A role for serine-175 in modulating the molecular conformation of calponin

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Calponin is an actin filament-associated protein found in smooth muscle and non-muscle cells. Calponin inhibits actin–myosin interaction in a manner that is prevented by protein kinase C (PKC)-catalysed phosphorylation of serine-175. To investigate the molecular basis of serine-175-mediated regulation, we examined the effect of phosphorylation on the conformation of calponin using monoclonal antibody (mAb) epitope analysis. Eight mAbs against different epitopes on chicken gizzard calponin were developed to monitor the conformational changes in calponin induced by PKC-mediated phosphorylation or serine-175 → alanine (S175A) substitution. The relative affinities of the mAbs for calponins immobilized on microtitre plates or bound to actin–tropomyosin thin filaments were determined, and epitope competitions between free and immobilized calponins were carried out. The changes in binding affinity between mAb paratopes and calponin epitopes demonstrate several serine-175 modification-induced conformational effects: (a) structures of calponin are reconfigured by serine-175 modification, supporting

the regulatory function of serine-175; (b) there are submolecular structures unaffected by modification of serine-175 in both free and thin filament-associated calponins, suggesting that the serine-175-based conformational modulation is a targeted allosteric effect; (c) significant conformational changes are detected between free and thin filament-associated calponins, indicating two functional states of the molecular conformation; and (d) the different epitope characteristics between thin filament-bound and free calponins suggest that calponin is a flexible molecule, and the modifications of serine-175 may also determine the structural flexibility to increase the epitope accessibility. These results provide novel information concerning the structure–function relationships of calponin and its regulation by phosphorylation.

Key words: actin binding, phosphorylation, protein conformation, protein kinase C, smooth muscle, tropomyosin.

INTRODUCTION

Calponin is a family of actin thin filament-associated proteins, first identified in smooth muscle [1,2] and later found also in non-muscle cells [3,4]. Biochemical studies showed that calponin binds to actin [1,5,6], Ca²⁺-calmodulin [1,2], S100 protein [7], caltropin (calyculin) [8], tropomyosin [2,9], myosin [10,11] and desmin [12,13]. Calponin's interactions with contractile and cytoskeletal proteins have led to its proposed role in modulating the Ca²⁺ signalling of contractile and cytoskeletal functions in smooth muscle and non-muscle cells.

The primary regulation of smooth-muscle contraction is through Ca²⁺-calmodulin-dependent phosphorylation of myosin light chain, which activates the actomyosin Mg-ATPase [14,15]. Nevertheless, during sustained contractions of tonic smooth muscle, force is maintained, while [Ca²⁺] and myosin phosphorylation are reduced [16,17]. Therefore it has been proposed that additional regulatory mechanisms may exist at the level of the thin filament [18–20], and calponin may play a role in this signal transduction pathway. For example, calponin inhibits the actin-activated Mg-ATPase activity of phosphorylated smooth muscle myosin, and this inhibition is regulated by phosphorylation-dephosphorylation of calponin [5,6,21–23]. *In vitro* motility assays demonstrated that calponin inhibits the relative movement of actin and myosin filaments [24,25]. It has also been shown that exogenously added calponin reduces the active tension and maximal shortening velocity in skinned smooth

muscle [26–29], and phosphorylation of calponin occurs concurrently with contraction of intact smooth muscle strips [23,30].

The structure–function relationships of calponin have been

Figure 1 Structural and functional domains of calponin

Regions of the proposed structural and functional domains of calponin are illustrated on a linear map together with the primary structure of the region flanking serine-175. The functional segments of calponin are identified according to *in vitro* biochemical studies as described in the text. None of these functional or structural domains are directly related to the serine-175 site that has been shown to regulate calponin's function in inhibiting the actomyosin ATPase. The amino acid sequences flanking the serine-175 region of chicken h1- [47], mouse h1-, h2-, porcine h1-, h2- [48], rat h1- [49], and rat acidic [3] calponins are aligned to demonstrate the highly conserved structure (the dash indicates a residue identical to that in chicken h1-calponin). CH domain, calponin homology domain.

Abbreviations used: ABTS, 2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulphonic acid); HRP, horseradish peroxidase; mAb, monoclonal antibody; PKC, protein kinase C.

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vigorously studied. Several functional domains of calponin have been identified (Figure 1). The N-terminal fragment of calponin (residues 1–52) is responsible for calmodulin binding. A second calmodulin-binding site is located within residues 153–163. The segment between residues 145 and 163 has been identified to interact with F-actin, and is involved in the inhibition of actomyosin ATPase activity [31]. Both the N-terminal and the 145–163 sites are also involved in tropomyosin binding. An approx. 100 amino acid hypothetical actin-binding domain with homologues found in many cytoskeletal proteins (the calponin homology) is located at residues 29–129 [32]. We have demonstrated that the phosphorylation of serine-175 by protein kinase C (PKC) plays a critical role in regulating calponin's function of inhibiting actomyosin ATPase activity [33]. While serine-175 is conserved among calponin isoforms (Figure 1), it lies outside of the above identified functional domains of calponin, and does not belong to either of the two proposed structural domains of calponin (residues 7–144 and 183–292 respectively) [31]. Bio-physical analysis demonstrated that calponin is a flexible elongated monomeric protein [34]. Therefore the serine-175-mediated regulation of calponin may involve a long-range conformational and functional modulation.

The lack of a high-resolution three-dimensional structure of calponin places a limitation on the investigation, and alternative approaches are needed to study the modulation of calponin conformation by phosphorylation. Antigen–antibody interaction is a protein–protein interaction depending on the conformational fits between the antigenic epitope and the specific antibody paratope. Using a panel of monoclonal antibodies (mAbs) [35] against multiple specific epitopes on calponin, we investigated in the present study the role of serine-175 in the modulation of calponin molecular conformation. Quantification of the interactions between the mAbs and calponin demonstrated that the conformation of calponin was allosterically modulated by phosphorylation of serine-175, supporting the regulatory role of this site. Conformational differences between the thin filament-bound and free calponins indicate two distinct functional states, and the modifications of serine-175 may also determine the molecular flexibility to increase the epitope accessibility. The data suggest a molecular mechanism for the serine-175-mediated regulation of calponin function.

MATERIALS AND METHODS

Recombinant chicken gizzard calponin and S175A mutant

Wild-type and S175A mutant calponins [33] were expressed in *Escherichia coli* and purified as described previously [33]. The purified calponins in 20 mM Tris/HCl (pH 7.5), 0.1 M KCl, 0.2 mM dithiothreitol, 2 mM MgCl₂ and 0.1 mM EGTA were stored in small aliquots at –80 °C before use. The calponins prepared were > 95% pure as shown by SDS/PAGE.

Purification of PKC, smooth muscle actin and tropomyosin

cPKC (a mixture of α , β and γ isoenzymes) was purified from rat brain as described previously [36]. Chicken gizzard actin and tropomyosin were purified as described previously in [37] and [38] respectively.

Protein concentration determination

Protein concentrations were determined by amino acid analysis using endogenous phenylalanine and exogenous norleucine for quantification.

In vitro phosphorylation of calponin

Wild-type or S175A mutant calponin (0.1 mg/ml) was incubated with PKC (1 μ g/ml) at 30 °C for 1 h in 50 mM Mops (pH 7.0), 5 mM MgCl₂, 0.3 mg/ml phosphatidylserine, 62 μ g/ml 1,2-diolein, 0.03% (w/v) Triton X-100, 0.25 mM ATP, and either 0.1 mM CaCl₂ or 10 mM EDTA in a reaction volume of 20 ml. Reactions containing Ca²⁺ were made 10 mM in EDTA at the end of the reaction. Data from our previous studies have demonstrated that similar reactions under identical experimental conditions produced effective phosphorylation of calponin at serine-175 [33].

Anti-calponin mAbs

Adult chicken gizzard calponin was used to immunize Balb/c mice. The hybridoma fusion and selective culture were performed as described previously [39]. The hybridomas were screened by indirect ELISA for the production of antibodies against chicken gizzard calponin immobilized on the microtitre plate. The stable hybridoma cell lines established were used to produce anti-calponin mAb-enriched ascites fluid in pristane-primed Balb/c mice. Interaction of the mAbs with native chicken calponin was verified by ELISA titration and, with SDS-denatured calponins, by Western blotting [39]. The immunoglobulin subclass of the anti-calponin mAbs was determined on the hybridoma culture media by a sandwich ELISA method using a mouse Ig isotyping kit (Pharmingen, San Diego, CA, U.S.A.).

By SDS/PAGE and Western blotting using ¹²⁵I-labelled rabbit anti-mouse IgG (ICN Biomedicals) secondary antibody, specificity of the anti-calponin mAbs was examined on gizzard smooth muscle total protein extracts homogenized in SDS gel sample buffer. By Western blotting using alkaline phosphatase-labelled goat anti-mouse IgG (Sigma) secondary antibody, calponin isoform specificity was analysed on h1-calponins from gizzard or stomach smooth muscles and cloned mouse h2-calponin [40] expressed in *E. coli*.

ELISA epitope analysis of serine-175 modification-induced conformational changes in immobilized calponin

An indirect ELISA-based solid-phase epitope analysis was developed to detect conformational changes in calponin induced by modification at the serine-175 site. As outlined in Figure 2(A), wild-type or S175A calponin at 5 μ g/ml in buffer A [0.1 M KCl, 10 mM Pipes (pH 7.0), 3 mM MgCl₂], with or without PKC treatment, was coated on 96-well microtitre plates (Falcon 3915) by incubation (100 μ l/well) at 4 °C overnight. After blocking the remaining plastic surface with 1% BSA and 0.05% Tween 20 in buffer A at room temperature for 2 h, and washing the plates with buffer A plus 0.05% Tween 20 (buffer T), the immobilized calponins were incubated with serial dilutions of the anti-calponin mAbs in buffer T plus 0.1% BSA at room temperature for 1 h. Following washes with buffer T to remove the unbound antibody, the plates were further incubated with horseradish peroxidase (HRP)-labelled goat-anti-mouse total Ig secondary antibody (Sigma) at room temperature for 1 h, followed by buffer T washes and H₂O₂-ABTS [2,2'-azinobis-(3-ethylbenzothiazoline-6-sulphonic acid)] substrate reaction. A_{405nm} development for each assay well was recorded by an automated microplate reader (Benchmark; BioRad) at a series of time points to quantify the interaction between specific mAb paratopes and the epitopes on the wild-type or S175A calponins before and after PKC phosphorylation. All of the experiments were done in triplicate and

Figure 2 ELISA-based epitope analyses

(A) Indirect ELISA analysis of epitope conformation in immobilized calponin. Unphosphorylated or phosphorylated wild-type and S175A mutant calponins were coated on microtitre plates. After blocking the remaining plastic surface with non-ionic detergent and BSA, the calponin immobilized in the wells was incubated with serial dilutions of an anti-calponin mAb. Following standard ELISA procedure using HRP-labelled anti-mouse Ig secondary antibody and ABTS- H_2O_2 substrate, reactivity between the mAb and the specific epitope on immobilized calponin was quantified by absorbance at 405 nm. (B) Competitive ELISA analysis of epitope conformation in free calponin. Unphosphorylated wild-type or S175A calponin was coated on microtitre plates and the wells were blocked as above. Serial dilutions of the competing wild-type, phosphorylated wild-type, S175A or phosphorylated S175A calponin were added to the plate just before the addition of a pretitrated concentration of an anti-calponin mAb. After incubation and washes to remove all mAb in solution, HRP-conjugated secondary antibody was used to detect the binding of the mAbs to the immobilized calponin in the presence of different concentrations of the competing free calponin via H_2O_2 -ABTS substrate reaction. In addition to free calponin, calponins bound to actin-tropomyosin were also used in the competition assays to analyse the epitope conformation of thin filament-associated calponin. (C) Protein-binding assay. Actin and/or tropomyosin were coated on microtitre plates. After blocking, the plates were incubated with serial dilutions of calponin, followed by anti-calponin mAbs to detect the bound calponin. The washing, secondary antibody reaction, and substrate colour development are as described in (A). In addition to quantifying calponin-thin filament interaction, this assay system was also used to immobilize calponin under a physiological condition for the epitope conformational analysis illustrated in (A).

data from the linear region of the $A_{405\text{nm}}$ time course were used to construct the titration curves.

ELISA epitope competition analysis of serine-175 modification-induced conformational changes in free and thin filament-associated calponins

To determine the conformational changes in free calponin induced by serine-175 modification, the interactions between calponin epitopes and the specific mAbs were analysed by a competitive ELISA procedure illustrated in Figure 2(B). Wild-type or S175A calponin without PKC treatment was diluted in buffer A at $5\text{ }\mu\text{g/ml}$ and coated on microtitre plates at $4\text{ }^\circ\text{C}$ overnight. After blocking and washing the wells as above, serial dilutions of wild-type or S175A calponin with or without PKC treatment, alone or together with chicken gizzard actin and tropomyosin [calponin/actin/tropomyosin (1:14:4), mol/mol for an effective association of the calponin to the actin filament], were added to the microtitre plate in buffer T containing 0.1% BSA. A predetermined dilution of each of the anti-calponin mAbs in buffer T containing 0.1% BSA was then added to the wells, followed immediately by 8 s shaking in the microplate reader to mix the reagents. The reactions were incubated at room temperature for 1 h. After washing the wells with buffer T as above, HRP-conjugated anti-mouse Ig secondary antibody was added and incubated at room temperature for 1 h. After final

washes with buffer T, the plates were developed with H_2O_2 -ABTS substrates, and $A_{405\text{nm}}$ was measured as described above in triplicate assays. The competition between the serially diluted free or thin filament-associated calponin and the constant amount of calponin immobilized on the microtitre plate for the mAb was plotted to reveal the changes in affinity of mAbs for the epitope on the competing calponin, which represents the conformational changes induced by the modification of serine-175 by PKC phosphorylation or Ala substitution.

ELISA-mediated solid-phase protein-binding experiments

Similar to the procedures which we have developed for studying troponin T isoforms [41], ELISA-based actin-calponin and tropomyosin-calponin binding assays were developed to characterize the interactions of calponin with the actin thin filament. As illustrated in Figure 2(C), microtitre plates were coated at $4\text{ }^\circ\text{C}$ overnight with purified chicken gizzard actin and tropomyosin (alone or together at a molar ratio of 7:2, corresponding to the association of seven actins to a tropomyosin dimer) in buffer A. After washes to remove the excess actin and/or tropomyosin with buffer T, the plates were incubated with serial dilutions of calponin in buffer T containing 0.1% BSA at room temperature for 2 h. The plates were then washed with buffer T to remove unbound calponin and the binding of calponin to actin and/or

tropomyosin was measured by an anti-calponin mAb (CP1, recognizing an epitope independent of the thin filament binding-associated or serine-175-modulated conformational changes; Figure 5). Following incubation at room temperature for 1 h and washes as above, HRP-conjugated anti-mouse Ig secondary antibody was incubated with the plate at room temperature for 45 min followed by final washes and H₂O₂-ABTS substrate colour reaction. The $A_{405\text{nm}}$ of triplicate assay wells was monitored at a series of time points as above. The actin-tropomyosin-coated microtitre plates were also used to immobilize calponin to a physiological matrix for epitope conformation analysis as described above.

Data analysis

The ELISA results were plotted using the Igor Pro computer program (Wavemetrics, Lake Oswego, OR, U.S.A.). The mAb dilutions required for 50 % maximum binding to the immobilized calponins, the calponin concentrations required for 50 % maximum binding to actin and/or tropomyosin, and the concentrations of free or actin-associated calponin resulting in 50 % inhibition of the maximum binding of the mAb to the immobilized calponin, were determined graphically from the titration curves, and the statistical significance of the differences was examined by Student's *t* test.

RESULTS

MAbs against multiple specific epitopes on chicken h1-calponin

Eight mouse mAbs (CP1, CP2, CP3, CP4, CP5, CP6, CP8 and CP9) raised by chicken gizzard calponin immunization were used in this study. Their immunological characterization is summarized in Table 1. The diverse reactivity of the eight mAbs indicates that they are against different epitopes on calponin, providing a battery of tools to monitor the global conformation of calponin. All of the mAbs react with immobilized calponin in ELISA showing a wide range of binding affinity. Due to the restricted availability of the secondary antibody, Western blotting was carried out only for the IgG mAbs. Among the five IgG mAbs, CP6 did not recognize the SDS/PAGE-resolved calponin, indicating that the CP6 epitope may be sensitive to SDS

Table 1 Epitope-specific anti-calponin mAbs

N/D, not determined.

mAb	Immunoglobulin subclass	Relative affinity in ELISA*	Western blot†	Dependency on integrity of calponin‡	Species cross-reactivity§
CP1	IgG- κ	High	+	High	Broad
CP2	IgA- κ	Low	—	N/D	N/D
CP3	IgG2b- κ	High	+	High	Intermediate
CP4	IgG- κ	High	+	Low	Narrow
CP5	IgM- κ	Intermediate	—	N/D	N/D
CP6	IgG- κ	Low	—	N/D	N/D
CP8	IgG- κ	High	+	Low	Broad
CP9	IgM- λ	Intermediate	—	N/D	N/D

* Determined by the titres in indirect ELISA.

† Using anti-mouse IgG second antibody.

‡ Deduced from Western blots on CNBr partially cleaved fragments of chicken gizzard calponin [40].

§ Among h1-calponins from amphibian, avian and mammalian smooth muscles [40].

Figure 3 Specificity of the anti-calponin mAbs for the purified wild-type and S175A calponins

(A) Total protein extracts from chicken gizzard smooth muscle were used in Western blotting to demonstrate the specificity of the anti-calponin mAbs. The autoradiographs via ¹²⁵I-labelled anti-mouse IgG secondary antibody show specific recognition of calponin by the mAbs. (B) Cloned wild-type and S175A mutant chicken gizzard calponins prepared from *E. coli* expression were examined by SDS/12%PAGE (acrylamide/bisacrylamide, 29:1) along with calponin purified from chicken gizzard tissue. The SDS gel shows the highly purified recombinant calponins with gel mobilities identical to that of gizzard calponin. The SDS gel-resolved proteins were transferred to nitrocellulose membrane and examined by Western blotting for their reactivity to the anti-calponin mAbs. The blots, via alkaline phosphatase-labelled secondary antibody and 5-bromo-4-chloro-3-indolylphosphate/Nitro Blue Tetrazolium colour reaction, verified the authenticity of the calponins expressed from cloned cDNA.

denaturation and/or is highly dependent on the native molecular conformation of calponin. Western blots showed that the mAbs CP1, CP3, CP4 and CP8 specifically recognize only calponin in the chicken gizzard total protein extracts (Figure 3A). The Western blots in Figure 3(B) further show that the S175A mutation did not abolish the mAb reactivity, indicating no overlap between the mAb epitopes and the serine-175 site. These

Table 2 Isoform specificity of anti-calponin mAbs

Four anti-chicken gizzard calponin mAbs were analysed for their specificity to calponin isoforms. Total protein homogenates from chicken gizzard, mouse stomach, rat stomach, pig stomach, cloned mouse h1- and h2-calponins purified from *E. coli* culture were analysed by SDS/PAGE and Western blotting [40]. The summarized results show that the mAbs had different reaction patterns to the calponin isoforms while their primary structures flanking the Ser175 region are identical or very similar (Figure 1). +, strength of reactivity of the mAbs; —, no detectable interaction.

Calponins	mAbs			
	CP1	CP3	CP4	CP8
Chicken h1	+	+	+	+
Mouse h1	+	—	+	+
Rat h1	+	+	+	+
Porcine h1	+	—	—	+
Mouse h2	—	—	—	—

anti-gizzard calponin (h1-calponin) mAbs did not cross-react with h2-calponin, and exhibited quantitative differences in cross-reactivity with h1-calponins from different species (Table 2). The data indicate that these anti-calponin mAbs all recognize epitopes outside the serine-175 site [since the primary structures flanking the serine-175 site are identical, or very similar, in all calponin isoforms sequenced to date (Figure 1)] and, therefore, can be used to monitor global conformational changes of calponin induced by the modification at serine-175.

Phosphorylation of serine-175 reduces calponin's actin-binding affinity

ELISA-mediated protein binding assays showed a high affinity binding between wild-type calponin and immobilized actin (Figure 4A). The higher affinity between calponin and immobilized actin (8 nM, Figure 4B) in comparison with that previously reported (6 μ M, [42]) may be due to the nature of the solid-phase assay conditions, which may facilitate the formation of the F-actin-calponin complex. Although detectable by the ELISA experiments, the binding between calponin and immobilized tropomyosin is significantly weaker in contrast to the binding to actin (Figure 4A). The inclusion of tropomyosin in the actin filament did not affect the affinity of calponin binding (9 nM, Figure 4C). Figures 4(B) and 4(C) further show that PKC phosphorylation of wild-type calponin reduced the binding affinity to actin and actin-tropomyosin filaments by 3-fold (approx. 25 nM, $P < 0.001$). S175A mutation resulted in the same effect and the PKC treatment of S175A calponin did not produce further change in the actin-binding affinity, indicating the presence of two functional states of calponin which are modulated by the phosphorylation of serine-175.

Changes of calponin epitopic structures by PKC-catalysed phosphorylation and S175A substitution

The PKC treatment and/or S175A mutation-induced changes in mAb epitope affinity against microtitre plate-immobilized or free calponin are summarized in Tables 3 and 4 respectively. Represented by the titration curves shown in Figure 5, the results demonstrated three classes of calponin structures with distinct

conformational responses to the serine-175 phosphorylation-based regulation. The free calponin competition assays against immobilized wild-type or S175A calponin showed identical results (results not shown), indicating that S175A mutation did not affect calponin's global folding and, in turn, the mode of coating on to microtitre plates.

Epitope structures modulated in immobilized and free calponins

The epitopes recognized by mAbs CP2, CP4 and CP6 on immobilized calponin showed decreased binding affinity to the mAbs in response to the structural modification at serine-175. Phosphorylation and S175A substitution had similar effects on the conformation and reactivity of the CP2 epitope. The S175A substitution permitted additional PKC-catalysed phosphorylation, as demonstrated by the further decrease in CP2 epitope affinity when compared with that of the PKC-treated wild-type calponin. On the other hand, the CP6 epitope was only slightly affected by PKC-catalysed phosphorylation, but a significant decrease in affinity for the mAb was observed when serine-175 was replaced by alanine. The further decrease in CP6 epitope affinity of the PKC-treated S175A calponin indicated an additional conformational effect of PKC-catalysed phosphorylation at other sites. These results demonstrate a role of serine-175 in regulating the molecular conformation of calponin.

The specific epitope recognized by mAb CP4 underwent a significant decrease in mAb binding affinity in immobilized calponins due to structural modification at serine-175 by PKC-catalysed phosphorylation (Figure 5). In contrast with the effects on the CP2 and CP6 epitopes, while S175A substitution resulted in a small decrease in mAb CP4 affinity, PKC phosphorylation was able to further lower the CP4 affinity to a level similar to that of PKC-treated wild-type calponin. This phenomenon suggests that the changes in CP4 epitope structure may reflect a two-step modulation in the serine-175-based regulation. In this case, a primary structural modification, e.g. PKC-catalysed phosphorylation or substitution of serine-175, induces an original conformational change that promotes secondary phosphorylation at an additional site(s), as shown by the further conformational changes of the CP4 epitope. The effect of serine-175 modification on the CP4 epitope conformation was also demonstrated by the free calponin epitope competition assays. PKC-catalysed phosphorylation and S175A substitution produced similar increases in CP4 epitope affinity of free calponin without the two-step effect seen with the immobilized calponins. The different conformational responses of the CP4 epitope in immobilized versus free calponin imply that this epitope structure may be sensitive to calponin's association with and dissociation from the actin filament. Knowing that phosphorylation of calponin will reduce the calponin-actin binding affinity (Figure 4), the increase in CP4 epitope accessibility may correspond to a related regulatory structure.

The CP3 epitope affinity titration showed that the serine-175 modification by PKC-catalysed phosphorylation or alanine substitution did not induce change in the immobilized calponins. However, PKC-catalysed phosphorylation of wild-type, but not S175A, calponin induced a small but significant increase in CP3 epitope-binding affinity, as shown by the free calponin epitope competition assays against immobilized wild-type and S175A calponins (Table 4). This conformational change may represent a physiological effect in the serine-175 phosphorylation-based regulation of calponin function, and the CP3 epitope may represent a target structure for the PKC-catalysed phosphorylation of serine-175-mediated signal transduction.

Figure 4 Phosphorylation of serine-175 reduces the actin-binding affinity of calponin

ELISA-mediated protein binding assays were carried out as illustrated in Figure 2(C). (A) The titration curves show a high-affinity binding between wild-type calponin and immobilized F-actin. In contrast, the binding between calponin and immobilized tropomyosin is significantly weaker. The inclusion of tropomyosin in the actin filament did not affect the affinity of calponin binding. (B) and (C) PKC phosphorylation of wild-type calponin significantly decreased the binding affinity to actin and actin–tropomyosin filaments respectively. S175A mutation resulted in the same effect, and the PKC treatment of S175A calponin did not cause further change in the actin-binding affinity.

Table 3 Dilutions of anti-calponin mAbs needed for 50% maximum binding to the immobilized calponins

The values are dilutions of specific mAb ascites required to produce 50% of the maximum binding to the four immobilized calponins. Statistical significance in paired comparisons using Student's *t* test: *, *P* < 0.02; †, *P* < 0.005; ‡, *P* < 0.005; §, *P* < 0.002; ||, *P* < 0.005; ¶, *P* < 0.0001; **, *P* < 0.0005; ††, *P* < 0.0005; ‡‡, *P* < 0.05; §§, *P* < 0.05; |||, *P* < 0.0005; ¶¶, *P* < 0.01; ***, *P* < 0.05.

mAb	Calponin			
	Wild-type	Wild-type + PKC	S175A	S175A + PKC
CP2	10 ^{-2.29} *,†	10 ^{-2.03} *	10 ^{-2.06} †,‡	10 ^{-1.68} †
CP6	10 ^{-2.35} §	10 ^{-2.22}	10 ^{-1.67} §,	> 10 ^{-1.48}
CP1	10 ^{-5.10}	10 ^{-4.92}	10 ^{-4.92}	10 ^{-4.92}
CP3	10 ^{-5.56}	10 ^{-5.48}	10 ^{-5.48}	10 ^{-5.48}
CP4	10 ^{-5.30} ¶,**	10 ^{-4.42} ¶	10 ^{-4.90} ††,‡‡	10 ^{-4.20} ††
CP9	10 ^{-4.79} †,§§	10 ^{-4.59} ††	10 ^{-4.64} §§,	10 ^{-4.02}
CP5	10 ^{-4.19} ¶¶	10 ^{-4.21}	10 ^{-3.84} ¶¶,***	10 ^{-3.43} ***
CP8	10 ^{-4.08}	10 ^{-4.01}	10 ^{-4.14}	10 ^{-4.11}

Table 4 Concentrations of free calponin needed for 50% inhibition of the mAbs' binding to immobilized calponins

Values are concentrations (nM) of the four free calponins required to reach 50% inhibition of the maximum binding of the mAbs to the immobilized calponin. Statistical significance in paired comparisons using Student's *t* test: *, *P* < 0.005; †, *P* < 0.01; ‡, *P* < 0.0005; §, *P* < 0.0005; ||, *P* < 0.00002.

mAb	Against immobilized wild-type calponin				Against immobilized S175A calponin	
	Wild-type	Wild-type + PKC	S175A	S175A + PKC	S175A	S175A + PKC
CP1	24	17.8	25.1	27.5	17.4	17.8
CP3	34.7*,†	19.5*	60.2†	56.2	21.9	17.8
CP4	> 500‡,§	363‡	380§	347	339	263
CP9	≥ 500	≥ 500	≥ 500	≥ 500	≥ 500	≥ 500
CP5	≥ 500	≥ 500	≥ 500	≥ 500	≥ 500	≥ 500
CP8	25.1	20	10	10.2	69.2	83.2

Modulation of molecular flexibility of calponin by serine-175 modification

Figure 5 shows that the specific epitope recognized by mAb CP8 on immobilized calponin had no response to the modification at serine-175 by phosphorylation or alanine substitution. While phosphorylation did not alter the conformation of the CP8 epitope on free calponins, S175A substitution significantly decreased the binding affinity of free calponin for the mAb (Table 4). The S175A-induced decrease of CP8 epitope accessibility in free calponin suggests a role for the serine-175 site in the determination of calponin molecular flexibility. The physiological significance of this mechanism was supported by the results that the S175A calponin associated with the actin filament did not show such an effect (the corresponding panel in Figure 5C).

Epitope structures unaffected by modification of serine-175 in both free and immobilized calponins

The binding affinity between mAb CP1 and its epitope did not change in microtitre plate-coated, free or actin filament-

Figure 5 Representative epitope conformational responses of calponin to modifications at serine-175

ELISA affinity titrations against immobilized calponins (A) and free calponin epitope competition assays against immobilized wild-type calponin (B) were performed for eight mAbs as illustrated in Figures 2(A) and 2(B) respectively. Three classes of conformational responses to the serine-175 modifications were detected as shown by the representative results of three mAbs. (1) Changes of epitope conformation in immobilized and free forms: the CP4 epitope in immobilized calponin exhibited significant decreases in affinity for the mAb, reflecting conformational changes induced by PKC phosphorylation and, to a lesser extent, by S175A substitution. PKC phosphorylation of the S175A calponin further reduced the CP4 epitope affinity to a level similar to that induced by PKC phosphorylation of wild-type calponin. The epitope competition analysis showed that PKC phosphorylation and S175A substitution produced similar increases in the CP4 epitope affinity in free calponin. (2) Serine-175 modification may have effects on the flexibility of calponin: the CP8 epitope in immobilized calponin exhibited no change in response to the structural modification at serine-175 by phosphorylation or alanine substitution (A). In contrast, phosphorylation or S175A substitution significantly decreased the binding affinity between free calponin and the mAb (B), suggesting a decreased molecular flexibility resulting in reduced accessibility to the epitope in free calponin, and this effect may have been eliminated in the immobilized calponins. This is supported by the fact that no CP8 epitope affinity change was detected in the serine-175 modified calponins associated with the actin thin filament (C). (3) Epitope structures independent of modifications of serine-175: the affinity between CP1 and its epitope was unchanged in the microtitre plate-immobilized, free or actin filament-associated calponins upon the structural modification at serine-175 induced by PKC phosphorylation or alanine substitution.

associated calponin upon the structural modification at serine-175 induced by PKC-catalysed phosphorylation or alanine substitution (Figures 5A–5C). The results suggest that, in contrast to a non-specific global disruption of the protein conformation, the epitope conformational changes detected by other anti-calponin mAbs represent a targeted allosteric response to the structural modification at serine-175. The fact that the CP1 epitope has a stable conformation in both free and immobilized calponins agrees with its conservation in h1-calponins across species [40], and may represent a core structural domain in the calponin molecule.

Different epitope characteristics of immobilized and free calponins

As described above, the CP3 (Tables 3 and 4) and CP4 (Figure 5) epitopes demonstrated different binding properties to the specific mAbs in immobilized and free calponins. More dramatic differences were seen for the mAbs CP9 and CP5 epitopes

(Figure 6, Tables 3 and 4). These epitopes on immobilized calponin responded to the structural modification at serine-175 by PKC-catalysed phosphorylation or S175A substitution in a similar way to that seen for the CP2 and CP6 epitopes (Figure 6A). However, the CP9 and CP5 epitopes on free calponin showed undetectably low affinity for the mAbs when competing against the immobilized calponins, with identical results from the wild-type and S175A mutant (Figure 6B). Similar to those on calponin immobilized to a microtitre plate, the CP5 and CP9 epitopes were accessible on calponins associated to the actin-tropomyosin filament (Figure 6C). PKC phosphorylation resulted in significant change in the affinity of the CP9 epitope of thin-filament-associated calponin, indicating a physiologically regulated structure that undergoes a conformational switch during the association-dissociation of calponin to the actin filament. It is worth noting that the S175A mutation resulted in a smaller change, and PKC-catalysed phosphorylation did not have a further effect (Figure 6C), supporting the regulatory role of

Figure 6 Calponin epitope structures sensitive to association-dissociation with the actin filament

(A) ELISA affinity titration against wild-type calponin on microtitre plate using mAb CP9 (and CP5, see Table 3) showed that the specific epitopes on immobilized calponins responded to the structural modification at serine-175 by phosphorylation or alanine substitution. (B) Competition analysis showed these epitopes on free calponin had undetectably low affinity for the mAbs against the immobilized calponin, demonstrating that immobilization reconfigures the molecular conformation of calponin. The association of calponin to actin filaments also confers the epitope structures in both affinity titration (C) and competition (D) assays, suggesting that they represent allosteric structures with physiological significance.

serine-175 phosphorylation. The configuration of the CP9 epitope upon calponin's association to actin was confirmed by the positive competition for the mAb by calponins bound to actin-tropomyosin (Figure 6D). The results provide evidence that thin filament-associated versus free calponins have significantly different molecular conformations, in agreement with calponin's role as a flexible allosteric regulatory protein. The mAbs recognizing actin-bound but not free calponin have provided useful tools for monitoring the functional states of calponin.

DISCUSSION

Antibody epitope analysis to monitor calponin conformational regulation

The nature of antigen-antibody recognition is a specific protein-protein binding interaction. Like other protein-protein inter-

actions, it depends on a conformational fitting between the antigenic epitope and the antibody paratope, both formed by protein three-dimensional structures. mAbs feature the recognition of a single antigenic determinant, i.e. a specific epitope [35]. Therefore the application of mAbs provides a novel approach to monitoring protein conformation, structural dynamics and folding [43]. It has been shown that the mAb titres determined by the solid-phase ELISA represent the relative affinity of the binding between the antibody and the antigen [44]. Solid-phase ELISA is a high efficiency method and can quantitatively analyse hundreds of antibody-antigen interactions in parallel with excellent reproducibility. Based on the affinity-dependent nature of the solid-phase immunosorbent method, the ELISA epitope analysis is highly sensitive and can detect small changes in the fitting between the antigen epitope and the antibody paratope. We have successfully applied this approach for structure-function analysis of troponin T [41,45]. The epitope analysis

developed in the present study for monitoring conformational changes of both immobilized and free calponins is an advancement of this application of mAb technology. For proteins like calponin, whose crystal or NMR three-dimensional structure is not yet available, the ELISA-based antibody epitope analysis is highly valuable to quantitatively monitor conformational changes of the protein under various conditions.

Serine-175 is a sensitive site for targeted conformational modulation of calponin conferring its regulatory role in modulating calponin's functional states

mAb epitope analysis clearly showed that the molecular conformation of calponin is reconfigured by phosphorylation and S175A substitution, supporting the regulatory function of serine-175. Furthermore, the presence of certain submolecular structures unaffected by the modification of serine-175 in both free and thin filament-associated calponins indicates that the serine-175 phosphorylation-based conformational modulation is a set of targeted allosteric effects. Although the S175A substitution eliminated this primary PKC phosphorylation site [33], and is not a natural regulatory mechanism for calponin structure and function, the change of calponin conformation due to the alanine mutation supports the critical role of serine-175 in determining calponin molecular conformation and functional state. These data also provide evidence that an alanine mutation is not a 'silent' substitution, and should be applied with caution in mutagenesis mapping of protein structure–function relationships.

Phosphorylation of serine-175 by PKC may reconfigure the molecular conformation and functional state of calponin via a two-step mechanism

The analysis of the CP3 epitope on free calponin showed that a domain of structures is modulated specifically by PKC phosphorylation (Table 4), implying a physiological-responding target. However, in the analysis of the CP4 epitope on immobilized calponin, S175A substitution also resulted in a decrease in mAb affinity, which is less than that produced by PKC phosphorylation of wild-type calponin (Figure 5 and Table 3). It is worth noting that PKC-catalysed phosphorylation of the S175A calponin further reduced the affinity to a level similar to that produced by PKC phosphorylation of wild-type calponin (Figure 5). The effect of serine-175 modification on the PKC phosphorylation of other sites in the calponin molecule may represent a mechanism by which serine-175 fulfills its role in the regulation of calponin function. In other words, when serine-175 is phosphorylated as the primary site of the PKC-mediated signalling, the induced primary change of calponin molecular conformation will produce a secondary effect to allow phosphorylation of other residues, further reconfiguring the molecular structure and functional state of calponin. This two-step regulation model may be able to explain the previous observations that other sites, for example threonine-184, may also be phosphorylated under certain experimental conditions [46].

Calponin is a flexible allosteric protein and serine-175 may modulate the flexibility of calponin

The different epitope characteristics of immobilized versus free calponins suggest that calponin is a flexible molecule of changing conformation when interacting with its environment. This feature is important for a regulatory protein whose function depends on the association-dissociation with the actin filament. The immobilization-related change of calponin epitope accessibility

found in this study may reflect a modulation of molecular flexibility, which may underlie the mechanism for calponin's function in the signal transduction pathway involving its on–off cycling of association with the actin filament. The fact that modifications of serine-175 by PKC-catalysed phosphorylation can increase epitope accessibility, as shown by the increase of CP3 and CP4 epitope affinity in free calponin (Figure 5 and Table 4) and S175A substitution-produced decrease of CP8 epitope affinity (Figure 5 and Table 4), suggest that this site may also regulate the flexibility of calponin.

The characterization of the serine-175-based conformational changes of calponin leads to a model whereby serine-175 confers its regulatory role by modulating the two conformational and functional states of calponin corresponding to its actin filament-associated and free states. To guide further investigations, this study revealed the first evidence for changes in calponin's molecular conformation during the phosphorylation-regulated cycling of association-dissociation with the actomyosin system. Together, the results provide novel information concerning the structure–function relationships of calponin and the molecular basis of serine-175-mediated regulation.

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