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Two domains of interaction with calcium binding proteins can be mapped using fragments of calponin

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Abstract

Native calponin is able to bind 2 mol of calcium binding protein (CaBP) per mole calponin. This study extends this observation to define the 2 domains of interaction, one of which is near the actin binding site, and the other in the amino-terminal region of calponin. Also, the first evidence for a differentiation in the response of calponin to interaction with caltropin versus calmodulin is demonstrated. The binding of caltropin to cleavage and recombinant fragments of calponin was determined by 3 techniques: tryptophan fluorescence of the fragments, CD measurements to determine secondary structure changes, and analytical ultracentrifugation. In order to delineate the sites of interaction, 3 fragments of calponin have been studied. From a cyanogen bromide cleavage of calponin, residues 2–51 were isolated. This fragment is shown to bind to CaBPs and the affinity for caltropin is slightly higher than that for calmodulin. A carboxyl-terminal truncated mutant of calponin comprising residues 1–228 (CP 1–228) has been produced by recombinant techniques. Analytical ultracentrifugation has shown that CP 1–228, like the parent calponin, is able to bind 2 mol of caltropin per mol of 1–228 in a Ca^{2+}-dependent fashion, indicating that there is a second site of interaction between residues 52–228. Temperature denaturation of the carboxyl-terminal truncated fragment compared with whole calponin show that the carboxyl-terminal region does not change the temperature at which calponin melts; however, there is greater residual secondary structure with whole calponin versus the fragment. A second mutant produced through recombinant techniques comprises residues 45–228 and is also able to bind caltropin, thus mapping the location of the second site of interaction to near the actin binding site.

Keywords: calmodulin; calponin; caltropin; smooth muscle; thin filament

Calponin has been identified as an actin, tropomyosin, and calmodulin binding protein (Takahashi et al., 1986, 1988) that is able to inhibit the actin activation of myosin ATPase by virtue of its binding to actin (Abe et al., 1990; Nishida et al., 1990; Winder & Walsh, 1990a). Two potential roles for calponin, as a component of the thin filament, have been identified in smooth muscle contraction, either as a regulator of the actin–myosin interaction as described above, or as a component of the cytoskeleton, potentially serving to regulate arrangement of the actin filaments (Takeuchi et al., 1991; North et al., 1994). Either of these mechanisms would work in concert with the phosphorylation of myosin light chain, which has been identified as a central regulatory event in smooth muscle contraction (Adelstein & Eisenberg, 1980; Hartshorne, 1987). It has been documented that, as contraction progresses, there is a loss of myosin light chain phosphorylation while the force of contraction continues (reviewed in Hai & Murphy, 1989), and an important objective of the research on calponin is to elucidate the mechanism for this behavior. Calponin, when bound to actin, serves to inhibit actin's interaction with myosin and may well inhibit actin's interaction with cytoskeletal components such as filamin. In order for contraction to occur, there must be a release of this inhibition. It has been shown that calponin can be phosphorylated in vitro and that phosphorylation prevents calponin's inhibitory activity (Winder & Walsh, 1990b). There is controversy over whether calponin is phosphorylated in vivo, and the biological relevance of this regulatory mechanism has yet to be established (Gimona et al., 1992; Bárány & Bárány, 1993; Winder et al., 1993). A second putative regulatory mechanism that has been suggested is modulation of calponin's inhibitory activity through interaction with calcium binding proteins (CaBPs), which releases calponin's inhibition of actin-activated myosin

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ATPase. The interaction of calponin with CaBPs has been shown to occur in a calcium-dependent fashion, and with a variety of CaBPs that expose hydrophobic patches upon binding calcium (Wills et al., 1993). Although questions have been raised as to whether the affinity between calmodulin and calponin is strong enough to be a biologically relevant interaction (Abe et al., 1990; Makuch et al., 1991), it has been demonstrated that caltropin (CT), another CaBP isolated from smooth muscle and characterized by Mani and Kay (1990), interacts with calponin with much greater affinity and is able to regulate calponin's activity at reasonable concentrations (Wills et al., 1994). This interaction between calponin and CT may take several forms: it may reverse calponin's inhibition of actin activation of myosin ATPase; it may prevent calponin's inhibition of an interaction between actin and a cytoskeletal protein such as filamin; or it may modulate a posttranslational regulatory event such as phosphorylation of calponin.

In order to more fully understand the interaction between calponin and calmodulin or CT, we have studied various fragments of calponin in order to map the sites of interaction on calponin. From the accessibility profile of calponin (Fig. 1), it is apparent there are 2 highly exposed regions of the molecule of 10 amino acids or more that may by involved in protein-protein interactions: 22-32 and 145-159. It was therefore of interest to isolate domains containing these residues to determine their binding specificity. A number of previous studies have been reported in an effort to identify sites of interaction on calponin. Winder and Walsh (1990b) used NTCB (2-nitro-5-thiocyanobenzoic acid), which cleaves at cysteine, to show that a 21-kDa fragment of calponin retained actin, tropomyosin, and calmodulin binding abilities in addition to the ability to inhibit the actin-activated myosin ATPase. Vancompernolle et al. (1990) isolated the amino-terminal 22-kDa chymotryptic fragment of calponin and reported it is able to bind tropomyosin. Finally, Mezgueldi et al. (1992) found that the carboxyl-terminal 13-kDa chymotryptic fragment of calponin did not bind actin, or calmodulin, whereas the amino-terminal 13-kDa and the amino-terminal 22-kDa fragments were able to bind actin and calmodulin. On the other hand, actin would only bind to the amino-terminal 22-kDa fragment that localized the actin binding region to between residues

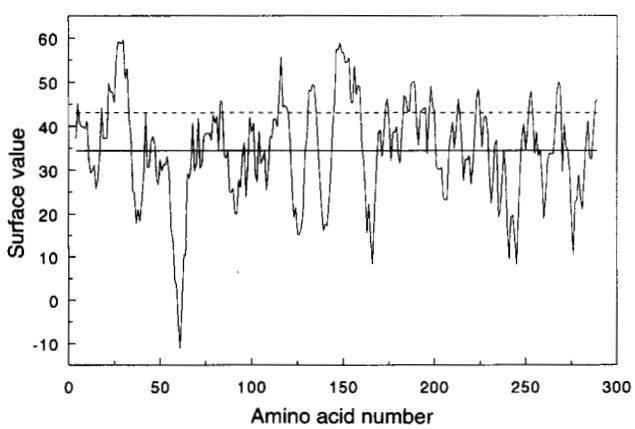

Fig. 1. Accessibility profile of calponin calculated according to the method of Parker et al. (1986). Solid line represents the mean value for the entire protein, and the dashed line is mean + 25% of the difference between the mean and the maximum.

144 and 184. When this work was carried out, assumptions on binding were made based upon 1 binding site on calponin for CaBPs, and it has been demonstrated that, in fact, 2 mol of CaBP can bind to each mol of calponin (Wills et al., 1993, 1994). Therefore, it is necessary to carry out fragment studies to delineate both sites of interaction. In order to accomplish this, we have studied the interaction of 3 fragments of calponin by their binding to CT and calmodulin. A carboxyl-terminal truncated calponin comprised of residues 1-228 was expressed in *Escherichia coli* through recombinant techniques in order to study the effect of the loss of the carboxyl-terminal portion of the molecule on stability and protein interactions. In addition, residues 2-51 were isolated from the cyanogen bromide (CNBr) digest of calponin, and residues 45-228 were produced through recombinant techniques in order to study the complementary portion of the 1-228 mutant. We have been able to separate the two calcium binding sites, assign 1 site to each of the smaller fragments, and characterize the interaction between these fragments and the CaBPs.

Results

Analytical ultracentrifugation of the carboxyl-terminal truncated calponin mutant comprising residues 1-228

In order to determine if the region from residue 229 to the carboxyl-terminal influenced the binding of calponin to the CaBPs, the carboxyl-terminal truncated mutant of calponin comprising residues 1-228 (CP 1-228) was combined with excess CT, and the molecular mass of the complex was determined in the analytical ultracentrifuge in the presence of calcium. This approach has been well established in 2 previous papers (Wills et al., 1993, 1994) as an efficient method of determining the stoichiometry of interaction of calponin in complex with a variety of CaBPs. Previously, we have shown that calmodulin and CT are able to bind to calponin in a ratio of 2 mol CaBP:1 mol calponin. It was logical, therefore, to apply this same method to determine whether CP 1-228 maintained the ability to bind 2 mol of CT. When CP 1-228 was run alone, a molecular mass of 25.7 kDa was observed (data not shown), which is within experimental error of the molecular mass calculated for CP 1-228 of 25.2 kDa, indicating that, as with the parent calponin, no aggregation of this component occurs. Previously, it had been demonstrated that CT does not undergo aggregation (Mani & Kay, 1990). When excess CT was combined with the 1-228 fragment, a molecular mass well above a 1:1 complex was observed (Fig. 2). There was not complete complex formation, as evidenced by a heterogeneous population in the centrifugation cell; molecular mass in the cell ranged from 25 kDa, which is indicative of the uncomplexed constituents, to 63 kDa, which is within experimental error of the expected mass of a 2 CT:1 CP 1-228 complex of 67 kDa. Thus, truncation of the carboxyl-terminal of calponin did not affect the ability of this mutant to bind 2 mol of CaBP, the same stoichiometry with which the parent calponin interacts.

Denaturation of native calponin and the fragment CP 1-228

The temperature denaturation of CP 1-228 was monitored by CD, and the resulting spectra were compared to the tempera-

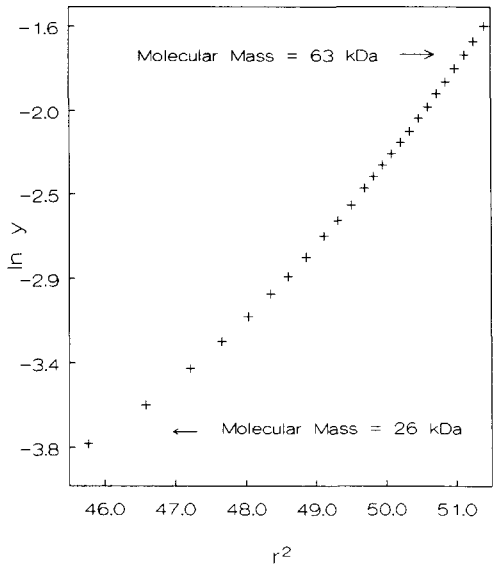

Fig. 2. Representative plot of ln y versus r^2, where y is the concentration in fringe displacement units and r is the distance from the axis of rotation in centimeters. The slope of the curve is used to determine the molecular mass distribution across the cell and the molecular mass shown is the limiting mass observed at the top and bottom of the cell. The sample was combined as 2 mol caltropin (CT):1 mol CP 1-228, and loaded at an initial concentration of 0.6 mg/mL. The speed was 12,000 rpm, and the buffer was comprised of 50 mM MOPS, pH 7.2, 100 mM NaCl, 1 mM DTT, and 3 mM $CaCl_2$.

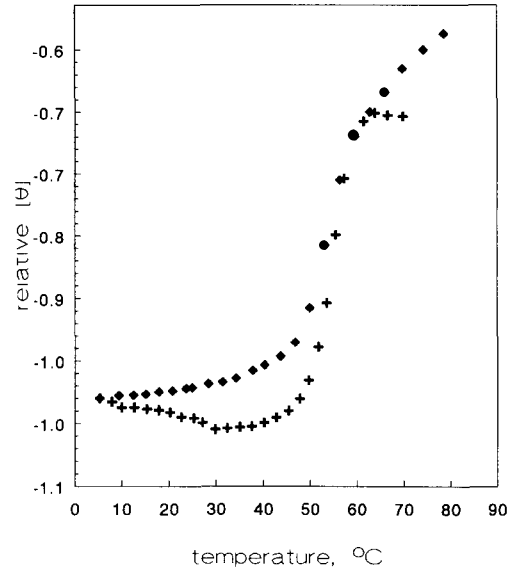

Fig. 3. Temperature denaturation of native calponin (+) and CP 1-228 (◆) monitored by CD at 221 nm. The concentration of protein was 1 mg/mL in 50 mM MOPS, pH 7.2, 100 mM NaCl, and 1 mM DTT.

ture denaturation of native calponin. As can be seen in Figure 3, both native calponin and the mutant 1-228 have a sharp melting transition at 55 °C. CP 1-228 has reduced stability during the early stages of the titration, and loses ~10% more of the ellipticity monitored at 221 nm, indicative of a reduction in the stability of the molecule due to the carboxyl-terminal truncation. This is supported by the calculated ΔH (enthalpy difference) values of 107 cal/mol for native calponin, and only 49.8 cal/mol for CP 1-228. However, the similarity of the 2 denaturation profiles suggests the core of the molecule remains intact.

Tryptophan fluorescence of the calponin fragments

Previously, we have used an acrylodan (6-acroyl-2-(dimethylamino)naphthalene)-labeled calponin in order to probe its interaction with CaBPs because the tryptophan signal of native calponin does not respond significantly to interaction with CaBPs. The acrylodan label is attached to Cys 273, the most carboxyl-terminal of the cysteines of calponin, and labeling the other cysteines was found to be detrimental to structural integrity (Wills et al., 1993, 1994). Because the fragments worked with in this study have had the carboxyl-terminal truncated, including cysteine 273, we examined the tryptophan fluorescence to determine if this signal would be sensitive to interaction in these fragments. We found that in all 3 fragments investigated the tryptophan signal did indeed respond to interaction with CaBPs.

CP 1-228 maintains both of the tryptophan residues of native calponin. Fluorescence studies on this tryptophan signal show that the peak wavelength is at 332.5 nm (Fig. 4A), similar to native calponin. Interaction with calmodulin or CT caused a quenching of the fluorescence. Calmodulin effected a 38% decrease in fluorescence at the peak wavelength and blue shifted the spectrum to 325 nm, indicating an overall movement of the tryptophans to a less polar, buried environment. CT decreased the fluorescence by 30% and caused a red shift in the spectrum to 335 nm, indicating that the tryptophans moved into a more polar, exposed environment. These fluorescence changes in CP 1-228 can be monitored as the protein is titrated with the CaBPs in order to determine relative affinities of these 2 proteins for CP 1-228. From the titrations shown in Figure 5A it is apparent that, although calmodulin may cause a slightly greater quenching of fluorescence, CT still has a much higher affinity for CP 1-228, in agreement with the previously published titrations monitoring the acrylodan label on whole avian calponin (Wills et al., 1994). The titration curves show a slight sigmoidal character, which we believe is due to opposing responses of the 2 tryptophans. From the analytical ultracentrifuge data, it is known that 2 mol of CaBP bind to each mol of CP 1-228, and also CP 1-228 contains 2 tryptophans. Initially, one of the tryptophans may be experiencing an increase in fluorescence, and then a lower affinity binding may be causing a decrease in fluorescence. This type of complicated situation, in which the contribution of the individual tryptophans cannot be quantified, precludes analysis of the data for binding constants.

The proteolytic fragment CP 2-51 possesses 1 tryptophan residue at position 37. The fragment was purified by binding in a calcium-specific manner to a calmodulin affinity column, demonstrating it was capable of binding to CaBPs. By monitoring the fluorescence of this tryptophan, the binding of CP 2-51 to both calmodulin and CT was confirmed. CP 2-51 has a peak wavelength of emission at 342 nm in the presence and absence of calcium, which indicates the tryptophan is in a highly exposed environment (Fig. 4B). When calmodulin is added to CP 2-51, there is a 50% increase in the fluorescence emission at the peak wavelength, together with a shift in the maximum to 330 nm.

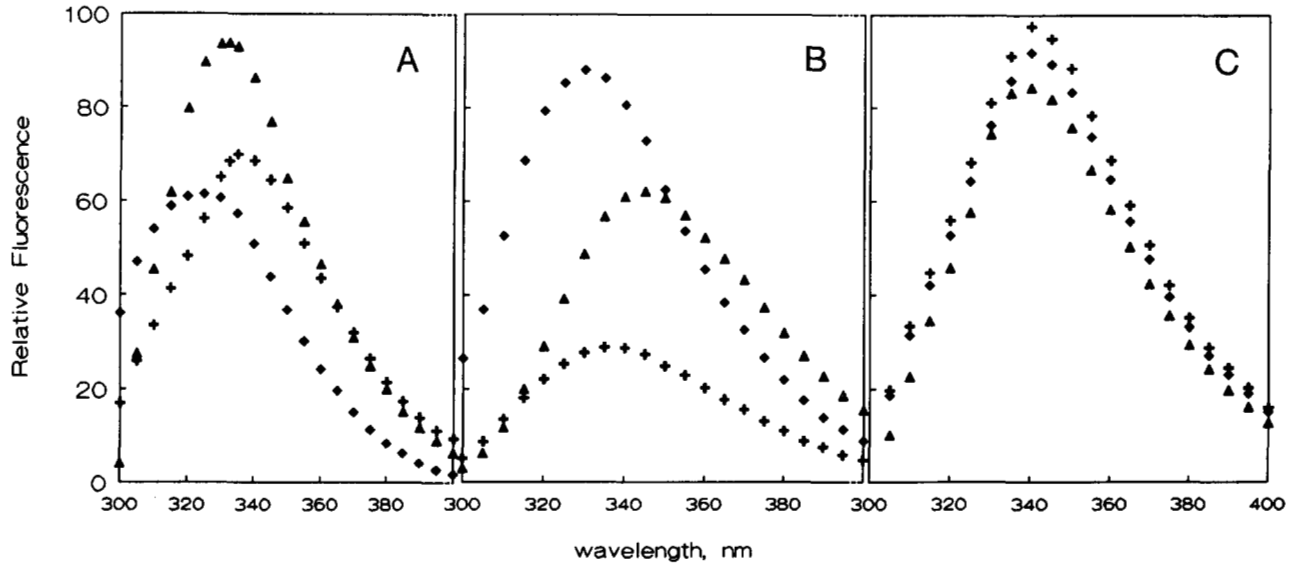

Fig. 4. Fluorescence emission spectra of calponin fragments in the apo state or in the presence of calcium (▲), complexed with CT (+), and in complex with calmodulin (◆). **A:** CP 1–228 excited at 295 nm. **B:** CP 2–51 excited at 295 nm. **C:** CP 45–228 excited at 300 nm. The buffer conditions were 50 mM MOPS, pH 7.2, 100 mM NaCl, 1 mM DTT, 1 mM EGTA, ±3 mM $CaCl_2$.

CT also causes a blue shift in the spectrum to 332 nm; however, rather than an increase in fluorescence intensity, CT quenches the fluorescence by 50%. Thus, in this case, there is a difference both in the quantitative response and the qualitative effect of these 2 proteins upon this fragment of calponin. These changes can be titrated by monitoring the tryptophan fluorescence as increasing amounts of the CaBPs are added. Figure 5B shows the results of this titration, normalized so that binding affinities can be compared directly. From the titration it is evident that more than 1 mol of CP 2–51 is able to interact with each mol of CaBP because 60% of the titration is over by the time a 0.5:1 ratio of CaBP to CP 2–51 is added. However, there is only a single class of binding sites apparent as the curve is not biphasic. The stoichiometry of the peptide binding to CT was confirmed using size exclusion chromatography to isolate the complex and reversed-phase chromatography to separate and quantitate the components (Fig. 6). CP 2–51 eluted at 18 min in 2 peaks representing the homoserine and homoserine lactone products of the CNBr cleavage. The area of these 2 peaks was combined for a total of 1,527 units. Caltropin, which eluted at 25 min, had an area of

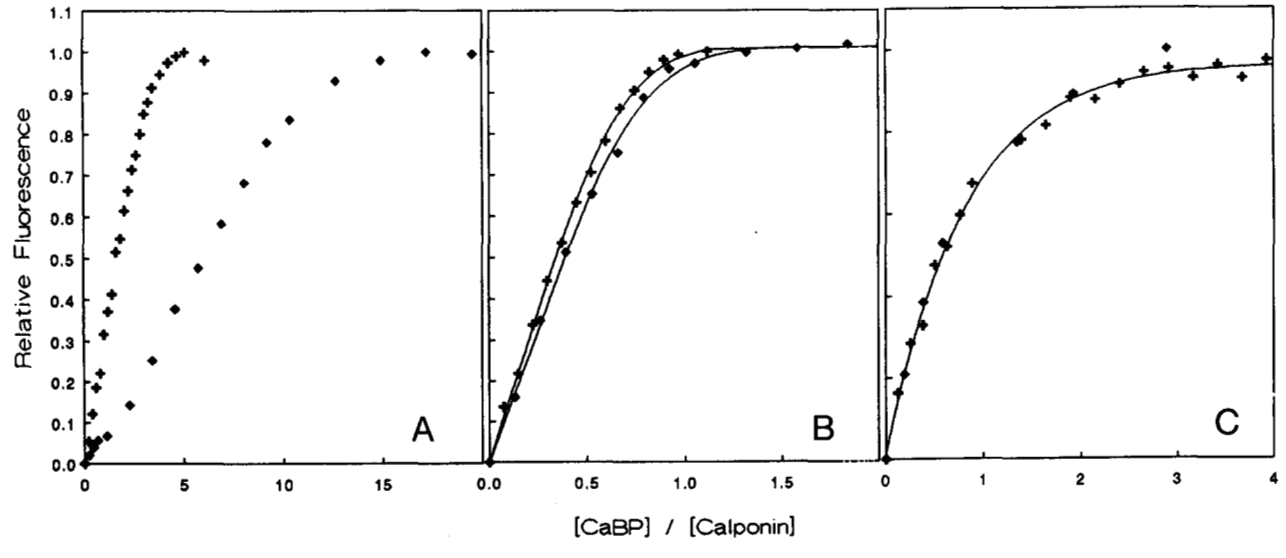

Fig. 5. Fluorescence of calponin fragments titrated with CT (+) or calmodulin (◆). **A:** CP 1–228 excited at 295 nm, and monitored at 332 nm at an initial protein concentration of 1.7 μM. **B:** CP 2–51 excited at 295 nm, monitored at 330 nm, at an initial protein concentration of 13.2 μM. **C:** CP 45–228 excited at 300 nm, monitored at 340 nm, at an initial protein concentration of 2.28 μM. The buffer conditions were 50 mM MOPS, pH 7.2, 100 mM NaCl, 1 mM DTT, 1 mM EGTA, and 3 mM $CaCl_2$.

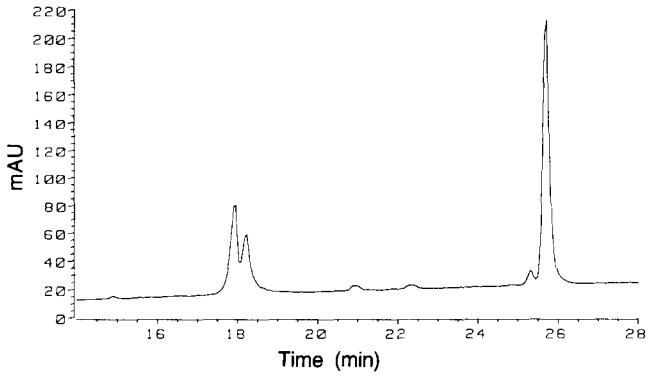

Fig. 6. RP-HPLC profile of the complex of CT with CP 2–51. A 1-mL/min linear AB gradient, where A is 0.1% TFA and B is 0.1% TFA/acetonitrile, was run at 2% B per min. The wavelength of detection was 210 nm. The peaks at 17.9 and 18.2 min are CP 2–51 and at 25.7 min is CT.

2,551 units. When these areas are normalized for the number of amino acids in each protein, there are 2 mol of CP 2–51 per mole of CT. This is not surprising because this peptide has a molecular mass of only 5,300 Da, whereas CT is a dimer resulting in 1 peptide interacting with each component monomer. Calmodulin also has 2 hydrophobic patches, one on each domain, each of which can bind to certain drugs with a hydrophobic ring structure (reviewed in Means et al., 1991). Binding affinities for such curves can be calculated by assuming that when 50% of the fluorescence change has occurred, 50% of the protein is bound, and then calculating the free CaBP at this point. Application of this method results in a K_d of 1.2 μM for CT, whereas 1.8 μM is found for calmodulin, indicating that this fragment is barely able to differentiate between calmodulin and CT by binding to one tighter than the other, unlike the parent calponin and the fragment CP 1–228, which show much greater affinity for CT over calmodulin (Wills et al., 1993, 1994).

The tryptophan fluorescence of the fragment 45–228 can be used to demonstrate that this portion of calponin is also able to bind to CaBPs. This fragment also possesses a single tryptophan at position 82, which has a peak of fluorescence at 340 nm, indicating that this fluorophore is in an exposed polar environment. The tryptophan responds to the interaction with calmodulin and CT by increasing its intensity with no concomitant wavelength shift. CT is able to increase the fluorescence of the fragment by 18%, whereas calmodulin is able to cause a 9% change (Fig. 4C). These are not the same qualitative effects observed with CP 2–51, supporting the hypothesis that the tryptophans of CP 1–228 are not experiencing the same environmental effects. When these changes are titrated with the CaBPs (Fig. 5C), and the results normalized, it is apparent that the binding affinity of CP 45–228 for these 2 CaBPs is very similar and, using the method referred to above to calculate binding constants, assuming 1 mol per mol interacts, a K_d of 0.07 μM can be calculated. It is apparent that the differential affinity the parent displays for the various CaBPs is not inherent in these smaller fragments, and further work must be done on the parent, with the full complement of possible interactions intact in order to further investigate this behavior. A ternary complex of CP 2–51, CP 45–228, and CT was not able to be isolated using size exclusion chromatography, which suggests the 2 calponin fragments binding to a single CT molecule may be mutually exclusive.

Secondary structure studies on calponin and its fragments

CD was performed on the fragments of calponin in order to compare their secondary structure with that of the intact calponin molecule. In addition, the CD spectra of the complexes of these fragments with CT were determined in the presence and absence of calcium and compared to the theoretical spectra, generated by summing up the constituent spectra, in order to determine the type of structural changes that occur upon complexation. In Figure 7A, the CD spectra of native calponin, calponin 1–228, the complex of calponin 1–228 with 2 mol of CT in the presence of calcium, and the theoretical complex of 1–228 with CT in the presence of calcium are presented. The CD spectrum of chicken gizzard calponin has been published previously (Wills et al., 1993), and here we present the spectrum of porcine calponin to demonstrate the CD spectrum of smooth muscle calponin does not significantly vary from species to species. The ellipticity at 221 nm varies by 328° between these species, which is within experimental error (±300°) and does not represent significant structural differences. The mutant mouse calponin fragments do not vary significantly in sequence from the porcine used as control; there are only 2 amino acid differences in the 1–228 fragment, an arginine to a glutamine and an arginine to a lysine (Strasser et al., 1993). Provencher-Glöckner analysis of the CD spectra was performed in order to evaluate the secondary structure content of the proteins and the complexes. The results are tabulated in Table 1. The results of the analysis of the 1–228 species indicate that this fragment still has a high level of structure despite the carboxyl-terminal truncation. Because we had established 2 mol of CaBP bind to each mole of CP 1–228, we used this ratio of proteins in order to establish the CD spectrum of the complex and compared it to the theoretical one in order to determine the type of secondary structure that changes upon complex formation. In the absence of calcium, the observed and theoretical spectra were superimposable, indicating that no secondary structural changes occurred (data not shown). In the presence of calcium, however, there was a 1,580° smaller ellipticity in the observed complex versus the theoretical spectrum at 221 nm, indicating 6% less α-helix and 11% more β-sheet in the observed versus the calculated complex.

Figure 7B shows the CD spectra of the CNBr proteolytic fragment of CP 2–51. This fragment has low α-helix, high β-sheet, β-turn, and remainder. In order to determine if more helix could be induced in this fragment under the appropriate conditions, we added 50% TFE (2,2,2-trifluoroethanol), a known helix inducer (Jasonoff & Fersht, 1994). The apparent helical content of CP 2–51 increased significantly to 48%, indicating that this fragment is certainly capable of taking on helical structure in the native protein, or upon interaction with another protein. The accessibility profile (Fig. 1) of calponin indicates that this region does not contain any significant nonaccessible regions, so it is not surprising that it is not fully structured when free in solution. When CP 2–51 was combined with CT in the absence of calcium, the CD spectrum was superimposable with the theoretical spectrum, indicating that no secondary structural changes occurred (data not shown). In the presence of calcium however, there was a 1,990° greater ellipticity at 221 nm than predicted, most likely due to the induction of helix in CP 2–51 upon in-

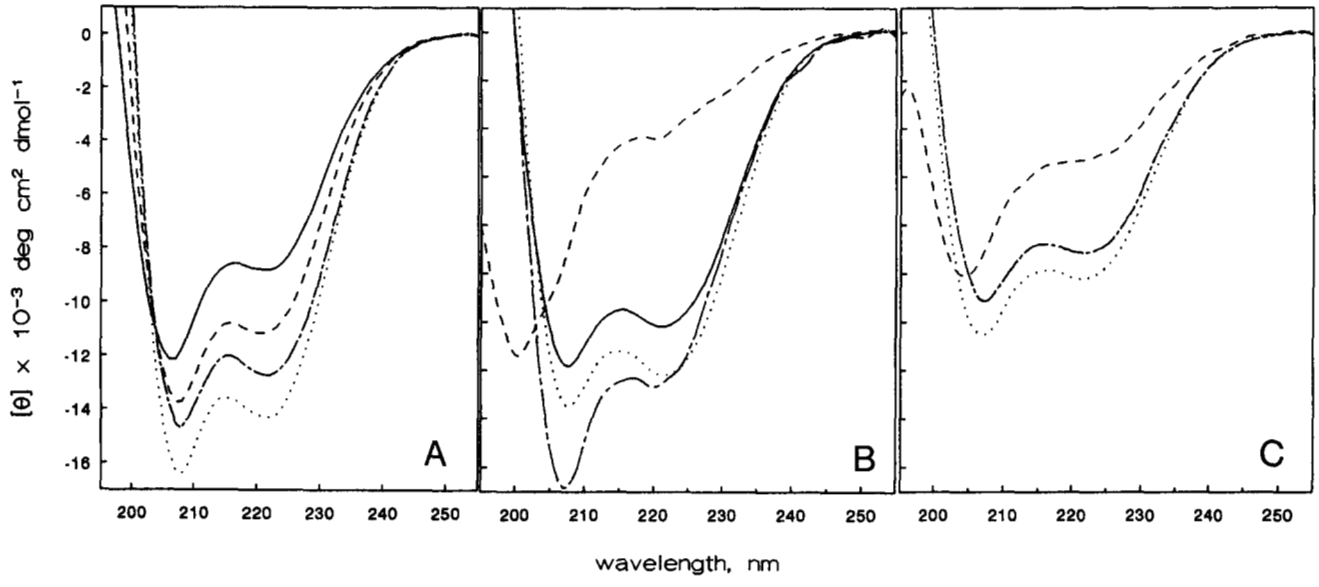

Fig. 7. Far-UV CD spectra of calponin fragments. **A:** Spectrum of CP 1-228 (- - - -), native porcine calponin (———), CP 1-228 in complex with CT (—— —), and the theoretical complex of CP 1-228 with CT (· · · ·). **B:** CP 2-51 (- - - -), 2-51 with 50% TFE (———), in complex with CT (—— —), and the theoretical complex with CT (· · · ·). **C:** CP 45-228 (- - - -), in complex with CT (—— —), and the theoretical complex with CT (· · · ·). The buffer conditions employed were 50 mM MOPS, pH 7.2, 100 mM NaCl, 1 mM DTT, 1 mM EGTA, and 3 mM $CaCl_2$ was present for the complexes.

teraction with CT. Provencher-Glöckner analysis indicated an increase of 9% helix over the predicted structure.

Figure 7C contains the CD spectra of the recombinant CP 45-228. The accessibility plot (Fig. 1) indicates that there are highly inaccessible residues in this region, indicating that it may form part of the hydrophobic core of the protein. When combined with CT in the absence of calcium, there was no change in secondary structure compared to the theoretical spectrum generated for this complex (data not shown). However, in the presence of calcium, there is a 1,090° smaller ellipticity at 221 nm than predicted, indicating that structural changes occurred upon interaction. The Provencher-Glöckner analysis indicates a 5% decrease in α-helix and a 10% increase in β-sheet. These changes in helix and sheet are very similar to the changes observed in the complex of native calponin with CT (Wills et al., 1994).

Table 1. *Secondary structure predictions*[a]

Protein	α-Helix	β-Sheet	β-Turn	Remainder
Porcine calponin (CP)	33	23	16	27
CP 1-228	42	15	11	31
CP 1-228 + caltropin (CT)	43	26	10	21
Theoretical	49	15	08	28
CP 2-51	13	43	22	22
CP 2-51 + 50% trifluoroethanol	48	27	03	22
CP 2-51 + CT	47	22	06	25
Theoretical	38	21	10	31
CP 45-228	17	29	26	28
CP 45-228 + CT	30	35	18	17
Theoretical	35	25	11	29

[a] Analysis of the CD spectra are expressed as percent total structure. The complexes are in the presence of Ca^{2+} as described with the individual experimental results, and the theoretical is the predicted amounts based on summing up the constituent proteins assuming no secondary structural changes occurred upon complexation.

Discussion

Progress is continuing on elucidating the details of calponin's biological role, especially with relation to its inhibition of the actomyosin interaction. Horiuchi and Chacko (1991) found that calponin causes a decrease in the V_{max} of the actomyosin ATPase, without a substantial decrease in the K_{ATPase}. This finding suggests that calponin is causing a structural change in actin that is affecting the catalytic state of the reaction, rather than sterically blocking the interaction of myosin and actin. A similar conclusion is indicated by studies that covalently crosslinked actin to S1, wherein calponin was still able to inhibit the ATPase (Miki et al., 1992). The work of Shirinsky et al. (1992) has shown, by monitoring actin filament motility over immobilized myosin, that calponin inhibited filament movement in an all-or-none fashion, rather than producing a graded inhibition of filament velocity, and this finding has been supported by the recent study of Haeberle (1994) in which calponin was found to inhibit filament velocity, while at the same time increasing the force of the crossbridges and strengthening the actin-myosin binding. Haeberle proposed that this indicates calponin is enhancing the strong binding state of actin and myosin, leading to a load-bearing complex, and could therefore contribute to the latch state of smooth muscle. Regulation of calponin's function is not well understood, and proposals such as phosphorylation of calponin as a mechanism of regulation are highly controversial. In

this study, the interaction of calponin with the CaBP CT has been probed. This interaction is of great potential interest in smooth muscle regulation because CT has been demonstrated to be capable of regulating calponin's activity in in vitro ATPase assays (Wills et al., 1994).

The present study has demonstrated the utility of calponin fragments in studying the interaction of CaBPs with calponin in terms of the characterization of the interactions, along with identifying the domains on calponin where the interactions occur. Native calponin tryptophan fluorescence does not respond significantly to the interaction of calponin with CaBPs; however, these fragments of calponin do respond with a change in the tryptophan fluorescence, which signifies that for the first time intrinsic fluorescence can be used to monitor the interactions, allowing investigation of complex formation in the absence of probes modifying the protein. Thus, although the deletion of residues 229 to the carboxyl-terminal does not affect the ability of calponin to inhibit ATPase as shown with studies of other fragments (Winder & Walsh, 1990b; Mezgueldi et al., 1992), or to bind to CaBPs, as shown in this study, the carboxyl-terminal fragment certainly interacts with other portions of the molecule in order to alter the tryptophan fluorescence response. This is also indicated by acrylodan-labeled cysteine 273 of calponin, which is located in this truncated carboxyl-terminal region, being extremely sensitive to interaction with CaBPs, despite this region not being required for interaction. The fluorescence of CP 1–228 indicated a different type of response with calmodulin that caused a blue shift, suggesting a more protected environment, as compared to CT, which caused a red shift, suggesting movement of the tryptophan into a more solvent-exposed, polar position. This differential response was not observed with the acrylodan-labeled calponin, in which all of the CaBPs caused a blue shift of the fluorescence due to acrylodan on cysteine 273, signifying its movement into a less exposed environment. This difference in the effect upon the tryptophans between these 2 CaBPs may be due to the dimeric nature of CT as opposed to the monomeric nature of calmodulin, and suggests that the tryptophans may be used in the future as a sensitive probe in studies dedicated to why CT binds with higher affinity to calponin than calmodulin does and the type of structural changes that this binding induces in calponin. The complicated tryptophan fluorescence response of CP 1–228, in which the 2 tryptophans may be experiencing opposing environments, together with the lack of tryptophan response of native calponin, may be further investigated by the replacement of the tryptophans with probes of altered fluorescent properties such as 5-hydroxytryptophan (Hogue et al., 1992), or the nonfluorescent analogue 4-fluorotryptophan (Bronskill & Wong, 1988). This would allow the response of the individual tryptophans to be examined, each one of which appears to be located in a domain of interaction with the CaBPs, and may well produce very informative results about the relative behavior of these 2 binding events.

Through the use of chemical cleavage of calponin and recombinant techniques, 2 smaller fragments of CP 1–228 were made, CP 2–51 and CP 45–228, each of which is able to bind to CaBPs. Thus, the 2 domains of interaction between calponin and the CaBPs have been isolated. The fluorescence titration of CP 2–51 with CT and calmodulin cannot be fit to a 1:1 binding, and 2 mol of this peptide are able to bind to each mol of CaBP. Although this ratio of interaction would not be expected based on the stoichiometry of interaction of the native parent proteins, it is not an unreasonable result considering that CT is a dimer and may well possess 2 equivalent sites of interaction, and calmodulin has a hydrophobic patch on both its amino-terminal and carboxyl-terminal domains (Strynadka & James, 1989). Because the peptide is relatively small, with a molecular mass of 5,300 Da, one would not expect the same steric hindrance to prevent both sites of interaction that one would expect to find in the parent molecule of 32 kDa. The phenomenon of 2 interaction sites has been observed before. The skeletal muscle protein troponin I has been shown to bind to either the amino- or carboxyl-terminal domain of both troponin C and calmodulin (Lan et al., 1989; Swenson & Fredricksen, 1992). In addition, the peptide melittin has also been shown to bind to both the amino- and carboxyl-terminal domains of calmodulin (Seeholzer et al., 1986), and the NMR structure of calmodulin in complex with a peptide of myosin light chain kinase (MLCK) shows contact with both the amino- and carboxyl-terminal domains of calmodulin (Ikura et al., 1992). Most recently, an analogous situation was demonstrated by Marston et al. (1994), in which 2 mol of a caldesmon peptide are able to interact with 1 mol of calmodulin, although this is not the stoichiometry achieved with the intact proteins. Interestingly, it has been demonstrated in some cases that a helix is the motif used for interaction with these CaBPs, as in the case of melittin mentioned above, and we have shown in this study that CP 2–51 is capable of having helix induced in it under the appropriate conditions. Further, the complex of CP 2–51 with CT exhibits much greater helix than would be predicted, suggesting that the peptide may indeed be taking on a helical conformation upon interaction.

The fluorescence titrations of CP 2–51 and CP 45–228 with the CaBPs did not show the large preference for interaction with CT over calmodulin that the native protein is capable of demonstrating (Wills et al., 1993, 1994). However, the mutant CP 1–228 was still able to exhibit this preference. This indicates that interaction between the various domains of the protein is an important determinant in these interactions, and a number of interactions from various parts of the molecule play a role. Thus, the information obtained in this study must be used to selectively modify various residues within the intact calponin molecule in order to delineate which site is the high-affinity site, which site is the low-affinity site, and the biological relevance of each of them.

The secondary structure analysis of the fragments of calponin indicate that the secondary structural content changes upon interaction with CT, which provides further evidence of complex formation. This study has looked at the amino-terminal 228 amino acids. In the future it will be of interest to pursue studies on the carboxyl-terminal regions of calponin, the function of which remains to be resolved. Secondary structure prediction algorithms indicate that this region contains β-sheet and turns with little or no α-helix (Strasser et al., 1993). In future studies involving the interactions and regulation of smooth muscle, these changes in structure should help to characterize the behavior of the calponin molecule when it inhibits actin activation of ATPase compared to when it is regulated.

As an increasing number of calponin isoforms are being identified, their sequences are being compared in order to determine the biological significance of various regions of the molecule (Vancompernolle et al., 1990; Takahashi & Nadal-Ginard, 1991; Nishida et al., 1993; Strasser et al., 1993; Applegate et al., 1994).

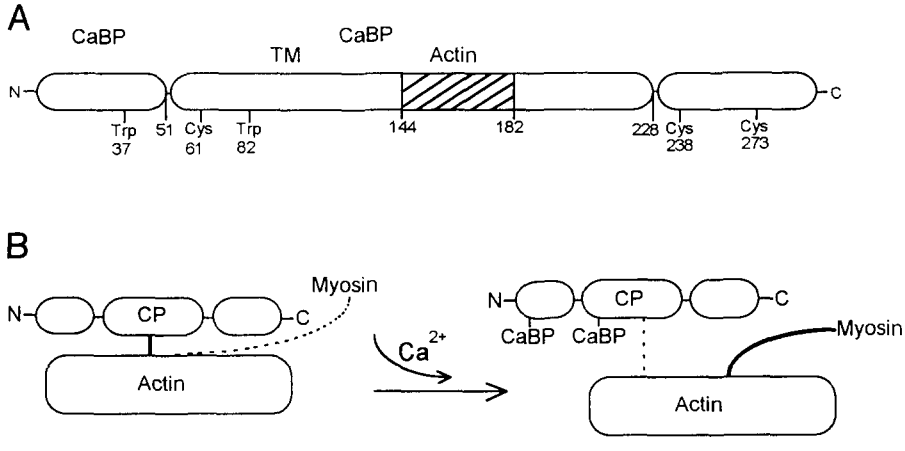

Fig. 8. A: Map of the domains of calponin as determined in this study using fragments of calponin, and the putative sites of interaction with actin, tropomyosin, and the calcium binding proteins (CaBPs). **B:** Proposed model for regulation of the actin-calponin interaction by the CaBPs.

It therefore becomes increasingly important to understand the domains of calponin and their sites of interaction with various proteins so that sequence variations can be related to function. In order to get an accurate picture of where these interactions are occurring, it is important to incorporate the new information presented in this study demonstrating 2 sites of interaction with CaBPs. Other work, in which mapping of binding sites for actin, tropomyosin, and calmodulin have been performed, produced a map of binding sites (Mezgueldi et al., 1992). Figure 8A shows our revised map based upon all the information to date. We have demonstrated that CP 1-228, which has the carboxyl-terminal truncated, is still able to bind 2 mol of CaBP, indicating that the loss of 64 amino acids does not affect this function. Furthermore, we have demonstrated that this truncation does not greatly affect the stability of calponin, indicating that it may not contribute to the core of the calponin molecule; however, the tryptophan fluorescence results indicate the carboxyl-terminal portion certainly has an effect upon the functional portion of calponin. Winder and Walsh (1990b) found that a 21-kDa cleavage product of NTCB was able to bind to actin, calmodulin, and tropomyosin, and this fragment would presumably represent residues 61-238. It has also previously been shown that a 22-kDa calponin fragment, from residues 1 to 184, maintains all of the binding interactions of calponin, and that the 13-kDa carboxyl-terminal portion of calponin did not interact with tropomyosin, actin, or calmodulin (Vancompernolle et al., 1990; Mezgueldi et al., 1992). Our results are in line with these studies, wherein we find that loss of the carboxyl-terminal does not cause loss of interaction with CaBPs. Previously, the information has been interpreted to mean that the CaBP interaction site can be localized to amino acids 61-144. We postulate that this is not so because that interpretation was based on 1 CaBP site of interaction. We show in this work that there is 1 site of interaction between residue 2 and 51, which would be observed in the studies on the 1-144 fragment, and the 1-184 fragment used in the work of Mezgueldi et al. (1992). Furthermore, the fact that 1-144 bound to calmodulin does not preclude the presence of a second binding site in the 144-184 region. The observation of Mezgueldi et al. (1992) that calmodulin bound to the crosslinked actin and calponin 52-168 complex would suggest 52-168 contains the second binding site we are detecting with our 45-228 fragment, and which Winder and Walsh observed with their 21-kDa fragment that was able to inhibit actin-activated myosin ATPase. This second CaBP interaction site may well be close to or even overlap the actin binding region of 144-184. The observation of Winder and Walsh (1990b) of a 9-kDa fragment that did not bind to actin, tropomyosin, or Ca^{2+}/calmodulin must presumably have been generated from the residues carboxyl-terminal of cysteine 238 and would not be contained within the fragments used in our study.

Figure 8B shows a model of how these proteins might interact. We propose that the binding site for CT on fragment 45-228 may overlap or interfere with the binding site for actin demonstrated to be in this region and as such is responsible for the ability of CT to interfere with calponin's ability to inhibit the actin-activated myosin ATPase. The observation of an additional site of interaction in the amino-terminal of calponin allows for the exploration of its function to be addressed. We suggest that the site of interaction contained within fragment 2-51 may serve to modulate either: (1) calponin's binding to actin; (2) a posttranslational regulatory event as suggested by Naka et al. (1990), who showed that phosphorylation of calponin was inhibited by the presence of calcium/calmodulin; or (3) another interaction in which calponin is involved and yet to be determined. If, as has been suggested, calponin is involved in cytoskeletal structure regulation (Takeuchi et al., 1991; North et al., 1994), CT binding to either of these 2 sites may well regulate or modulate interaction with other cytoskeletal proteins. This study lays the groundwork for future work, which will involve further delineation of both sites of interaction and their respective functions. Determination of the functional abilities of calponin with specific sites, such as the 2 CaBP interaction sites, appropriately modified, will aid in resolving the questions of calponin's function and its role in smooth muscle contraction.

Materials and methods

Protein purifications

Calponin was purified as previously described (Wills et al., 1994). CT was isolated from turkey gizzards according to the method of Mani and Kay (1990). Calmodulin was isolated from turkey gizzards as a by-product of the CT purification in which peak IV off the Q-Sepharose column is calmodulin (Mani & Kay, 1990), which was then subjected to reversed-phase HPLC

(RP-HPLC) from which it elutes at ~44% acetonitrile. The fragment comprising CP 2–51 was isolated from a CNBr digest of turkey gizzard calponin in 70% formic acid. The digest was lyophilized, then taken up in 10 mM 3-(*N*-morpholino)propanesulfonic acid (MOPS), pH 7.0, 1 mM $CaCl_2$, 1 mM dithiothreitol (DTT), and 0.01% NaN_3 and applied to a calmodulin affinity column. The fragment eluted when the column was washed with 2 mM ethylene glycol bis(β-aminoethyl ether)-*N,N,N,N*-tetraacetic acid (EGTA). This pool was then applied to a C18 RP-HPLC column from which it eluted at 30% acetonitrile. The identity of the fragment was confirmed by amino acid compositional analysis and sequencing. For amino acid analysis, protein samples were hydrolyzed for 1 h at 160 °C in 6 N HCl/0.1% phenol. The samples were analyzed on a Beckman 6300 ion-exchange HPLC amino acid analyzer. Amino acids were detected by postcolumn reaction with ninhydrin. Sequencing was performed on an Applied Biosystems 473A pulsed liquid/gas-phase protein sequencer. The sample was loaded on a precycled polybrene-coated glass fiber disc and subjected to standard Edman degradation chemistry. The expression system employed for producing the recombinant mouse calponin fragments 1–228 and 45–228 used in this study was as described previously (Strasser et al., 1993). Given that there are differences in sequence between avian and mouse calponin, we used porcine calponin as a control because it has a very similar sequence to mouse calponin (Strasser et al., 1993) and was more readily available than full-length mouse calponin. Secondary structure analysis controls were run between avian and porcine calponin to demonstrate that they had similar properties. The calponin constructs were designed using existing restriction sites in the cDNA. To maintain the correct reading frame, the ends of the restriction fragments had to be modified in some cases prior to subcloning into the expression vector (blunt-ending by Mung bean nuclease, filling in by Klenow fragment, inserting of linkers). All products were checked by sequencing. In order to purify these fragments, bacterial cells were centrifuged at 5,000 rpm and the pellet dissolved in 5 mM KH_2PO_4, 5 mM K_2HPO_4, 10 mM NaCl, 2 mM EDTA, 2 mM EGTA, pH 7.0. A French press was used to open the cells and the suspension clarified by centrifugation at 18,000 rpm. Ammonium sulfate cuts from 0 to 30 and 30 to 60% saturation were performed. The 30–60% pellet was redissolved in the same phosphate buffer as above and applied onto a hydroxylapatite column (2.5 × 10 cm) equilibrated in the same buffer. A linear phosphate gradient (2 × 150 mL, 10–250 mM PO_4) was used to elute the recombinant protein, which eluted at ~180 mM. The peak fractions were pooled and precipitated with ammonium sulfate at 60% saturation. The pellet was dissolved in 20 mM 2-(*N*-morpholino)ethanesulfonic acid, 10 mM NaCl, 2 mM EGTA, 2 mM EDTA, pH 5.4, and loaded onto an S-Sepharose column equilibrated in the same buffer. Bound protein was eluted in a single step by applying 1 M NaCl in the same buffer and the peak fractions were collected. After concentration by ammonium sulfate precipitation the pellets were dissolved in 50 mM MOPS, pH 7.1, 100 mM NaCl, 1 mM DTT, and purified on a fast protein liquid chromatography (FPLC) Superose 12 column (1 × 30 cm). The 45–228 fragment was not soluble after the French press treatment, so 4 M urea was added to the crude French press extract and the hydroxylapatite column was run in 4 M urea from which the protein eluted at ~20–40 mM PO_4. Four molar urea was also used in the phosphate buffer to purify this fragment on an S-100 Sepharose fast flow column and then this fragment was purified on the FPLC column as described above. Protein concentrations were determined from the following extinction coefficients: calponin $A^{1\%}_{276nm} = 7.5$ (Wills et al., 1993); calmodulin $A^{1\%}_{277nm} = 1.95$ (Klee, 1977). The following extinction coefficients used were calculated from the sequences using $E^{1M}_{279.8nm} = 5{,}600$ for tryptophan and $E^{1M}_{279.8nm} = 1{,}420$ for tyrosine (Gratzer, 1989): CP 1–228, $A^{1\%}_{276nm} = 7.7$; CP 45–228 $A^{1\%}_{276nm} = 5.16$; CP 2–51 $A^{1\%}_{276nm} = 15.92$; porcine calponin $A^{1\%}_{276nm} = 8.9$. The concentration of CT was determined from amino acid analysis using the composition of Mani and Kay (1990). The accessibility plot of calponin was calculated using the values compiled by Janin (1979), according to the method of Parker et al. (1986), wherein the average surface hydrophilicity is the mean of the profile values, and any residue having a profile value greater than 25% of the difference between the mean and the maximum value was determined to be exposed. The surface profile values were determined by summing up the accessibility value for each residue of a 7-residue segment and assigning that value to the fourth residue in the segment. These values were plotted against the amino acid residue number.

Analytical ultracentrifugation

Studies were performed on a Beckman Spinco Model E analytical ultracentrifuge. Molecular masses were determined by low-speed sedimentation equilibrium employing Raleigh interference optics according to Chervenka (1969). Proteins were loaded at an initial concentration of 0.6–0.8 mg/mL, in 50 mM MOPS, pH 7.2, 100 mM NaCl, 1 mM DTT, and 3 mM $CaCl_2$. Molecular masses were calculated from the slope of the ln y versus r^2 plot.

Fluorescence studies

Studies were performed on a Perkin-Elmer MPF 44B recording spectrofluorimeter as described previously (Wills et al., 1993). The solvent used was 50 mM MOPS, pH 7.2, 100 mM NaCl, 1 mM DTT, and 1 mM EGTA.

CD measurements

CD measurements were carried out on a Jasco J-720 spectropolarimeter as described previously (Wills et al., 1993). The computer averaged 10 scans, and the signal due to solvent was subtracted. The solvent used was 50 mM MOPS, pH 7.2, 100 mM NaCl, 1 mM DTT, 1 mM EGTA with and without 3 mM $CaCl_2$, and the protein concentrations were in the range of 0.3–0.9 mg/mL. Secondary structure analysis was performed using the algorithm of Provencher and Glöckner (1981). Theoretical curves were generated by adding the contributions of the constituent proteins in the ratios of their relative mass present during the experiment. The temperature denaturation profile was monitored at 221 nm. ΔH values were calculated from Van't Hoff plots employing the relation $\Delta H = -R(d \ln K)/d(1/T)$ where R is the gas constant, T is the temperature in K, ΔH is the enthalpy difference, K is the apparent equilibrium constant (McCubbin et al., 1980) and was calculated according to Pace (1975).

Size exclusion and reversed-phase chromatography

The stoichiometry of the CT in complex with the calponin fragment 2–51 was determined according to Van Eyk and Hodges (1991) with the following modifications. The complex was isolated from a Superdex 75 size exclusion column (Pharmacia LKB Biotechnology, Uppsala, Sweden) attached to a Vista 5000 HPLC. The buffer consisted of 25 mM Tris-HCl, 100 mM KCl, 3 mM $CaCl_2$, and 1 mM DTT, and was run at 0.3 mL/min. A 3× excess of CP 2–51 was incubated with CT for 10 min in the presence of calcium prior to application to the column. The protein concentration was 0.5 mg/mL in a volume of 225 μL. The peak was lyophilized before applying to a Zorbax analytical RP column SB-C8 (4.6 × 150 mm). To calculate the peptide/protein ratio in the complex, the peak areas of peptide and CT, following separation of the complex by RP-HPLC, were obtained, then corrected for the number of amino acid residues per protein in order to obtain relative ratios of the 2 proteins.

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