Purification and Characterization of an Actin-, Calmodulin- and Tropomyosin-Binding Protein from Chicken Gizzard Smooth Muscle

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An actin-binding protein (p33) has been purified from chicken gizzard smooth muscle. The homogenous protein has a molecular weight near 33000 as determined by both sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and size exclusion chromatography. Its binding ability to F-actin remained after heating at 95 °C for 4 min. Immunoblot analyses indicated that p33 was not a degradation product from higher molecular components. The binding of p33 to F-actin was saturable in a molar ratio of about one p33 to 2–3 actin molecules with an apparent binding constant of $6.6 \times 10^7 \text{ M}^{-1}$. p33 also bound to calmodulin and tropomyosin. The bindings of p33 to F-actin and tropomyosin were regulated by calmodulin in a Ca^{2+} -dependent fashion. In addition to actin, caldesmon and tropomyosin, p33 was contained in the native thin filaments prepared from smooth muscle. Other actin-binding proteins, including α -actinin, caldesmon and filamin, had little effect on p33 binding to actin filaments. These results demonstrate that p33 may function in actin-based cellular processes which are mediated by Ca^{2+} and calmodulin.

Keywords actin; calmodulin; tropomyosin; binding protein; smooth muscle; native thin filament; binding site

Actin-containing microfilaments are found in nearly all eukaryotic cells and are responsible for a number of cellular activities including cell motility, cell shape change, cytokinesis, secretion and adhesion to substratum.^{1–3)} The organization of actin filaments is modulated by a variety of actin-binding proteins that have been classified into several categories based on their biochemical properties. In a variety of smooth muscle, it is generally considered that Ca^{2+} can initiate and modulate the contractile response derived from actin and myosin filaments, and calmodulin acts as a transducer of the intracellular Ca^{2+} signal. There are dual regulatory mechanisms of smooth muscle contraction. One is the phosphorylation of myosin light chain catalyzed by myosin light chain kinase,^{4–6)} and the other is actin-mediated regulation by caldesmon.^{7,8)} Ca^{2+} and calmodulin can lead to the activation of actomyosin adenosine triphosphatase (ATPase) activity via the activation of myosin light chain kinase activity and the inhibition of caldesmon–actin interaction. Myosin light chain kinase and caldesmon also bind to actin^{9,10)} and tropomyosin,^{11–13)} respectively, and their bindings are regulated by Ca^{2+} and calmodulin. Taken together, these observations suggest that calmodulin-dependent actin-binding proteins would participate in the contractile systems. In addition, it is not clear whether or not a troponin-T-like protein is present in smooth muscle.

During the isolation of caldesmon from chicken gizzard smooth muscle, we observed a low-molecular-weight protein which could bind to F-actin, calmodulin and tropomyosin. In this paper, we describe the purification procedure and functional characterization of this protein.

Materials and Methods

Preparation of Proteins Actin was prepared from rabbit skeletal muscle by the method of Spudich and Watt,¹⁴⁾ and was further gel filtered through Sephadex G-150.¹⁵⁾ The following proteins were isolated from chicken gizzard smooth muscle using published methods: caldesmon,¹⁶⁾ tropomyosin,¹⁷⁾ α -actinin¹⁷⁾ and filamin.¹⁷⁾ Calmodulin was purified from porcine brain according to the method of Isobe *et al.*¹⁸⁾ Native thin filaments were prepared from chicken gizzard muscle as described by Marston and Lehman.¹⁹⁾

Purification of an Actin-Binding Protein (p33) Unless otherwise noted, all purification procedures were carried out at 4 °C. Fresh chicken gizzard muscles were homogenized in 6 volumes of 50 mM imidazole–HCl buffer

(pH 6.9) containing 2 mM MgCl_2 , 0.5 mM phenylmethanesulfonyl fluoride (PMSF), 5 mM ethylene glycol bis(2-aminoethyl ether)tetraacetic acid (EGTA) and 0.3 M KCl by Polytron[®], heated at 95 °C for 4 min, and then chilled rapidly on ice. The suspension was centrifuged at $50000 \times g$ for 30 min and the precipitate was discarded. Solid ammonium sulfate was added to give 30% saturation and the resulting precipitate collected by centrifugation was dialyzed overnight against 20 mM Tris–HCl buffer (pH 7.5) containing 0.5 mM dithiothreitol (DTT), 0.5 mM PMSF, 1 mM EGTA and 50 mM KCl (buffer A). The dialysate was clarified by centrifugation and applied to a carboxymethyl (CM)-cellulose column (2×14 cm) equilibrated with buffer A. The column was washed with the same buffer and the bound protein was eluted with a linear concentration gradient of KCl, ranging from 0 to 350 mM of buffer A. The p33 fractions were combined and concentrated using Aquacide II. The concentrated solution was subjected to gel filtration on an ultrogel AcA-44 column (3×85 cm), which was equilibrated and eluted with buffer A containing 350 mM KCl. p33 fractions were collected and stored on ice.

Preparation of Antiserum Antisera against p33 were raised in rabbits by subcutaneous injection of about 300 μg of protein in complete Freund's adjuvant. Rabbits were boosted bimonthly with 150 μg of protein in incomplete Freund's adjuvant. The rabbits were bled 2 weeks after the last injection.

Electrophoresis and Immunoblotting Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was performed according to Laemmli,²⁰⁾ using 0.8 mm-thick slabs stained with Coomassie blue R-250. Immunoblots were performed by a modification of the method of Towbin *et al.*²¹⁾ Proteins were electrophoretically transferred to a nitrocellulose membrane (Schleicher & Schuell). After incubating the filter in 5% bovine serum albumin and 1% calf serum in 10 mM Tris–HCl buffer (pH 7.5) containing 150 mM NaCl (Tris-buffered saline) for 4 h at 25 °C to block nonspecific binding sites, it was incubated for 2 h at 37 °C with diluted antisera (1:3000) to p33 in 5% bovine serum albumin in Tris-buffered saline. The filters were then treated with alkaline phosphatase-conjugated anti-rabbit immunoglobulin G (IgG) for 2 h at 37 °C. The immunoreactive bands were visualized by using nitro blue tetrazolium and 5-bromo-4-chloro-3-indolylphosphate as a substrate for alkaline phosphatase.¹⁶⁾

Other Methods The interaction of p33 with F-actin was measured by the cosedimentation assay.^{16,22)} Calmodulin and tropomyosin were coupled to cyanogen bromide-activated Sepharose 4B following the procedures outlined by Pharmacia. About 5 mg calmodulin and 2.5 mg tropomyosin were linked to 1 ml of Sepharose. Protein concentration was determined by a Bio-Rad coomassie blue protein assay using bovine serum albumin as a standard, or spectrophotometric measurements using $E_{\text{cm}}^{1\%}$ at 290 nm of 6.5 for actin,²³⁾ $E_{\text{cm}}^{1\%}$ at 278 nm of 2.9 for tropomyosin²⁴⁾ and $E_{\text{cm}}^{1\%}$ at 276 of 1.8 for calmodulin.²⁵⁾

Results

Purification of p33 from Chicken Gizzard Smooth Muscle When crude extract from chicken gizzard was mixed

Fig. 1. Sedimentation Analysis of Actin-Binding Proteins in Chicken Gizzard Smooth Muscle

Chicken gizzards were extracted in buffer A and centrifuged at $100000 \times g$ for 60 min at 4°C . Obtained supernatant was incubated with F-actin for 60 min at 25°C in 5 mM 1,4-piperazinediethanesulphonic acid (PIPES)-NaOH (pH 7.0), 1 mM MgCl_2 , 1 mM ATP, 100 mM NaCl in a final volume of 200 μl . The supernatants (s) and pellets (p) were separated and analyzed by SDS-PAGE. The arrowheads indicate the positions of molecular weight markers: phosphorylase b (94000), bovine serum albumin (67000), ovalbumin (43000), carbonic anhydrase (30000) and lysozyme (14400), from top to bottom. CDM, TM, and a) indicate caldesmon, tropomyosin and 33000 protein, respectively.

with skeletal muscle F-actin, incubated for 60 min at 25°C and then centrifuged at $100000 \times g$ for 30 min at 25°C , a number of components including filamin, caldesmon and tropomyosin cosedimented with F-actin. In addition to these proteins, a 33000 kilodaltons (kDa) polypeptide (p33) estimated by SDS-PAGE also bound to F-actin (Fig. 1). Since p33 was soluble after heating at 95°C for 4 min and retained its actin binding activity, we utilized this characteristic for its purification.

Figure 2A shows the elution profile of CM-cellulose chromatography. Fractions 39 through 43 were collected and used for further purification. The sample was applied to an ultrogel AcA-44 column equilibrated with buffer A containing 350 mM KCl. The elution profile and the polypeptide composition are shown in Fig. 2B. Fractions 81 through 86 were collected and used as the purified p33. Analysis by microplate procedure showed that the yield was about 35% (12 mg of purified p33 from 200 g of chicken gizzard).

The preparation gave a single band with a molecular weight of 33000 on SDS-PAGE (Fig. 3) and we called it p33. The molecular weight of the native protein was estimated to be 30000 by gel filtration (Protein pak 125 HPLC and Sephadex G-100 columns) indicating p33 exists as a monomer. Immunoblot analysis showed that the polyclonal antibodies against p33 reacted specifically with p33 and did not crossreact with other polypeptides in chicken gizzard smooth muscle.

Interaction of p33 with Actin, Tropomyosin and Calmodulin

The binding of p33 to F-actin is examined by sedimentation assay and the results are plotted in the form of a Scatchard plot (Fig. 4). Control experiments showed that p33 did not sediment by itself and also had no influence on the amount of actin sedimented after this centrifugation. The amount of p33 in actin pellet progressively increased by increasing

Fig. 2. Chromatographic Purification Steps of p33

A) The 0–30% ammonium sulfate fraction was applied to a 2×10 cm CM-cellulose column equilibrated with buffer A. After washing with the buffer, the column was eluted with 140 ml gradient from 0 to 350 mM KCl of buffer A at a flow rate of 10 ml/h. Fractions of 3 ml each were collected and analyzed for protein using the Bio-Rad dye binding assay. The dotted line represents the KCl concentration. B) The concentrated CM-cellulose fractions were applied at a flow rate of 12 ml/h to a 3×85 cm ultrogel AcA-44 column equilibrated with buffer A containing 350 mM KCl. Fractions (5 ml) were collected and analyzed for protein. The inset shows the profile for SDS-PAGE for applied sample (a) and fractions 2 and 40 (A) and fraction 85 (B). The small arrowheads on the right indicate the positions of the molecular weight markers described in the legend to Fig. 1.

Fig. 3. SDS-PAGE and Immunoblot

Chicken gizzard homogenate (a, c) and purified p33 (b, d) were separated in 12.5% SDS-PAGE. (a, b) and (c, d) indicate coomassie blue-stained gels and immunoblots with anti-p33 polyclonal antibodies, respectively.

Fig. 4. Scatchard Plot of the Binding of p33 to F-Actin

F-Actin ($7 \mu\text{M}$) was incubated with various concentrations of p33 as indicated for 30 min at 25°C . The details of the cosedimentation assay are described in the legend to Fig. 1. The protein bands were stained with coomassie blue and scanned in a densitometer (Shimadzu CS9000) at 570 nm .

Fig. 5. Tropomyosin- and Calmodulin-Sepharose 4B Affinity Chromatographies of p33

A) The $0.6 \times 9 \text{ cm}$ column was equilibrated with 20 mM Tris-HCl ($\text{pH } 7.0$) containing 0.1 mM DTT (buffer C), and purified p33 (1 mg) was applied. The column was washed with the above buffer and the bound protein was eluted with a linear gradient of $0-150 \text{ mM}$ KCl (---). B) Purified p33 (0.5 mg) was applied to a calmodulin-Sepharose column ($0.6 \times 11 \text{ cm}$) equilibrated with buffer B. The column was washed with buffer B and at the point indicated by arrow, the bound protein was eluted with 1 mM EGTA of buffer B without 0.5 mM CaCl_2 . Both fractions (1 ml) were collected and analyzed for protein. The insets show the SDS-polyacrylamide gel profiles for fractions 35 (A) and 33 (B).

the concentration of p33. Saturation was achieved at an approximate molar ratio of one p33 to 2–3 actin monomers and an apparent association constant was $6-7 \times 10^7 \text{ M}^{-1}$.

We investigated whether p33 could bind to tropomyosin and calmodulin by affinity chromatography. When purified p33 was applied to a tropomyosin-Sepharose column, most of the protein was retained by the column (Fig. 5A). The binding appeared to be relatively weak because bound p33 was eluted at $60-80 \text{ mM}$ KCl under our experimental conditions. Purified p33 was also applied to a calmodulin-Sepharose 4B column which had been equilibrated with 10 mM Tris-HCl buffer ($\text{pH } 7.0$) containing 0.1 mM DTT, 50 mM KCl and 0.5 mM CaCl_2 (buffer B) and washed with buffer B (Fig. 5B). The bound protein was eluted with 1 mM EGTA of buffer B without 0.5 mM CaCl_2 . Electrophoretic analysis showed that p33 was recovered in the bound fractions. These results demonstrated that p33 was an actin-, calmodulin- and tropomyosin-binding protein.

Effects of Ca^{2+} and Calmodulin on p33-Actin and p33-Tropomyosin Interactions Calmodulin is well known to be a Ca^{2+} -dependent regulatory protein. F-actin sedimentation experiments were also done to examine the effect of calmodulin on the binding of p33 to F-actin (Fig. 6). After centrifugation of the three proteins at p33:actin:calmodulin = $1:6:5$ (molar ratio), calmodulin released about 50% of p33 from actin filaments in the presence of Ca^{2+} (pair d), while this release was not observed in the presence of 0.2 mM EGTA (pair c). Similar results were obtained when tropomyosin was present in the assay mixture (data not shown).

We also examined the effect of Ca^{2+} /calmodulin on the interaction between p33 and tropomyosin by affinity chromatography (Fig. 7). The binding of p33 to tropomyosin was not affected by 0.2 mM EGTA or 0.5 mM CaCl_2 , and calmodulin did not bind to the column irrespective of Ca^{2+} concentration. In the presence of EGTA, p33 adsorbed on the column was not released by calmodulin and most of the protein was eluted by 200 mM KCl. On

Fig. 6. Sedimentation Analysis of the Interaction between p33 and F-Actin in the Presence or Absence of Ca^{2+} and Calmodulin

The concentrations of p33, F-actin and calmodulin were $3, 6$ and $15 \mu\text{M}$, respectively. Pair a, 0.2 mM EGTA; pair b, 0.5 mM CaCl_2 ; pair c, 0.2 mM EGTA + calmodulin; pair d, 0.5 mM CaCl_2 + calmodulin.

Fig. 7. Effect of Ca^{2+} and Calmodulin on the Binding of p33 to Tropomyosin-Sepharose 4B

p33 (1 mg) was applied to the tropomyosin-Sepharose column as described in the legend to Fig. 5. The column was washed with buffer C containing 0.2 mM EGTA (A) or 0.5 mM CaCl_2 (B). At the first point, calmodulin (1 mg) which was dissolved in buffer C containing 0.2 mM EGTA (A) or 0.5 mM CaCl_2 (B) was applied to the column. Then bound protein was eluted with the buffer containing 250 mM NaCl at the second point. Fractions of 1 ml each were collected and analyzed for protein. The inset shows the SDS-polyacrylamide gel profiles for the applied sample (a) and fractions 19 and 42 (A) and fractions 19 and 40 (B).

the other hand, the addition of calmodulin in the presence of Ca^{2+} released most of the p33 bound to the column.

p33 is a Component of Native Thin Filaments Since p33 can bind to F-actin, calmodulin and tropomyosin, and its bindings to actin and/or tropomyosin are regulated by Ca^{2+} and calmodulin, this protein is most likely to be associated with the contractile processes of smooth muscle. We prepared myofibrils and native thin filaments from chicken gizzard, and their constituents were analyzed by SDS-PAGE (Fig. 8). In addition to actin, tropomyosin, caldesmon and myosin (only in myofibrils), a 33000 polypeptide was found in the myofibrils and the native thin filament fractions (lanes 1 and 2), and the polyclonal antibodies raised against p33 cross-reacted with the polypeptide (lanes 3 and 4).

Effects of Actin-Binding Proteins on the Interaction between p33 and F-Actin In order to determine whether p33 binding site(s) on actin directly involved the binding regions of other actin-binding proteins in smooth muscle, we examined the effects of α -actinin, caldesmon and filamin

Fig. 8. SDS-PAGE and Immunoblot of Myofibrils and Native Thin Filaments

Chicken gizzard myofibrils (a, c) and native thin filaments (b, d) were separated in 12.5% SDS-PAGE. (a, b) and (c, d) indicate coomassie blue-stained gels and immunoblots with anti-p33 polyclonal antibodies, respectively. MHC, myosin heavy chain.

Fig. 9. Sedimentation Analysis of the Interaction between p33 and F-Actin in the Presence of α -Actinin, Caldesmon and Filamin

The experimental conditions were the same as for Fig. 6. The concentrations of α -actinin, caldesmon and filamin were 0.06, 0.08 and 0.05 mg/ml, respectively.

on p33 binding to F-actin by sedimentation analyses (Fig. 9). The binding of p33 to F-actin was significantly changed in the presence of these proteins, and behaved *vice versa*.

Discussion

In the present experiments, we describe the purification and characterization of an actin-, calmodulin- and tropomyosin-binding protein (p33) with a molecular weight of 33000 from chicken gizzard. The properties of p33 reported here are apparently very similar to those of caldesmon (M.W. 120000 on SDS-PAGE), an abundant F-actin-, calmodulin- and tropomyosin-binding protein whose F-actin and tropomyosin binding abilities can be modulated by Ca^{2+} and calmodulin.^{7,8,12,16,22,26,27} Moreover, this protein exhibit a similar apparent molecular weight on SDS-PAGE as an actin-, calmodulin- and tropomyosin-binding fragment (M.W. 35000–38000) digested from caldesmon.^{22,26,27} Immunoblot analysis showed that the antibodies raised against p33 are specific for p33 and do not recognize other proteins in chicken gizzard muscle (Fig. 3). The apparent association constants of caldesmon, filamin, villin, vinculin and synapsin I to F-actin have been calculated to be 10^5 – 10^6 M^{-1} ,²⁸ $1.3 \times 10^7 \text{ M}^{-1}$,²⁹ 7×10^5

M^{-1} ,³⁰⁾ $2.5 \times 10^7 \text{M}^{-1}$ ³¹⁾ and $1.2\text{--}1.9 \times 10^6 \text{M}^{-1}$,³²⁾ respectively. The maximum bindings reach 1 mol of protein to 6–28 mol (caldesmon), 12 mol (filamin), at least 10 mol (villin), 100 mol (vinculin) and 2–3 mol (synapsin I) of actin monomers as determined by sedimentation analyses. The estimated K_a value ($6.6 \times 10^7 \text{M}^{-1}$) for the binding of p33 to F-actin is adequate as an actin-binding protein and the maximum amount of p33 to bind F-actin is relatively high compared with those of other actin-binding proteins.

This protein is likely to be basic as judged from its elution profile from a CM-cellulose column (Fig. 2A), while actin, calmodulin and tropomyosin are acidic proteins.^{24,34)} However, the bindings of p33 to these proteins seem to be caused not merely by ionic interactions. This is supported by the following observations. The binding of p33 to calmodulin was dependent on Ca^{2+} concentrations (Fig. 5B), and p33 bindings to actin and tropomyosin were regulated by calmodulin in a Ca^{2+} -dependent fashion (Figs. 6 and 7). Although tropomyosin has been reported to block the binding of actin-binding proteins such as α -actinin,³⁴⁾ filamin,³⁵⁾ gelsolin,³⁶⁾ villin³⁰⁾ and heat shock protein³⁷⁾ to F-actin and their functions, the interaction between p33 and F-actin was also observed in the presence of tropomyosin. In addition, α -actinin, caldesmon and filamin from chicken gizzard smooth muscle did not affect the binding of p33 to F-actin and p33 *vice versa* (Fig. 9).

It has been reported that two Ca^{2+} -dependent membrane-binding proteins, calcimedins, with molecular weights of 67000 and 35000, are isolated from chicken gizzard muscle and belong to the annexin family.^{38,39)} Some proteins of this family can bind to F-actin.^{40,41)} However, the bindings are not observed in the absence of Ca^{2+} , and calmodulin does not confer Ca^{2+} -sensitivity on these interactions. Recently, Takahashi *et al.*^{42,43)} isolated an actin- and calmodulin-binding protein from chicken gizzard and called this protein calponin. Immunologically, calponin reacted with the antibodies against troponin T from skeletal muscle. However, we could not detect any polypeptides in skeletal muscle crude extract to crossreact with antibodies to p33 (data not shown). The elution of p33 from calmodulin-Sepharose was only dependent on Ca^{2+} concentrations, while calponin release occurred at over 100 mM NaCl in addition to EGTA. At present, we have no definite evidence that p33 is distinct from calponin. More recently, Lehman⁴⁴⁾ has shown that an actin-binding protein with a molecular weight of 35000 is not associated with chicken gizzard thin filaments. As shown in Fig. 8, p33 is included in the myofibrils and native thin filaments. A similar polypeptide has also been observed in the native thin filaments reported by Ngai *et al.*⁴⁵⁾ It may be due to differences in experimental conditions, especially in the concentrations of Triton X-100 and ATP. Further experiments are necessary to examine the distribution and localization of p33. The present results demonstrate that p33 may play a role in microfilament- and calmodulin-mediated cellular processes in smooth muscle.

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