Characterization of the Smooth Muscle Calponin and Calmodulin Complex†

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Received July 14, 1992; Revised Manuscript Received November 18, 1992

ABSTRACT: Calponin interacts with several Ca^{2+} binding proteins in a Ca^{2+}-dependent manner. In order to determine the possible biological relevance of these interactions in smooth muscle function, it is necessary to characterize the strength and stoichiometry of the complexes formed. The interaction between calponin and calmodulin can be monitored through an acrylodan label on a cysteine of calponin. The fluorescently labeled calponin possesses the same biological function and physical behavior in binding to calmodulin as the native calponin. This probe is very environment-sensitive and responds to the calponin–calmodulin interaction by the emission peak blue-shifting 20 nm and by the fluorescent quantum yield increasing 3.5 times at 460 nm. The stoichiometric nature of this complex has been determined using analytical ultracentrifugation and is two calmodulins to one calponin, and the interaction is Ca^{2+}-sensitive with a K_{d_1} of ≤ 0.22 μM and a K_{d_2} of 2.5–3.4 μM. Calmodulin is not the only protein which interacts with calponin in this manner, but rather this interaction seems to be a general feature attributable to all hydrophobic patch exposing proteins, suggesting that it may be nonspecific, occurring because of a generalized mode of interaction. Two other proteins, S-100b from bovine brain and SMCaBP-11 from smooth muscle, had stronger affinities for calponin, and in particular interaction of SMCaBP-11 with calponin may be biologically relevant. In determining the nature of calponin's interaction with these Ca^{2+} binding proteins, it was apparent there was no effect of Ca^{2+} upon calponin itself and physical studies could find no evidence that calponin interacts with calcium.

Regulation of contraction in smooth muscle is known to occur at the level of the thick filament by phosphorylation of the myosin light chain which promotes the activity of the actomyosin ATPase (Adelstein & Eisenberg, 1980). It is apparent, however, that additional control of muscle contraction occurs at the level of the thin filament (Marston & Smith, 1985). Several proteins have been isolated from smooth muscle in an attempt to elucidate the complete mechanism of smooth muscle regulation [see reviews by Hartshorne (1987) and Walsh (1991)]. In particular, the isolation of calponin from avian smooth muscle has been described by Takahashi et al. (1986), and the sequence of two isoforms has been determined (Takahashi & Nadal-Ginard, 1991). Calponin was identified as binding to F-actin and F-actin–tropomyosin in a Ca^{2+}-independent fashion, and interacts in a Ca^{2+}-dependent manner with calmodulin (Takahashi et al., 1986). Initially calponin was suggested to be a possible Ca^{2+} binding protein although our results indicate calponin does not interact with Ca^{2+}. These properties, together with calponin's location on the thin filament (Takahashi et al., 1988a), identified it as a potential regulator of smooth muscle contraction. Winder and Walsh (1990b) demonstrated that calponin was indeed able to inhibit actomyosin ATPase activity up to 80%. This inhibition has been shown to be reversible both by phosphorylation of calponin (Winder & Walsh, 1990b) and by addition of Ca^{2+}–calmodulin (Abe et al., 1990; Makuch et al., 1991). Due to the ability of calmodulin to interfere with calponin's biological activity, it was of great interest to characterize the interaction between these two proteins.

Calmodulin is a member of the EF-hand superfamily originally described by Kretsinger [see review by Moncrief et al. (1990)] in reference to the helix–loop–helix motif used by members of this group to bind Ca^{2+}. Calmodulin is known to interact in a Ca^{2+}-dependent fashion and regulate a number of other proteins including myosin light chain kinase, cyclic nucleotide phosphodiesterase, Ca^{2+}/calmodulin-dependent protein kinase II, and phosphorylase kinase. A partial list of other proteins in this EF-hand superfamily includes troponin C (TNC)[1] calcineurin, oncomodulin, parvalbumin, S-100, myosin light chains, calpain (Moncrief et al., 1990), and SMCaBP-11 (Mani & Kay, 1990). Since calponin had been described to interact with two other members of this group, TNC (Takahashi et al., 1988b) and calpain (Tsunekawa et al., 1989), in addition to calmodulin, it suggested there may be a common determinant among these proteins conferring upon them the ability to interact with calponin. In particular, we wanted to establish whether calponin specifically interacted with calmodulin, or if it was a nonspecific interaction due to a reaction site on calponin that would optimally be used to bind a similar but separate protein.

In our pursuit of this question, the stoichiometry and strength of calponin's interaction with calmodulin have been characterized and the dissociation constants compared with those of four other members of the EF-hand family: troponin C, the Ca^{2+} binding subunit of the troponin complex which confers Ca^{2+} sensitivity upon cardiac and skeletal muscle contraction; S-100b, the β–β dimer form of the S-100 isoforms whose functions are not fully delineated; SMCaBP-11 a Ca^{2+} binding

† This research was supported by a grant from the Medical Research Council of Canada. F.L.W. is the recipient of an AHFMR studentship.

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[1] Abbreviations: TNC, troponin C, Ca^{2+} binding subunit of troponin; TNI, troponin I, inhibitory subunit of troponin; SMCaBP-11, smooth muscle calcium binding protein; M_r 11 000; kDa, kilodalton(s); EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; Tris, tris(hydroxymethyl)aminomethane; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); UV, ultraviolet; CD, circular dichroism; TCA, trichloroacetic acid; DTT, dithiothreitol; HMM, heavy meromyosin; pCa^{2+}, $-\log [Ca^{2+}]_{free}$; MOPS, 3-(*N*-morpholino)propanesulfonic acid; HPLC, high-performance liquid chromatography; TFA, trifluoroacetic acid; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

0006-2960/93/0432-2321$04.00/0

protein isolated from chicken gizzard which forms a dimer of 21 kDa; and parvalbumin, whose proposed function is as a relaxing factor of muscle by binding to Ca^{2+} as it is released from regulatory proteins following contraction. In addition to the fact that all of these proteins bind Ca^{2+}, all of them except parvalbumin expose hydrophobic patches upon Ca^{2+} binding. These hydrophobic patches are believed to be the site of interaction with many target proteins (Strynadka & James, 1989). Parvalbumin was thus included to test the necessity of the hydrophobic patch for interaction. A major finding was that this area does indeed seem to be required for interaction and it is not simply a complexation based upon the basic nature of calponin and the acidic nature of the Ca^{2+} binding proteins which enables interaction. Furthermore, it would seem the interaction between calponin and calmodulin may be nonspecific since we found there are other hydrophobic patch exposing proteins which interact with calponin with greater affinity.

MATERIALS AND METHODS

Protein Purification. Calponin was purified from turkey gizzards by a modification of the method of Takahashi et al. (1986) in which the Sulphopropyl-Zetaprep modular from Cuno, Inc., Life Sciences was used in place of an SP-C50 cation-exchange column. The modular was equilibrated with 6 M urea, 25 mM solution acetate, pH 4.5, 50 mM NaCl, 1 mM DTT, 1 mM EGTA, and 0.01% NaN_3, and the calponin was eluted with a 400-mL 50–350 mM NaCl gradient at 1.5 mL/min. The buffer for the 2.5 cm × 120 cm ACA 44 gel filtration column was 6 M urea, 10 mM NaH_2PO_4, pH 6.0, 100 mM NaCl, 1 mM DTT, 0.01% NaN_3, and 1 mM EGTA at a flow rate of 20 mL/h. Bovine brain calmodulin, rabbit skeletal troponin C, rabbit skeletal myosin and actin, and pike parvalbumin were prepared by routine procedures used in this laboratory (Cachia et al., 1986; McCubbin et al., 1982; Margossian & Lowey, 1982; Pardee & Spudich, 1982; Henryl et al., 1985, respectively). Bovine brain S-100b was prepared using the procedure for purifying the α-chain of S-100a (Leung et al., 1986). Peak IV obtained from the DEAE-Sephadex A-25 column was subjected to reversed-phase HPLC on a C_{18} column employing a solvent of 0.1% TFA with a 1%/min acetonitrile gradient to yield S-100b. Chicken gizzard SMCaBP-11 protein was prepared as described by Mani and Kay (1990). Final purification and removal of metal ions from calmodulin and troponin C were also achieved using the reversed-phase HPLC according to the procedure of Ingraham and Hodges (1988).

Absorption Spectroscopy. Absorption and UV difference spectroscopy was performed on a Perkin Elmer Lambda 5 spectrophotometer. UV difference spectroscopy for detecting Ca^{2+} binding was performed over the wavelength range 250–350 nm in 1-cm path-length cells. With 18 μM protein solutions in both cells, additions from 0.1 μM to 2 mM free Ca^{2+} were added to the sample cell while equal volumes of H_2O were added to the blank cell, and the spectrum was then scanned to detect any changes. The buffer was 25 mM Tris, pH 7.5, 100 mM NaCl, 1 mM DTT, and 1 mM EGTA.

Analytical Ultracentrifugation. Ultracentrifugation studies were performed on a Beckman Spinco Model E analytical ultracentrifuge. Molecular weights were determined by low-speed sedimentation equilibrium employing Raleigh interference optics according to Chevenka (1969). Protein concentrations were in the range of 1–2 mg/mL, and the molecular weights were calculated from the slope of the ln y vs r^2 plot.

Acrylodan Labeling. Calponin was labeled with a 1.5-fold excess of acrylodan (Molecular Probes, Eugene, OR) over calponin in 50 mM MOPS, pH 7.15, and 100 mM NaCl at a protein concentration of 1 mg/mL. The labeling was allowed to proceed overnight at 4 °C, the reaction stopped by the addition of excess DTT, and the solution dialyzed to remove excess label.

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 ninhydin.

Protein Concentrations. An extinction coefficient for calponin was determined by comparing the magnitude of the absorption peak observed at 276 nm with the results of an analytical ultracentrifuge experiment on the same sample to determine a fringe count. The relationship 4.1 fringes = 1 mg/mL protein solution was used (Babul & Stellwagen, 1969). This concentration was confirmed by amino acid analysis of the sample using the amino acid composition of Takahashi and Nadal-Ginard (1991) for the 32-kDa isoform. An $A^{1\%}_{276nm}$ of 7.5 was determined. This extinction coefficient was used to determine the protein concentration for experiments and was routinely confirmed by amino acid analysis. The following extinction coefficients were used to determine protein concentrations: calmodulin, $A^{1\%}_{277nm}$ = 1.95 (Klee, 1977); TNC, $A^{1\%}_{277nm}$ = 1.75 (McCubbin et al., 1982); parvalbumin, $A^{1\%}_{259nm}$ = 1.54 (Henryl et al., 1985); S-100b, $A^{1\%}_{278nm}$ = 2.4 (Mani et al., 1982); SMCaBP-11 protein, $A^{1\%}_{278nm}$ = 6.8 (Mani & Kay, 1990); myosin, $A^{1\%}_{280nm}$ = 5.88 (Verpoorte & Kay, 1966); and actin, $A^{1\%}_{290nm}$ = 6.3 (Lehrer & Kerwar, 1972). The acrylodan label concentration on the calponin was determined by A^{1M}_{387nm} = 16 400 (Prendergast 1983). The labeled calponin concentration was determined by amino acid analysis.

Quin 2 Fluorescence Titrations. Monitoring of Ca^{2+} binding with the fluorescent Ca^{2+} indicator Quin 2 was achieved by using the Amicon Centricon 10 ultracentrifugation device for rapid flow dialysis. Protein solutions were dialyzed in acid-washed cylinders to reduce Ca^{2+} contamination, and in the presence of Chelex-100, in the buffer 25 mM Tris, pH 7.5, 100 mM NaCl, and 1 mM DTT. Known amounts of Ca^{2+} were added to 40 μM protein samples in the top chambers of prewashed Centricons and centrifuged at 4200 rpm for 30 min to filter a portion of buffer through the 10-kDa cutoff membrane. Calponin which has a mass of 32 kDa was retained in the upper portion of the Centricon. Aliquots of flow-through buffer were added to a solution of 20 μM Quin 2 and 100 mM MOPS, pH 7.2, to determine Ca^{2+} levels. The increase in fluorescence was monitored at an excitation wavelength of 339 nm and an emission wavelength of 492 nm. From the known amount of Ca^{2+} added, the determined free Ca^{2+} levels, and the amount of protein present, it is possible to calculate the amount of Ca^{2+} bound to the protein. The experiment was initially performed on TNC to ensure the method was accurate and on calponin to test for Ca^{2+} binding.

Equilibrium Dialysis. Equilibrium dialysis was performed in 50-mL centrifuge tubes. A 150-μL protein sample at 1.3 mg/mL calponin was placed in 10–12-kDa molecular mass cut off dialysis tubing and dialyzed vs 10 mM MOPS, pH 7.0, 150 mM KCl, 100 μM EGTA, 2 mM DTT, 0.26 μM $^{45}Ca^{2+}$, and 200, 300, or 600 μM cold Ca^{2+} in triplicate. Samples were placed on a shaker and left at 4 °C overnight. The free Ca^{2+} concentration was determined by the concentration of label in a 20-μL sample of dialysis buffer. The bound Ca^{2+} concentration was determined by measuring the total concentration in a 20-μL sample from the dialysis tubing and subtracting the free Ca^{2+} concentration.

DTNB Reaction. DTNB titrations of calponin were used to determine the number of sulfhydryls exposed in the benign

solvent 50 mM MOPS, pH 7.15, 100 mM NaCl, and 1 mM EGTA, which had the DTT removed by running the sample over a gel filtration column. The absorption spectrum of calponin was first determined to establish the protein concentration, then a 10-fold excess of DTNB was added to the blank and protein-containing cell. The increase in absorption at 412 nm was monitored to determine the concentration of free sulfhydryls. The released thiobenzoate ion was quantitated using A^{1M}_{412nm} = 13 600 (Means & Feeney, 1971).

Fluorescence Studies. Fluorescence emission and excitation spectra were measured on a Perkin-Elmer MPF 44B recording spectrofluorometer equipped with a DCSU-2-corrected spectra accessory which allows for automatic background fluorescence correction. The temperature was maintained at 20 °C, fluorescence measurements were at 90° to the source, and slit widths were set at 5 nm. The instrument was operated in the ratio mode. Absorption of the solution at the wavelength of excitation never exceeded 0.1, so no correction for the inner filter effect was necessary. The intrinsic tryptophan fluorescence of calponin was monitored by exciting at 295 nm and monitoring the emission at 335 nm. Acrylodan-labeled calponin was excited at 388 nm, and titrations with both Ca^{2+} and Ca^{2+} binding proteins were performed by monitoring at 460 nm, the wavelength of greatest change. The concentration of acrylodan-labeled calponin in the cell was 0.5 μM, and Ca^{2+} titration of calponin–calmodulin was performed with 10 mole ratios of calmodulin over calponin in order to saturate calponin. Overall effects were followed by scanning the fluorescence emission from 400 to 600 nm. The buffer used was 50 mM MOPS, pH 7.15, 100 mM NaCl, 1 mM DTT, and 1 mM EGTA.

Circular Dichroism Measurements. CD measurements were performed on a Jasco J-720 spectropolarimeter. The cell as maintained at 25 °C with an RMS circulating water bath (Lauda, Westbury, NY). Far-UV scans from 255 to 195 nm employed a cell with a path length of 0.01 or 0.02 cm. The computer averaged 10 scans, and the signal due to solvent was subtracted. The instrument was routinely calibrated with *d*(+)-10-camphorsulfonic acid at 290 nm and with pantoyl lactone at 219 nm by following procedures outlined by the manufacturer. The data were plotted as mean residue weight ellipticity (expressed in degrees square centimeters per decimole) vs wavelength (in nanometers). The concentration of calponin and acrylodan-labeled calponin was 30 μM, while 10 μM calponin was used in the complexation experiment with a 1:1 mole ratio of calmodulin. The mean residue weight of calponin used was 110.7 Da/residue calculated from the sequence published by Takahashi and Nadal-Ginard (1991).

ATPase assays were performed using 0.125 mg/mL rabbit skeletal myosin, 0.5 mg/mL rabbit skeletal actin, 5 mM $MgCl_2$, 60 mM KCl, 25 mM Tris, pH 7.5, and 0.5 mM DTT. The reaction was started by adding 2 mM ATP and ended after 5 min by the addition of TCA. Inorganic phosphate was measured using the colorimetric assay with malachite green according to Itaya and Ui (1966).

Computer-Assisted Analysis. The secondary structure predictions from the far-UV CD spectra of calponin, calmodulin, and the calponin–calmodulin complex were determined using the FORTRAN program CONTIN, developed by Provencher and Glöckner (1981), which analyzes CD spectra as a combination of CD data collected from 16 proteins whose structures are known from X-ray crystallography.

Curve-fitting of fluorescence titrations to predict K_d's was accomplished by fitting the data to a cubic equation describing the reaction P + L $\rightleftharpoons$ PL + L $\rightleftharpoons$ PL_2, in which two ligands (L) bind to one target molecule (P) (Williams et al., 1986).

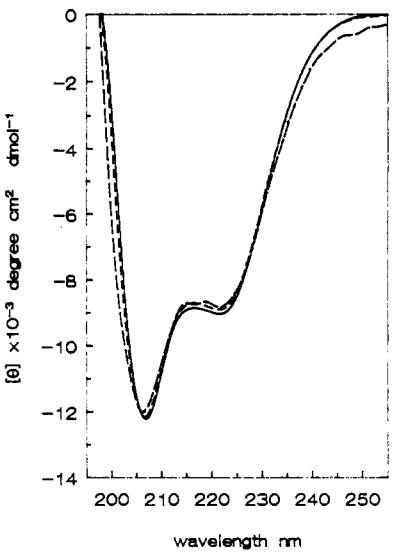

FIGURE 1: Far-ultraviolet CD spectra of native calponin in 50 mM MOPS, pH 7.15, 100 mM NaCl, 1 mM DTT, and 1 mM EGTA (—) or 1 mM free Ca^{2+} (- - -). Far-UV CD spectrum of acrylodan-labeled calponin (– –).

The dissociation constants, as well as the fractional fluorescence change attributable to each ligand binding, were allowed to be determined individually for each titration. The reported range of values includes the mean ± the standard deviation.

Free Ca^{2+} concentrations were calculated by an iterative method described by Perrin and Sayce (1967) using a program written for the Apple Macintosh (Courtesy of Dr. B. D. Sykes and R. Boyko, Department of Biochemistry, University of Alberta). This program includes constants for binding of Ca^{2+} to the protein. The sum of the negative log of the individual binding constants used was as follows: EGTA + H $\rightleftharpoons$ EGTA·H, 9.58; EGTA·H + H $\rightleftharpoons$ EGTA·H_2, 8.97; EGTA·H_2 + H $\rightleftharpoons$ EGTA·H_3, 2.8; EGTA·H_3 + H $\rightleftharpoons$ EGTA·H_4, 2.12; EGTA + Ca $\rightleftharpoons$ EGTA·Ca, 10.96; EGTA·H + Ca $\rightleftharpoons$ EGTA·H·Ca, 5.33 (Golosinska et al., 1991); Calm + 2Ca $\rightleftharpoons$ Calm·Ca_2, 13.4; Calm·Ca_2 + 2Ca $\rightleftharpoons$ Calm·Ca_4, 11.4 (Wang, 1985). Ca^{2+} titration data were fitted to a sigmoidal curve equation using Tablecurve 3.1 (Jandel Scientific).

RESULTS

Calponin–Calcium Interaction. There have been reports in the literature that calponin binds Ca^{2+} (Takahashi et al., 1988b; Winder & Walsh, 1990b); however, under our conditions, we were unable to find any signs of Ca^{2+} interaction. Calcium addition to calponin had no effect upon the circular dichroism spectrum, indicating there was no change in secondary structure upon Ca^{2+} addition (see Figure 1). Provencher–Glöckner analysis of the far-UV circular dichroism spectrum of calponin indicated the protein is comprised of 41% α-helix, 17% β-sheet, 12% β-turn, and 29% random coil in the presence or absence of Ca^{2+}. In addition, the UV difference spectrum (not shown) and the fluorescence of acrylodan-labeled calponin showed no change upon Ca^{2+} addition (see Figure 2). To further explore the possibility of an interaction, Quin 2 fluorescence studies and equilibrium dialysis in the presence of $^{45}Ca^{2+}$ were performed which indicated that there was no Ca^{2+} bound to calponin under the conditions used. Thus, by all the criteria explored, we were unable to find any evidence that calponin interacts with Ca^{2+} which is supported by the fact that the published calponin sequence does not contain any EF-hand-like regions (Takahashi & Nadal-Ginard, 1991).

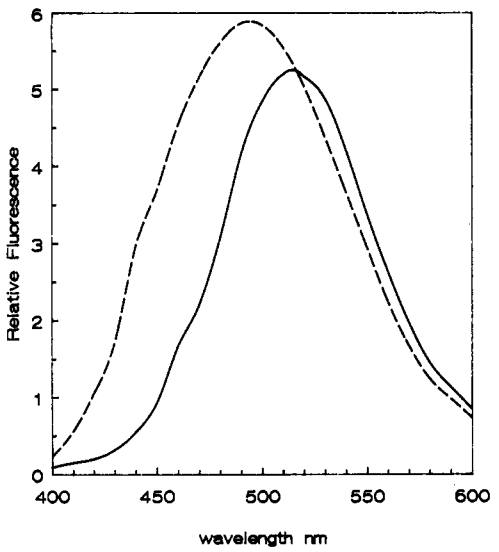

FIGURE 2: Fluorescence spectra of acrylodan-labeled calponin, acrylodan-labeled calponin + Ca^{2+}, and acrylodan-labeled calponin + calmodulin (—) and of acrylodan-labeled calponin + Ca^{2+}–calmodulin (- - -).

Stoichiometry of the Calponin–Calmodulin Complex. Analytical ultracentrifugation of the calponin–calmodulin complex indicated molecular masses ranging from the molecular masses of the individual proteins to well above the calponin (32.3 kDa) and calmodulin (16.7 kDa) theoretical 1:1 complex mass of 49 kDa. Neither calponin nor calmodulin alone, either in the presence or in the absence of calcium, demonstrated molecular masses above their monomer masses, indicating there was no aggregation of either protein alone (see Figure 3). The slope of the ln y vs r^2 plot was used to determine the molecular mass, and deviation from the straight line near the meniscus indicates the presence of lower molecular mass species at this position in the cell. In the case of calponin which shows a molecular mass of 32.1 kDa, these lower molecular mass species are breakdown products which are produced over the 48-h course of the experiment and can be seen by SDS–PAGE (data not shown). Calmodulin does not exhibit any deviation from linearity and shows a molecular mass of 17.2 kDa, well within experimental error of calmodulin's monomer mass of 16.7 kDa. Thus, the high molecular masses for the mixture must be due to complex formation. In order to determine the stoichiometric amounts of calponin and calmodulin in the complex, varying ratios of calponin and calmodulin were mixed to see if an excess of either protein favored complex formation (see Table I). When calponin was in excess, a molecular mass of only 50 kDa was seen, indicating no more than a 1:1 complex. However, when excess calmodulin was present, a molecular mass of 63.3 kDa was observed across the bottom third of the cell, indicating the nature of the complex to be 2 calmodulin molecules to 1 calponin (see Figure 3C). The fact that the slope of the ln y vs r^2 plot curved near the meniscus indicated not all the protein was complexed and suggested the interaction was not a strong one. When the experiment was carried out in the absence of Ca^{2+}, no molecular masses were observed above those of the constituent proteins, indicating the interaction was dependent upon the presence of Ca^{2+}. In addition, a 3 calmodulin to 1 calponin mixture in the presence of Ca^{2+} was used to determine if higher molecular masses could be observed; however, the molecular masses across the bottom third of the cell still remained at 63.4 kDa, indicating this is truly the upper limit of complexation. This experiment was repeated with other calcium binding proteins to see if they formed the same type of complex, and evidence indicates TNC, S-100b, and SMCaBP-11, all of which expose hydrophobic patches upon Ca^{2+} binding, also form this complex of 2 mol of the relevant Ca^{2+} binding protein to 1 mol of calponin in a Ca^{2+}-dependent manner. In the case of S-100b and SMCaBP-11 which exist as dimers, molecular mass data indicated 2 mol of dimer bound to 1 mol of calponin. On the other hand, parvalbumin, which does not expose hydrophobic patches upon Ca^{2+} binding, showed no signs of binding to calponin.

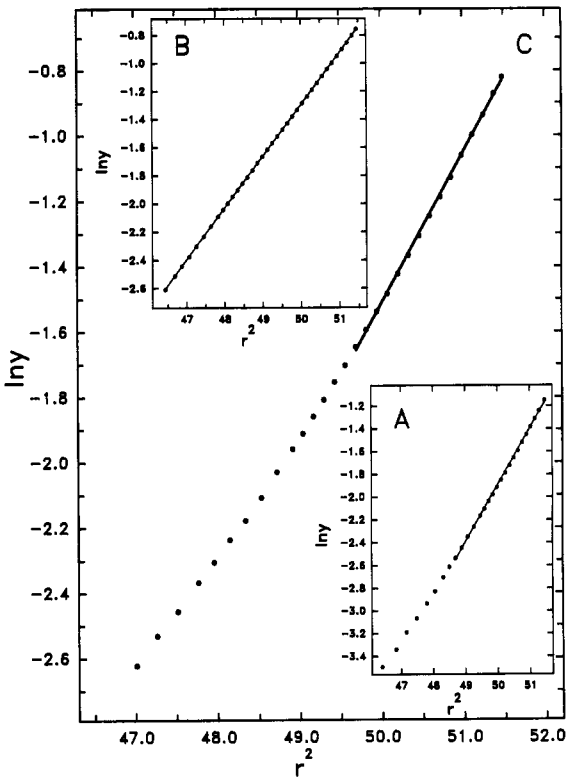

FIGURE 3: Representative plots of ln y vs r^2, where y is the concentration in fringe displacement units and r is the distance from the axis of rotation in centimeters. The slope of the line was determined from a least-squares fit of the data points through which the line passes and is used to calculate the molecular mass. In all cases, the fit to the data had an R value of >0.999. (A) Calponin at an initial loading concentration of 1.1 mg/mL, run at 16 000 rpm, molecular mass 32.1 kDa; (B) calmodulin at an initial loading concentration of 1.91 mg/mL, run at 18 000 rpm, molecular mass 17.3 kDa; (C) a 2 mol of calmodulin to 1 mol of calponin ratio at an initial loading concentration of 1.7 mg/mL, run at 11 000 rpm, molecular mass 63.3 kDa. The buffer used was 50 mM MOPS, pH 7.15, 100 mM NaCl, 1 mM DTT, and 1 mM $CaCl_2$, and the temperature was maintained at 5 °C.

Table I: Analytical Ultracentrifugation of the Calponin–Calmodulin Complex

protein mole ratios	calcium	molecular mass observed (kDa)
calponin	±	32.1
calmodulin	±	17.3
calponin + calmodulin	–	29.0
2 calponin + 1 calmodulin	+	50.8
1 calponin + 2 calmodulin	+	63.3
1 calponin + 3 calmodulin	+	63.4

Acrylodan Labeling of Calponin. Since the intrinsic tryptophan fluorescence of calponin did not respond to interaction with calmodulin, the fluorescent label acrylodan was used to label the exposed cysteine of calponin, thereby introducing a probe that is sensitive to ligand binding. DTNB titration of calponin indicated that although there is more than one cysteine per calponin molecule, two or three depending upon the isoform (Takahashi & Nadal-Ginard, 1991), when

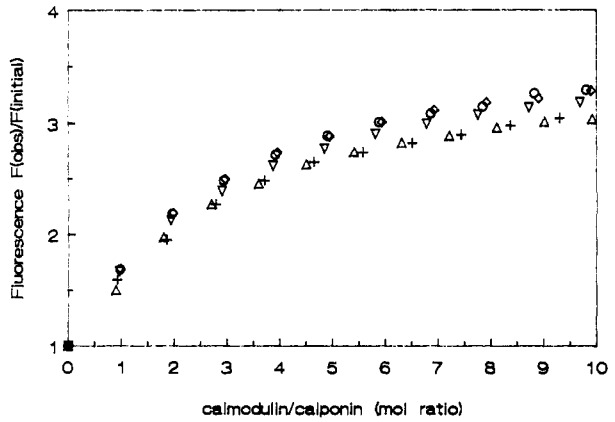

FIGURE 4: Titration with calmodulin of various ratios of acrylodan to native calponin. (+) 12%, (△) 22%, (○) 46%, (▽) 70%, and (◇) 84% of total calponin is labeled with acrylodan. The buffer used was 50 mM MOPS, pH 7.15, 100 mM NaCl, 1 mM DTT, 1 mM EGTA, and 3 mM $CaCl_2$.

DTNB was reacted with calponin under nondenaturing conditions there was 1.3 mol of cysteine/mol of calponin which reacted immediately with DTNB, while it required more than 30 min for 2 mol of cysteine/mol of calponin to interact. This indicates that there is one cysteine in a highly exposed environment on the calponin, while the other cysteines are more buried. We have observed that the integrity of the other cysteines must remain intact by the following observations: reaction with DTNB of more than one cysteine per calponin leads to instability of the protein and precipitation; labeling of calponin under denaturing conditions with acrylodan leads to loss of secondary structure as detected by far-UV CD; carboxymethylation of the cysteines under denaturing conditiosn also leads to loss of secondary structure; and maintenance of a reduced environment is very important for the function and structural integrity of calponin. Therefore, since the nondenaturing conditions used for labeling with acrylodan lead to a calculated average of one acrylodan label per calponin molecule, it is likely that it is only the highly exposed cysteine which is labeled. This singly labeled calponin retains its secondary structure as seen by CD (see Figure 1) and its biological activity in inhibiting the ATPase activity of actomyosin (data not shown). In order to determine if acrylodan-labeled calponin interacted with calmodulin in an identical fashion to native calponin, titrations with calmodulin of various ratios of labeled vs unlabeled calponin were performed. The resulting fluorescent curves are superimposable, indicating the labeled calponin and unlabeled calponin indeed behave in a similar fashion (see Figure 4).

Fluorescence of the Calponin–Calmodulin Complex. The acrylodan probe on calponin is a very sensitive monitor of the interaction beween calponin and the calcium binding proteins. The emission maximum of acrylodan bound to β-mercaptoethanol in the polar environment of water is 540 nm, while the very nonpolar environment of dioxane shifts the emission maximum to 435 nm (Prendergast, 1983). As shown in the fluoresence scan (Figure 2), acrylodan-labeled calponin alone has an emission maximum at 515 nm which would indicate the acrylodan probe is in a polar, exposed environment. The position of this peak is unaffected by calcium or by calmodulin in the absence of calcium (see Figure 2). However, when calmodulin is added in the presence of calcium, there is a shift in the emission peak to 495 nm, indicating the probe has moved into a less polar, less exposed environment. This indicated that after the complexation, the hydrophobic fluorophore becomes buried either within the protein or within the calponin–calmodulin interface. In addition to this peak shift,

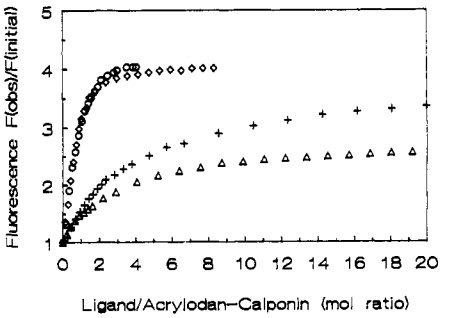

FIGURE 5: Fluorescence titration of calponin with Ca^{2+} binding proteins in the presence of Ca^{2+}. (△) TNC; (+)-calmodulin; (◇) S-100b; (○) SMCaBP-11. Mole ratios for S-100b and SMCaBP-11 are calculated for the dimer.

Table II: Dissociation Constants for Calponin–Ligand Complexes

ligand	K_{d_1} (μM)[a]	F_1[b]	K_{d_2} (μM)[a]	F_2[b]	F_{obs}/F_o
TNC	≤0.68	0.46 ± 0.05	4.5–8.5	0.54 ± 0.09	2
calmodulin	≤0.22	0.27 ● 0.07	2.5–3.4	0.73 ± 0.08	3.5
SMCaBP-11	≤0.13	0.94 ± 0.1	0.18–1.60	0.06 ± 0.15	4
S-100b	≤0.03	0.83 ± 0.03	0.36–0.77	0.17 ± 0.04	4
parvalbumin	no interaction				

[a] The range shown represents the mean ± the standard deviation. [b] F_1 and F_2 represent the fractional fluorescence change observed for each ligand binding.

there is also an increase in the fluorescence of the acrylodan-labeled calponin, and these two effects combine to produce the greatest change at 460 nm where the fluorescence, in the presence of saturating amounts of calcium–calmodulin, is 3.5 times the fluorescence of acrylodan-labeled calponin alone. This blue shift in the emission maximum and the increase in fluorescence also occur in an analogous fashion with the hydrophobic patch exposing proteins TNC, S-100b, and SMCaBP-11 in a calcium-dependent fashion. On the other hand, parvalbumin did not cause any change in the fluorescence.

The large increase in fluoresence at 460 nm of acrylodan-labeled calponin can be monitored as the calponin is titrated with the calcium binding proteins in the presence of calcium (see Figure 5). From these curves, it is evident that TNC has the weakest affinity for calponin of the proteins tested and causes the smallest (2-fold) increase in the fluoresence. Calmodulin has an intermediate affinity and causes a 3.5-fold increase in fluorescence, but it takes approximately a 40-fold molar excess of calmodulin over calponin to do so. S-100b and SMCaBP-11, however, show 4-fold increases in fluorescence, requiring only 2 molar ratio excess over calponin to do so with the highest affinities for calponin.

On the basis of the stoichiometry determined with the analytical ultracentrifuge of two calcium binding proteins binding to one calponin to form a complex, and the fact that the calmodulin and TNC fluorescence curves did not fit a 1:1 binding curve, the fluorescence data curves for the interaction between calponin and these other proteins can be curve-fitted for a 2:1 interaction to determine binding constants (see Table II). The results clearly indicate that TNC binds with the overall weakest affinity with a K_{d_1}, the binding of the first TNC to calponin, of ≤0.68 μM and a K_{d_2}, the binding of the second TNC to calponin, of 4.5–8.5 μM. Calmodulin demonstrated an intermediate affinity with a K_{d_1} of ≤0.22 μM and a K_{d_2} of 2.5–3.4 μM. S-100b and SMCaBP-11 demonstrated higher affinities, with SMCaBP-11 having a K_{d_1} of ≤0.13 μM and a K_{d_2} of 0.18–1.6 μM while S-100b exhibited a K_{d_1} of ≤0.03 μM and a K_{d_2} of 0.36–0.77 μM. It is evident that these latter two proteins show considerably

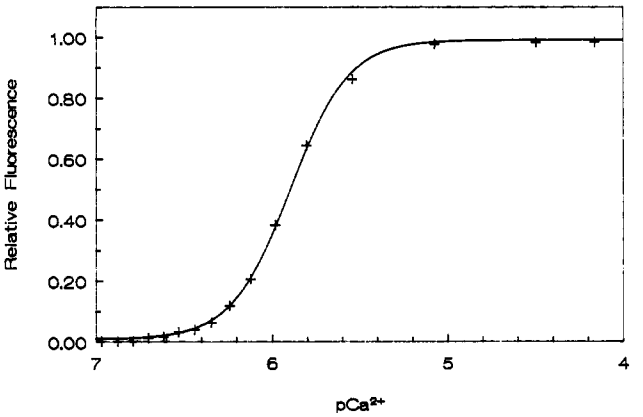

FIGURE 6: Fluorescence titration of the calponin–calmodulin complex with Ca^{2+} (+). The data were curve-fitted to a sigmoidal transition equation(—).

higher affinity for calponin than calmodulin does, and cause a larger increase in the quantum yield of the probe (F_{obs}/F_o in Table II) upon complexation. In addition, the first binding of SMCaBP-11 or S-100b to calponin causes a much greater increase in fluorescence than the first binding of TNC or calmodulin.

Calcium Titration of the Calponin–Calmodulin Complex. The interaction betwwen calponin and calmodulin is dependent upon calmodulin binding to Ca^{2+} as has been published previously (Takahashi et al., 1986) and as demontrated by the lack of an effect of calmodulin on the fluorescence spectrum of calponin in the absence of Ca^{2+} (see Figure 2). The dependence of complex formation upon Ca^{2+} can be demonstrated by titration by monitoring the increase in fluorescence as the complex forms (see Figure 6). From this titration, 50% of the fluorescence change occurs with a pCa^{2+} of 5.9 which is very close to the pCa^{2+} of the low-affinity sites on calmodulin of 5.7, found by Wang (1985). This suggests calponin binding to calmodulin is dependent upon a change occurring in calmodulin upon calcium filling the low-affinity sites, and does not respond to calcium filling of the high-affinity sites which occurs at a pCa^{2+} of 6.7. Preliminary experiments indicated TNC also required binding of Ca^{2+} to the low-affinity sites for interaction with calponin (data not shown).

Far-UV CD of the Calponin–Calmodulin Complex. In order to determine whether secondary structural changes occur upon complexation of calponin with calmodulin, the far-UV circular dichroism spectra of the two proteins in the presence of calcium were determined, and then a theoretical curve of the two proteins together was calculated assuming no structural changes occur. This theoretical curve is then compared to the observed curve for the complex to see if any changes actually occurred upon complexation (see Figure 7). This was done both in the presence and in the absence of Ca^{2+}. In the absence of Ca^{2+}, the difference in ellipticity between the observed and calculated spectra for the two proteins was 440° 222 nm, which is just out of experimental error for this technique. However, in the presence of Ca^{2+}, there is 1450° decrease in negative ellipticity at 222 nm in the observed spectrum vs the theoretical curve indicating a change in secondary structure in one or both components occurred upon complexation. Provencher–Glöckner secondary structure prediction analysis of both the theoretical and observed spectra was performed to determine the nature of the secondary structure changes. The observed spectra for the complex had 25% α-helix, which indicates a 5% decrease occurred, and 28% β-sheet, which indicates an 8% increase occurred upon complexation. Results from the analytical ultracentrifuge data indicate that when

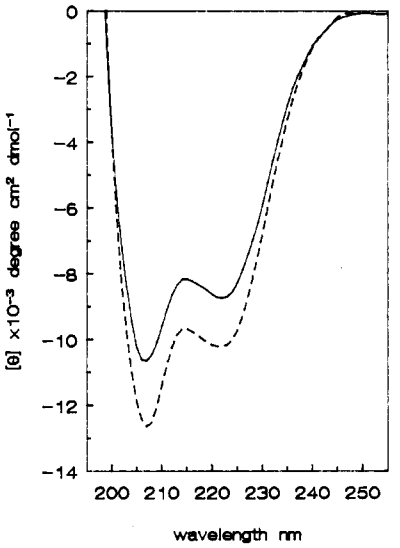

FIGURE 7: Far-ultraviolet CD spectra of the calponin–calmodulin complex in the presence of Ca^{2+} (—) and the calculated theoretical curve for the two proteins assuming no interaction (- - -).

calponin and calmodulin are mixed 100% of the protein does not form a complex as evidenced by the presence of lower molecular mass species at the top half of the cell (see Figure 3C). Therefore, the changes observed in the CD spectra are only an indication that secondary structural changes occur, and the spectrum observed does not represent the pure complex, but rather a mixture of complexed and free protein.

DISCUSSION

Calponin was originally described as a calcium, calmodulin, and actin binding troponin T-like protein (Takahashi et al., 1988b; Winder & Walsh, 1990b). In view of this initial suggestion that calponin bound calcium, we decided to investigate the relationship more closely. These studies on the physical properties of calponin were unable to demonstrate any evidence of calponin binding to calcium. This is not surprising since it has previously been reported that Ca^{2+} has no effect upon ATPase inhibition by calponin (Winder & Walsh, 1990a), or upon binding to actin or tropomyosin (Takahashi et al., 1986). While calponin does not bind Ca^{2+} directly, it interacts in a calcium-dependent fashion with proteins which expose a hydrophobic patch upon themselves binding Ca^{2+}. It has been previously reported that calponin is a substrate for the calcium-dependent protease calpain which is known to digest calmodulin binding proteins (Tsunekawa et al., 1989). This, together with the fact that calponin will bind both calmodulin and TNC (Takahashi et al., 1986), suggested that there may be a common determinant among these proteins which conferred their ability to interact with calponin. Of the group tested in this study, TNC, S-100b, calmodulin, and SMCaBP-11, all expose these hydrophobic patches in a Ca^{2+}-dependent fashion as shown by TNS titrations of these proteins (Tanaka & Hidaka, 1981; Mani & Kay, 1990). TNS interacts with hydrophobic regions, resulting in a dramatic increase in quantum yield of the TNS. We have shown that all these hydrophobic patch exposing proteins interact with calponin in a calcium-dependent manner. Parvalbumin, conversely, binds calcium but does not expose these hydrophobic patches which are postulated to provide the interface for interaction with target proteins (Strynadka & James, 1989). In agreement with our theory that it is these hydrophobic patches with which calponin is interacting, calponin does not bind to parvalbumin. The interaction between calponin and calmodulin was shown to be 50%

complete at a pCa^{2+} of 5.9 which coincides with the strength of calmodulin's low-affinity sites for Ca^{2+} (Wang 1985). Tanaka and Hidaka (1980) showed that the dramatic increase in exposed hydrophobic residues in calmodulin occurred with a pCa^{2+} of 5.5, which also agrees with the amount of Ca^{2+} required for calponin–calmodulin complexation. Calmodulin exhibits stepwise changes in structure with the number of moles Ca^{2+} bound, and the changes in calmodulin at these various Ca^{2+} levels can be used to modulate various processes within the cell (Klee et al., 1986). It has been shown that calmodulin-interacting proteins may variously depend upon Ca^{2+} binding to the high-affinity sites alone for activation, for example, phosphorylase kinase and plasma membrane ATPase, or upon Ca^{2+} binding to both the high- and low-affinity sites, as with cyclic nucleotide phosphodiesterase and myosin light chain kinase. Thus, different levels of Ca^{2+} filling are required for various functions, and calponin requires all four Ca^{2+} sites to be filled for interaction. What is surprising, however, is that proteins which require Ca^{2+} binding to the low-affinity sites for complexation with calmodulin generally demonstrate an increased Ca^{2+} affinity in the complex than calmodulin does alone; i.e., the low-affinity sites fill with Ca^{2+} at a lower concentration of free Ca^{2+} when the receptor protein is present than in its absence. This increase in affinity may be as much as 2 orders of magnitude (Klee, 1988). That calmodulin, while interacting with calponin, does not display this increased affinity for Ca^{2+} does indicate the free energy of interaction between these two proteins is not strong enough to affect the Ca^{2+} binding affinity and that this interaction may not be biologically significant.

The nature of the complex between calponin and calmodulin was probed through the use of circular dichroism spectroscopy and analytical ultracentrifugation. From the far-UV CD, we found that there was a change in secondary structure resulting in decreased α-helix and increased β-sheet as complexation occurs compared to the two proteins individually. These changes do not occur in the absence of Ca^{2+}, once again emphasizing the Ca^{2+} sensitivity of the complex. Analytical ultracentrifuge experiments indicated a complex between calponin and calmodulin was being formed which had a mass well above the theoretical mass for a 1:1 complex. Molecular masses obtained in the analytical ultracentrifuge indicate that in the presence of excess amounts of calponin a 1:1 complex between calmodulin and calponin is formed with an observed molecular mass of 50.8 kDa. However, in the presence of excess calmodulin, a molecular mass of 63.3 kDa was seen. This suggests the nature of the complex is two calmodulin molecules to one calponin, producing a complex of 66 kDa in the presence of calcium. However, when there is excess calponin present, the binding of the first calmodulin is stronger than the binding of the second calmodulin such that each calponin having one calmodulin bound is energetically more favorable than 50% of the calponin molecules having two calmodulins bound, with the result that only a 1:1 complex is observed. It has previously been observed in gel filtration experiments that an average of 1.2 molecules of calmodulin were eluting with each molecule of calponin, suggesting that at least 2 calmodulins could bind per molecule of calponin (Takahashi et al., 1986). Our analytical ultracentrifuge data indicate that even a 3-fold excess of calmodulin over calponin yields the 66-kDa molecular mass species, indicating the nature of the complex is two calmodulins per calponin. TNC, S-100b, and SMCaBP-11 also appear to interact with calponin in the same ratio, suggesting that all these proteins which are able to expose hydrophobic patches in the presence of Ca^{2+} bind with the stoichiometry of two Ca^{2+} binding proteins per calponin molecule. Since parvalbumin did not show any sign of interaction with calponin either in the presence or in the absence of Ca^{2+}, the reaction between these proteins and calponin is not simply due to the acidic nature of the calcium binding proteins and the basic nature of calponin. While parvalbumin is similar to the other proteins in the EF-hand superfamily in being an acidic binding protein which binds Ca^{2+} with EF-hand-like structures, it does not expose hydrophobic patches upon Ca^{2+} binding, and this seems to be the determining factor in the ability to bind calponin. The lack of interaction with parvalbumin underlines the importance of hydrophobic patches for calponin interaction.

The hydrophobic patches exposed upon calcium binding by calmodulin and TNC have been implicated in the interaction with their target proteins (LaPorte et al., 1980). It is believed that in order to interact, presentation of both hydrophobic and basic determinants is necessary, and some target proteins may form an amphipathic α-helix which is able to bind to the hydrophobic patches (Cachia et al., 1986). Some degree of flexibility in the interaction of target proteins with nonspecific hydrophobic patches has already been demonstrated (Cachia et al., 1985), and from this study, it is evident that calponin demonstrates this flexibility in binding to a variety of hydrophobic patch proteins, albeit with varying affinities.

Much controversy has surrounded the question as to whether the caldesmon–calmodulin interaction is strong enough that caldesmon would bind to calmodulin in vivo due to the fact that calmodulin binding to other target proteins, for example, myosin light chain kinase and calcineurin, demonstrates K_d's in the low nanomolar range compared to a K_d of 250 nM for caldesmon (Mills et al., 1988). Also the large mole ratios of calmodulin to caldesmon required to reverse caldesmon's inhibition of the actomyosin ATPase would argue against such a regulation being feasible (Marston, 1990). These same arguments apply even more so to the issue of calponin–calmodulin interaction in the cell. The present study would argue against the calponin–calmodulin interaction being biologically important as not only is the K_d for this interaction too weak, but also two other proteins, S-100b and SMCaBP-11, have demonstrated much greater affinities. In addition, two separate groups have shown that as much as 10 mole ratios of calmodulin to calponin are required to reverse its inhibition (Abe et al., 1990; Makuch et al., 1991). Thus, it would appear the calmodulin–calponin interaction, like the calponin–TNC interaction, is nonspecific. With reference to S-100b and SMCaBP-11, the SMCaBP-11 is isolated from smooth muscle cells and appears to be a likely candidate for interaction with calponin in a biologically significant manner. S-100 was originally thought to be primarily a nervous tissue protein as it was derived from bovine brain; however, varying isoforms of S-100 have now been reported in a variety of cell types including melanocytes, Langerhans cells, slow-twitch skeletal muscles, heart, and kidney tissue (Donato, 1991). Although S-100 has not been specifically isolated from smooth muscle tissue, it cannot be ruled out that calponin and S-100b or an S-100b-like protein could be present in the same tissue. Pritchard & Marston (1991) have attempted to isolate an S-100-like protein with regulatory abilities from smooth muscle; however, to date, this effort has been unsuccessful.

The ability of calponin to interact in a nonspecific fashion with these Ca^{2+} binding proteins can be related to its function in inhibiting the ATPase of actomyosin. Horiuchi and Chacko (1991) showed that while HMM bound to actin and the ATPase was active in the absence of calponin, increasing ratios of calponin/actin not only caused a drop in ATPase activity but also caused a concomitant decrease in the HMM bound

to actin. Since it is known that calponin binds to actin (Takahashi et al., 1986), it seems that actin is able to bind either calponin or HMM, but their interaction is mutually exclusive. This suggests the method by which calponin inhibits the ATPase is by binding to a site on actin such that myosin is no longer able to bind to actin. Makuch et al. (1991) showed that a 10-fold excess of Ca^{2+}–calmodulin over calponin can displace calponin from actin in ultracentrifugation experiments and also restore ATPase activity. This would be directly analogous to the findings of Van Eyk et al. (1991) in the skeletal muscle case. These researchers found that the TNI inhibitory peptide can bind either to actin in the absence of Ca^{2+}, which confers inhibition of ATPase activity and contraction, or to TNC in the presence of Ca^{2+}, which leaves actin free to interact with myosin, allowing ATPase activity and contraction to occur. Van Eyk et al. (1991) showed that actin and TNC compete for the same site on TNI, indicating a similar determinant on TNI must be recognized by both these proteins. Thus, it would appear the myosin–actin–TNI–TNC system in skeletal muscle is a reasonable model for myosin–actin–calponin–calmodulin behavior in smooth muscle. It, therefore, is not unreasonable that calponin's ability to bind to actin with high affinity (Winder et al., 1991) would confer upon it the ability to interact with proteins such as calmodulin, S-100b, and SMCaBP-11 which are similar to TNC. It will be of great interest to pursue these relationships and determine which proteins are biologically important in their interactions with calponin.

ACKNOWLEDGMENT

We thank K. Oikawa, A. Keri, L. Hicks, and M. Nattriss for invaluable technical assistance and Dr. R. Mani for helpful discussions and the generous gift of SMCaBP-11.

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