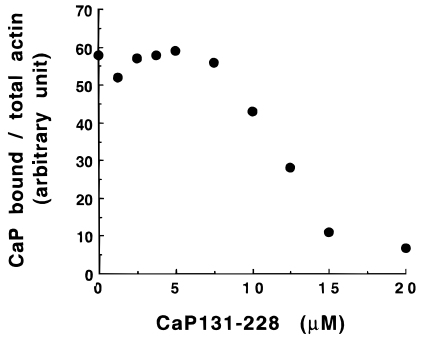


FIGURE 4: Competition between CaP131−228 and native calponin for binding to F-actin. Cosedimentation assays were performed as described under Materials and Methods using F-actin−calponin complex (10 μM F-actin and 2.5 μM calponin) and increasing concentrations of CaP131−228 up to 20 μM. After centrifugation, the pellets were analyzed by gel electrophoresis, and the amount of calponin bound to actin was measured by densitometry. Displacement was observed when the total calponin material exceeded the actin concentration.

The actin-binding activity of the recombinant fragments was also tested by chemical cross-linking achieved by the efficient EDC-NHS-catalyzed reaction. The experiments were conducted in the absence or presence of Ca^{2+}-calmodulin. The results are illustrated in Figure 5. After a 30-min reaction the recombinant fragment CaP45−228 cross-linked to F-actin (Figure 5B, lane b) to the same extent as the chymotryptic fragment spanning residues 7−182 (CH22) (Figure 5A, lane b). The cross-linking of the actin-CaP45−228 complex generated a novel product of about 64 kDa. The addition of Ca^{2+}-calmodulin (fragment/calmodulin molar ratio = 1:6) abolished the cross-linking process of either CH22 (Figure 5A, lane c) or CaP45−228 (Figure 5B, lane c) while the subsequent introduction of the Ca^{2+} chelator EGTA completely restored it (Figure 5A,B, lanes d). CaP1−228 could also be cross-linked to F-actin in a Ca^{2+}-calmodulin sensitive manner (data not shown). Figure 5C illustrates the time course of the cross-linking reaction between F-actin and the smallest recombinant fragment

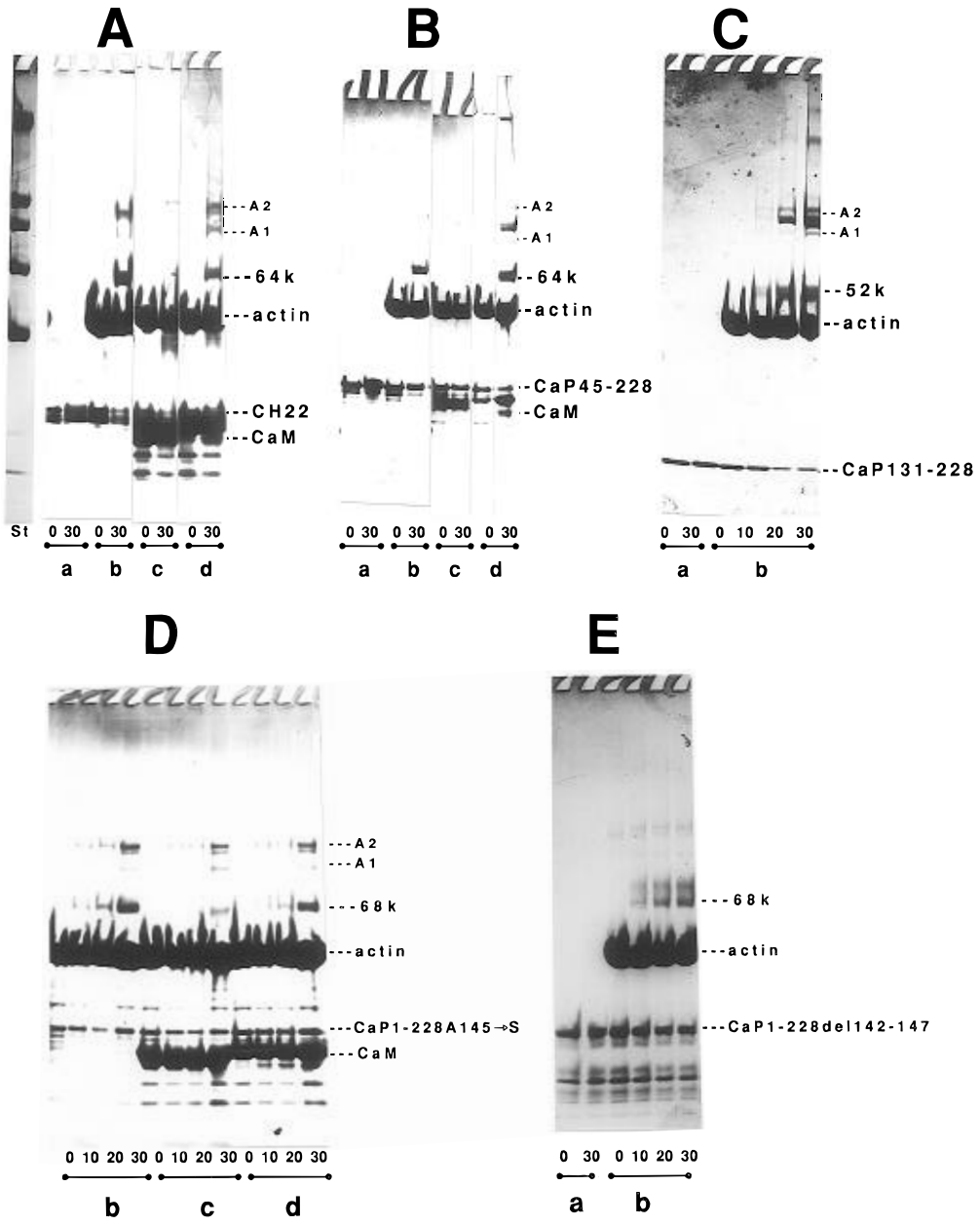


FIGURE 5: EDC-cross-linking of F-actin to calponin mutants modulated by Ca^{2+}-calmodulin. F-actin was mixed with chymotryptic fragment CH22 (panel A), CaP45−228 (panel B), CaP131−228 (panel C), CaP1−228A145S (panel D), or CaP1−228del142−147 (panel E) at a 3:1 molar ratio, and the protein complexes were covalently coupled with EDC-NHS, as specified under Materials and Methods. At the time indicated, the cross-linking reactions were analyzed by 5−18% gradient acrylamide gel electrophoresis. The reactions were conducted in the absence (lanes b) or in the presence of 6-fold molar excess of calmodulin (CaM) + 1 mM $CaCl_2$ (lanes c) or Ca^{2+}-calmodulin + 5 mM EGTA (lanes d); control EDC-NHS-treated fragments are run in parallel (lanes a). A1 and A2 = actin dimers. Lane St = molecular mass markers. The doublet band of CaM reflects the influence of Ca^{2+} on the protein mobility.

CaP131−228. A new species of 52 kDa appeared progressively, and its formation was also suppressed by Ca^{2+}-calmodulin (data not shown). The treatment of any fragment alone with the EDC-NHS reagent was without any effect (Figure 5A−C, lanes a). Figure 5D shows the Ca^{2+}-calmodulin sensitive cross-linking between F-actin and the mutant CaP1−228A145S. A new adduct of 68 kDa progressively appeared but with a lower yield than the cross-linking between actin and the wild-type recombinant fragments or CH22 (Figure 5D, lane b). The cross-linking observed was specific as it was alleviated by calmodulin in the presence of Ca^{2+} (Figure 5D, lane c) but not in its absence (Figure 5D, lane d). Finally, the pattern of the cross-linking reaction between actin and the mutant carrying the VKYAEK deletion, CaP1−228del142−147, is depicted in Figure 5E, lane b. In this case also the efficiency of cross-linking with a resultant 68 kDa band was decreased in agreement with the loss of actin-binding noticed for this mutant and was modulated by Ca^{2+}-calmodulin (data not shown). Its conjugation to actin indicates that the cross-linking reaction displayed by this fragment was mediated neither by K143 nor by K147.

Crucial Participation of the 142−147 Segment in the Inhibitory Activity of Calponin. The most important functional role of calponin is its inhibitory activity caused by actin-binding and modulated by Ca^{2+}-binding proteins. Since the recombinant proteins showed actin-binding activity, we explored their effect on the skeletal actomyosin ATPase

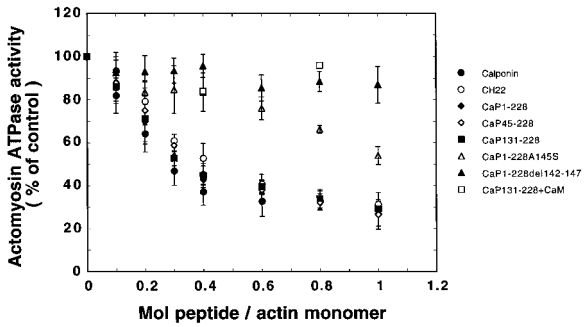


FIGURE 6: Influence of calponin mutants on the acto-S1 ATPase activity. ATPase assays were carried out, as indicated under Materials and Methods, using increasing concentrations of calponin (●), CH22 (○), CaP1−228 (◆), CaP45−228 (◇), CaP131−228 (■), CaP1−228A145S (△), and CaP1−228del142−147 (▲). For CaP131−228, the acto-S1 ATPase was also measured in the presence of 1 mM $CaCl_2$ + 20-fold molar excess of calmodulin (CaM) over the fragment (□). 100% ATPase = 0.490 μmol of P_i/(min:mg). Error bars indicate the standard deviation between four sets of experiments.

activity. As illustrated in Figure 6, increasing concentrations of wild-type recombinant fragments CaP1-228, CaP45−228, and CaP131−228 led to a progressive inhibition of the skeletal acto-S-1 ATPase. The extent of inhibition was slightly lower than for the parent calponin but greater than for the chymotryptic fragment CH22. At a 1:1 molar ratio of actin/recombinant fragment, a maximum inhibition of about 70% was observed. Furthermore, the inhibitory activity of CaP131−228 (Figure 6), CaP1−228, and CaP45−228 (data not shown) was completely abolished by Ca^{2+}-calmodulin. Hence this work describes, for the first time, a calponin peptide missing the large N-terminal and C-terminal domain but still exhibits the calponin actin binding and inhibitory activity in a concentration range comparable to that of the parent calponin.

Since the alteration of the VKYAEK motif by either the point mutation A145 to S or the deletion of the entire sequence decreased the calponin actin-binding property, we investigated the effects of these mutations on actomyosin ATPase inhibition. Substitution of A145 with S impaired the inhibitory activity. At a 1:1 molar ratio of actin/recombinant fragment, only 45% of inhibition was obtained with the mutant CaP1−228A145S while the wild-type recombinant fragment, CaP1−228, displayed about 70% inhibition of the acto-S-1 ATPase (Figure 6). Most interestingly, deletion of the hexapeptide 142−147 totally abolished the inhibitory function of calponin (Figure 6) while only weakening the actin-binding activity (Figure 3A,B). These investigations enabled us, for the first time, to dissociate the actin-binding property of calponin from its actomyosin ATPase inhibition.

Specific Binding of Ca^{2+}-Calmodulin and Tropomyosin to the Sequence 131−228. We studied the interaction of Ca^{2+}-calmodulin with the recombinant fragments by affinity chromatography using calmodulin-Sepharose column. The experiments were initiated in the presence of 1 mM $CaCl_2$ and under the high ionic strength conditions (0.1 M KCl) previously employed to assess the interaction of the chymotryptic CH 22 fragment with immobilized calmodulin (Mezgueldi et al., 1992). All the recombinant fragments bound to the calmodulin-Sepharose column and were eluted only in presence of the calcium chelator EGTA (data not shown). These results are consistent with the above described ability of Ca^{2+}-calmodulin to modulate the EDC-catalyzed cross-linking to F-actin and the inhibitory activity of the recombinant fragments. Interestingly, in contrast to its disturbing influence on the actin-binding and inhibitory function, the modulation of the VKYAEK at position 142−147 by either substitution or deletion had no effect on calmodulin recognition since both CaP1−228A145S and CaP1−228del142−147 bound to the calmodulin-Sepharose column.

We analyzed the interaction of the recombinant fragments with tropomyosin using affinity chromatography at neutral pH and low ionic strength. All the recombinant fragments were completely retained by the tropomyosin-affinity column (data not shown). The findings indicate that the sequence 131−228 also harbors a potent tropomyosin-binding site which was not affected by substitution of A 145 with S or the deletion of the hexapeptide 142−147.

DISCUSSION

To understand the mechanism by which calponin inhibit the acto-S-1 ATPase activity, precise information about the surface contact between calponin and actin are necessary. Therefore, we have used three complementary approaches, i.e., proteolysis, peptide synthesis, and recombinant technology, to build up a detailed picture on the calponin−actin interface. Earlier, we localized the actin binding and inhibitory activity of calponin in the 22 kDa N-terminal domain spanning residues 7−182 and postulated that the sequence A145−Y182 contains the major structural determinants for F-actin binding (Mezgueldi et al., 1992). More recently, using synthetic peptides, we defined the sequence A145−I163 as the minimum sequence able to display the regulatory function of calponin (Mezgueldi et al., 1995). In the present work, we have used recombinant technology and site-directed mutagenesis to highlight the nature of the inhibitory amino acids of calponin and their positioning within the regulatory actin and calmodulin binding domain, in order to gain insight into the molecular basis of its regulatory function.

All of the wild-type recombinant fragments we expressed here, CaP1−228, CaP45−228, and CaP131−228, comprise this actin- and calmodulin-binding domain and display all the physical and functional properties of calponin including heat stability, expected electrophoretic mobility, immunological cross-reactivity, and actin-, calmodulin-, and tropomyosin-binding capacity as well as zero-length cross-linking to F-actin and ATPase inhibitory activity modulated by calmodulin in a Ca^{2+}-dependent fashion. In this respect, they behave as excellent peptide models of the parent calponin extracted from smooth muscle (Takahashi et al., 1988; Winder & Walsh, 1990b) as well as of the fragments obtained by proteolysis (Mezgueldi et al., 1992; Vancompernolle et al., 1990), chemical cleavage (Winder & Walsh, 1990a), and peptide synthesis (Mezgueldi et al., 1995; Li et al., 1994). As illustrated in Figure 7, this regulatory domain lies at the N-terminal end of our 98-residue calponin mutant, CaP131−228.

We propose that the regulatory actin-binding domain of calponin would comprise the 38 amino acids 145−182 and probably extends over the exposed regions of Y144 and Y182, for the following reasons: (i) The binding of the mutant missing the hexapeptide 142−147 at the N-terminal

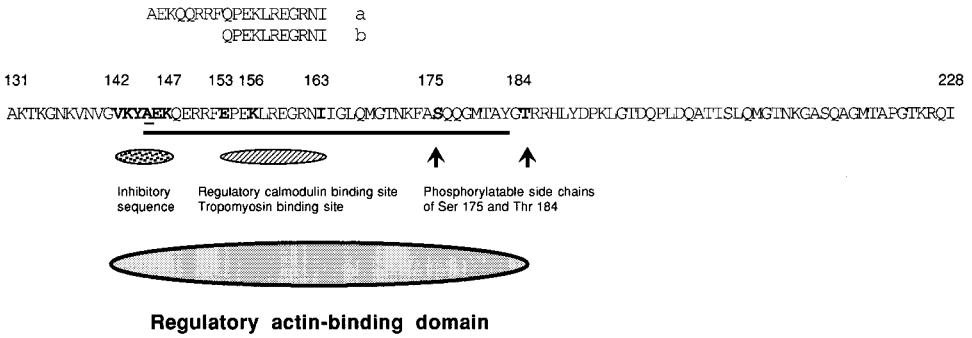


FIGURE 7: Organization of the regulatory actin-binding domain of calponin. The primary structure of the segment A 131−I 228 of mouse h1-calponin is shown. Within this region, the inhibitory actin-binding domain is proposed to extend from the mutated stretch VKYAEK (solid type), between residues 142−147 and representing the inhibitory sequence, through the phosphorylatable S175/T184. It comprises the regulatory calmodulin-binding site as well as a tropomyosin recognition site which both lie on the stretch Q153−I163 as predicted from recent studies employing the synthetic peptide analogs, a and b, corresponding to gizzard calponin sequence (Mezgueldi et al., 1995).

region of this domain is weaker than the binding of the entire calponin or the recombinant fragments comprising the stretches immediately adjacent to Y144 and Y182 (this work). (ii) The recombinant fragment CaP131−228, although devoid of the N- and C-terminal calponin segments but encompassing the sequences upstream to Y144 and downstream to Y182, displayed binding to F-actin comparable to the parent molecule (this work). (iii) F-actin has been shown to highly protect both Y144 and Y181 against chymotryptic cleavage (Mezgueldi et al., 1992). (iv) The high excess of the synthetic peptide A145−I163 needed for full binding to actin indicates the contribution of the C-terminal extended part of the actin-binding domain in the recognition of actin (Mezgueldi et al., 1995). (v) A calponin synthetic peptide spanning residues 144−175 bound to actin with an affinity much lower than that of the parent calponin (Li et al., 1994). (vi) Point mutation of S175 located in the C-terminal part, and outside the sequence A145−I163, markedly decreases actin binding (Tang et al., 1995). (vii) The *in vitro* phosphorylation of calponin at S175 and/or T184 is markedly inhibited by F-actin (Nagumo et al., 1994), and phosphorylation causes dissociation of calponin from actin filaments (Winder & Walsh, 1990b).

The actomyosin ATPase inhibitory activity requires the N-terminal part of this domain at positions 142−147 since modulation of this region by substitution of A145 with S (CaP1−228A145S) severely disturbs the inhibitory property while deletion of the entire peptide (CaP1−228del142−147) totally obliterates this function with only a moderate decrease in actin-binding ability. Thus, the VKYAEK motif at position 142−147 seems to play a crucial role in the ATPase inhibitory activity of calponin while representing only part of the actin-binding domain. Accordingly, the site-directed mutagenesis has permitted the separation of the actin-binding property of calponin from its inhibitory activity. Similarly, deletion mutants of actin at the N-terminus were earlier shown to cause the loss of the inhibitory function of caldesmon with maintenance of its binding activity (Crosbie et al., 1994). Most importantly, it has been shown that deletion of the troponin I sequence 104−119, corresponding to the regulatory domain, completely abolishes the troponin I inhibitory activity (Farah et al., 1994), just like the deletion of the calponin sequence 142−147 we report here. It is worth noting that the disrupted inhibitory function of the mutants is a specific consequence of the modulation of the hexapeptide 142−147 and was probably not caused by alteration of the native structure of their polypeptide chains since these fragments displayed a full range of biochemical properties including expected electrophoretic mobility, heat stability, actin, calmodulin, and tropomyosin binding, and zero-length cross-linking to F-actin switched by calmodulin in a Ca^{2+}-dependent manner similarly to the wild-type recombinant fragments.

On the basis of the observation that all the calponin point mutations, at A145 (this work), K156 (Gong et al., 1993), and S175 (Tang et al., 1995) reduce its actin binding property, it is reasonable to infer that the calponin−actin interface involves multiple contact sites. This would explain why abolition of one of these contacts reduces but does not totally abolish the calponin actin binding capacity. This hypothesis is supported by the implication of three separate actin sequences spanning residues 1−226 (Winder et al., 1992), 326−355 (Mezgueldi et al., 1992), and 373−375 (Bonet-Kerrache & Mornet, 1995) in calponin binding. Such binding may be initiated by anchoring calponin to actin filament followed by establishment of contact site (s) that interferes with actin activation of S-1-ATPase activity. In contrast to the binding to F-actin, calponin inhibitory activity was strongly affected by structural modulations of the hexapeptide V142−K147 supporting the direct participation of amino acid side chains within this contact site in acto-S-1 ATPase inhibition by calponin. This may mean that the other acto−calponin contacts remaining in the deletion mutant do not overlap with S-1 binding sites on actin involved in acto-S-1 ATPase activity. The attribution of the inhibitory activity to the N-terminal part of the actin binding domain is in agreement with the reported loss of the calponin inhibitory activity following treatment with the arginine-directed protease clostripain, which probably has led to the dissociation of the inhibitory amino acids from the remaining actin binding domain by cleavage at arginine peptide bonds (Mezgueldi et al., 1995).

Previously, we described the EDC cross-linking of actin to calponin or to its chymotryptic fragment of residues 7−182 (Mezgueldi et al., 1992) and, more recently, to a synthetic peptide spanning residues 145−163 (Mezgueldi et al., 1995); however, the residue(s) of calponin or its peptides engaged in this cross-linking was not identified. The cross-linking, observed herein, of the deletion mutant CaP1−228del142−147 missing the two lysines at positions 143 and 147, in combination with the reported cross-linking of the synthetic peptide 145−163, indicates that K156 is likely implicated in the conjugation reaction. Of course, the eventual participation of K143 and/or K147 in the cross-linking of native

calponin is not excluded. This conclusion is consistent with the previously reported partial loss of inhibitory activity found for a mutant of chicken calponin modified at K156 (Gong et al., 1993). On the other hand, point mutations of calponin at S175 have been recently reported to decrease ATPase inhibition (Tang et al., 1995). However, these effects are likely resulting from a decrease in actin binding, as reported by those authors, rather than from a specific contribution of side chains of K156 and S175 in the inhibitory activity of calponin.

The point mutation A145S found to moderate but not to abolish the ATPase inhibitory activity also represents a naturally occurring event since it is present in the sequence of the mouse and pig h2-calponin variant (Strasser et al., 1993). We would expect the corresponding protein to display a decreased actin binding and inhibitory activity.

Finally, we have included in the actin binding domain the regulatory calmodulin binding site spanning residues 153−163 that we recently identified (Mezgueldi et al., 1995). The presence of the calmodulin binding site just adjacent to the inhibitory amino acids could account for the reversal of the inhibitory function of calponin by calmodulin while the location of K156 within this sequence could explain the switching off of the acto−calponin cross-linking reaction by calmodulin. The presence of the phosphorylatable amino acids S175 and T184 is in accordance with the observed *in vitro* dissociation of calponin from actin filaments by phosphorylation (Winder & Walsh, 1990b) and the competitive blocking of phosphorylation by F-actin (Nakamura et al., 1993).

In conclusion, using expressed mutant fragments here and synthetic peptides elsewhere (Mezgueldi et al., 1995), we have been able to characterize the essential structural components of the regulatory domain of calponin; the combined results provide an experimental support to the earlier suggested localization of this domain within the sequence A145−Y182 (Mezgueldi et al., 1992). This domain functionally mimics the regulatory domain of gizzard caldesmon, N675−W722 (Mezgueldi et al., 1994), and also the troponin I 96−117 region involved in the regulation of the skeletal actomyosin ATPase (Talbot & Hodges, 1981).

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