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ISOLATION AND CHARACTERIZATION OF A 34000-DALTON CALMODULIN- AND F-ACTIN-BINDING PROTEIN FROM CHICKEN GIZZARD SMOOTH MUSCLE

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We isolated a 34000-dalton protein from the heat-soluble fraction of avian smooth muscle using the procedures of ammonium sulfate fractionation, cation exchange chromatography and gel filtration. The amount of 34000-dalton protein in the muscle homogenate was as much as tropomyosin. The 34000-dalton protein bound to F-actin and F-actin-tropomyosin in a Ca^{2+}-independent manner, but it Ca^{2+}-dependently interacted with calmodulin. We tentatively named the 34000-dalton protein gizzard p34K.

In vertebrate smooth muscle, calmodulin is recognized to be a Ca^{2+}-sensitizing factor common to the myosin- and actin-linked regulatory mechanisms for controlling the actin-myosin interaction. The myosin-linked regulation (1-4) involves the phosphorylation of myosin by Ca^{2+}- and calmodulin-dependent myosin light chain kinase. Recent studies (5-8) suggest that the regulation of actin filament by a specific actin-linked mechanism is necessary for the full activation of actin co-factor function. Calmodulin, caldesmon and tropomyosin constitute regulatory components of such an actin-linked mechanism. During the preparation of thin filament-linked regulatory components from chicken gizzard smooth muscle, we recognized a 34000-dalton protein which binds to calmodulin and F-actin. This protein was easily solubilized in large amounts in a Ca^{2+}-independent manner. Here we showed the purification procedures and biochemical characteristics of the protein. We tentatively named the 34000-dalton protein gizzard p34K.

MATERIALS AND METHODS

a) Purification of p34K: The trimmed smooth muscle from fresh chicken gizzard was immediately boiled in a boiling water bath for 2 min. The heated

Abbreviations: EGTA, ethylene glycol bis (β-aminoethyl ether)-N,N,N',N'-tetraacetic acid; DTT, dithiothreitol.

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muscle was chilled on ice and minced. All subsequent procedures were carried out at 4 °C. The minced muscle (200 g) was finely homogenized in 3.5 vols of extraction buffer (300 mM KCl, 1 mM DTT, 5 mM EGTA, 50 mM Imidazole-HCl (pH 6.9), 0.5 mM phenylmethyl sulfonyl fluoride, 10 ug/ml leupeptin and 1 mM sodium tetrathionate) by a blendor operated at a high speed twice for 30 sec. The homogenate was centrifuged at 150000 x g for 30 min. To 100 ml of filtered supernatant was added 16.4 g ammonium sulfate and stirred for 30 min. The resulting precipitate was collected by centrifugation at 12000 x g for 30 min. The pellet was dissolved in 25 ml of buffer A (6 M urea, 50 mM KCl, 0.1 mM EGTA, 0.5 mM DTT and 20 mM Tris-HCl (pH 7.8)) and dialyzed twice against 1 l of buffer A for 8 h. The solution was clarified by centrifugation at 150000 x g for 1 h and applied to a 2 cm x 25 cm SP-Sephadex C-50 (Pharmacia Fine Chemicals, Uppsala, Sweden) ion exchange column pre-equilibrated with buffer A. The column was eluted with a linear gradient produced by 250 ml of buffer A and 250 ml of buffer A containing 300 mM KCl. The p34K fractions eluted at 80-100 mM KCl were collected, concentrated, and gel chromatographed on a Ultrogel AcA 44 (LKB, Bromma, Sweden) column (2.6 cm x 90 cm) in buffer B (6 M urea, 400 mM KCl, 0.1 mM EGTA, 0.5 mM DTT. 0.02 % NaN_3 and 20 mM Tris-HCl (pH 7.5)).

b) Preparation of other proteins: Chicken gizzard actin was prepared from acetone powder according to the method of Spudich and Watt (9) and further purified by Ultrogel AcA 44 column chromatography after three cycles of polymerization and depolymerization procedures. Chicken gizzard tropomyosin was prepared as described by Ebashi et al. (10). Calmodulin was purified from bovine brain as described by Kakiuchi et al.(11).

c) Analytical gel chromatography: Estimation of Stokes radius of p34K and experiment for the interaction of p34K with calmodulin were performed by FPLC system (Pharmacia Fine Chemicals) on a Superose 12 column (1 cm x 30 cm). Protein preparations were applied to the column in 100 mM KCl, 2 mM $MgCl_2$, 0.2 mM DTT, 1 mM $CaCl_2$ or 1 mM EGTA, 0.02 % NaN_3 and 20 mM Imidazole-HCl (pH 7.2) at room temperature. The Stokes radius of p34K was calculated from a standard curve of $(Kd)^{1/3}$ versus Rs as in (12). Molecular weight standards were purchased from Pharmacia Fine Chemicals.

d) Chromatofocusing: Chromatofocusing was carried out at room temperature by the FPLC system on a Mono P column (Pharmacia Fine Chemicals). The purified p34K (2.5 mg) was applied to the column equilibrated with 75 mM Tris-acetate buffer (pH 9.3) and eluted with Polybuffer 96 (Pharmacia Fine Chemicals) diluted 1:10 at pH 6.0.

e) Analytical ultracentrifugation: Binding of p34K to F-actin was monitored by co-sedimentation with F-actin by ultracentrifugation. F-actin (460 μg/ml), p34K (135 μg/ml) and mixtures of p34K (135 μg/ml) with F-actin (460 μg/ml) and/or tropomyosin (180 μg/ml) were incubated in 100 mM KCl, 2 mM $MgCl_2$, 1 mM ATP, 0.2 mM DTT, 0.2 mM $CaCl_2$ or 1 mM EGTA, and 20 mM Imidazole-HCl (pH 7.2) at 25 °C for 30 min in a total volume of 0.2 ml. When the effect of KCl concentrations on the binding of p34K to F-actin was examined, F-actin (460 μg/ml) and p34K (135 μg/ml) were incubated under the same assay conditions (0.2 mM $CaCl_2$) except for the KCl concentrations (100, 200, 300, 400, and 500 mM). After centrifugation at 120000 x g for 60 min at 25 °C, equivalent amounts of the separated supernatants and pellets were analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Gels were stained with Coomassie Brilliant Blue R-250 and the protein contents distributed in the supernatant and pellet were quantified by densitometric scanning.

f) Calmodulin-Sepharose affinity chromatography: Calmodulin-coupled Sepharose 4B was prepared by the method of Klee et al. (13). The purified p34K was applied to the column (1 cm x 4 cm) equilibrated with 20 mM Imidazole-HCl buffer (pH 7.0) containing 100 mM KCl, 2 mM $MgCl_2$, 0.2 mM DTT and 1 mM $CaCl_2$ at room temperature and eluted from the column with the above Imidazole buffer containing 1 mM EGTA.

g) Miscellaneous procedures: SDS-PAGE was carried out in slab gels (12, 12.5 and 15 % acrylamide) in the buffer system of Laemmli (14). Densitometric scanning was performed by Toyo digital densitol DMU-33C at 620 nm. Protein concentration was determined by the method of Bradford (15) using γ-globulin as the standard. Molecular weight used are chicken gizzard actin, 43000 and tropomyosin, 70000.

RESULTS AND DISCUSSION

A summary of a purification scheme of p34K is presented in Fig. 1. The p34K was released into soluble fraction with the extraction buffer described in the text in the presence (10 mM $CaCl_2$) and absence (5 mM EGTA) of Ca^{2+}. The isolated p34K was completely heat-soluble for 10 minutes' boiling at 95 °C and even after heat treatment for 40 min, 46 % of the protein remained soluble. The amount of p34K in the pellet of ammonium sulfate fractionation (0-30 % saturation) was more than 70 % of the total proteins. Tropomyosin was completely separated by this procedure (Fig. 1, lane C). The final purification was carried out by gel filtration (lane F) after SP-Sephadex C-50 ion exchange chromatography (lane D and E). Starting with 200 g of minced muscle, about 80 mg of p34K was obtained. The quantity of caldesmon, tropomyosin and p34K relative to actin in muscle homogenate estimated by the scanning of gels (12.5 % acrylamide) stained with Coomassie Brilliant Blue were 0.19±0.01, 0.41± 0.03 and 0.22 ± 0.01 (n=5, mean ± S.D.), respectively. The estimated molar ratio of

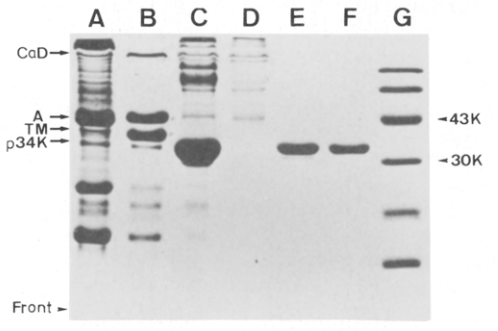

Fig. 1. Purification of gizzard p34K; lane A, muscle homogenate; lane B, heat-soluble proteins; lane C, 30 % ammonium sulfate-precipitated proteins; lane D, flow-through fraction of the SP-Sephadex C-50 column; lane E, materials subjected to the Ultrogel AcA 44 column; lane F, purified p34K. CaD: caldesmon A: actin, TM: faster-moving component of tropomyosin. Molecular weight markers (lane G): phosphorylase b (94000), BSA (67000), Ovalbumin (43000), carbonic anhydrase (30000), trypsin inhibitor (20100), α-lactoalbumin (14400). The gel contains 15 % acrylamide.

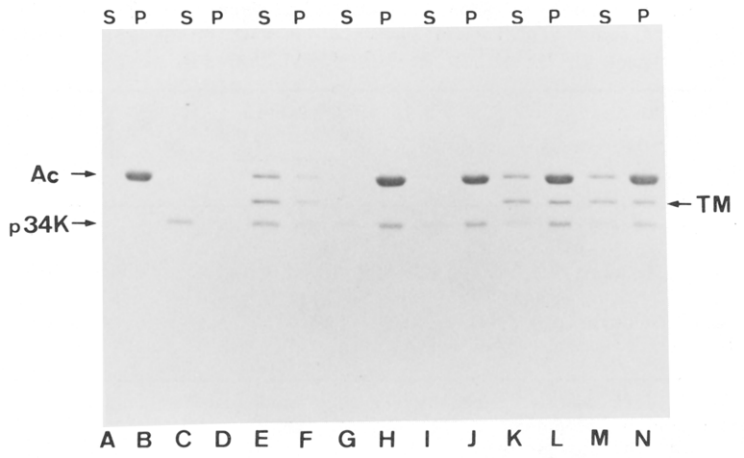

Fig. 2. Binding of p34K to F-actin: F-actin (460 μg/ml), p34K (135 μg/ml) or combinations of mixtures of p34K, F-actin and tropomyosin (180 μg/ml) were incubated in the presence (A-F, I, J, M and N) or absence (G, H, K and L) of Ca^{2+}. Lane A and B show the results of actin alone. Lane D and F show non-specific sedimentation of p34K and tropomyosin. Lane G-J and K-N show the results obtained with protein mixtures comprized of F-actin and p34K, and F-actin, tropomyosin and p34K, respectively. The gel contains 12.5 % acrylamide. s: supernatant, p: pellet, Ac: actin, TM: faster-moving component of tropomyosin.

tropomyosin and p34K was 1 : 1, assuming that the two protiens have the same dye-binding constants and molecular weight of 70000 and 34000, respectively.

The molecular size of p34K was estimated by SDS-PAGE and analytical gel chromatography. SDS-PAGE (15 % acrylamide) provided a single band of an apparent molecular weight of 34000 (Fig. 1). The Stokes radius determined by gel chromatography was found to be 27 Å in the presence and absence of Ca^{2+}. This value corresponds to an approximate molecular weight of 35000, demonstrating that the protein exists as a monomer in the described buffer condition.

Chromatofocusing of the purified protein showed distinctive two peaks, a major one at pH 8.2-8.3 and a minor one at pH 7.9-8.0. SDS-PAGE (12 % acrylamide) of each fraction confirmed that the separated components had the same molecular weight of 34000. This minor fraction may be an isoelectric variant of the major peak as suggested by Gerke and Weber in the case of intestinal p36.

The p34K bound to F-actin and F-actin-tropomyosin in a Ca^{2+}-independent manner (Fig. 2). In all assay conditions, 93-97 % of actin was sedimented in

TABLE I. Binding of p34K to F-actin and F-actin-tropomyosin
The gel stained with Coomassie Brilliant Blue R-250 (Fig. 2) was quantified by densitometric scanning

	% Distribution of p34K		Mass ratio p34K/actin pelleted
	s	p	
p34K alone + (Ca)	92.0	8.0	
p34K + Tropomyosin + (Ca)	88.1	11.9	
Actin + p34K - (Ca)	19.6	80.4	0.23
Actin + p34K + (Ca)	23.1	76.9	0.24
Actin + Tropomyosin + p34K - (Ca)	22.6	77.4	0.23[a]
Actin + Tropomyosin + p34K + (Ca)	20.4	79.6	0.20[a]

[a]Area of actin was estimated by scanning of fused bands of actin and slower-moving component of tropomyosin.
s: supernatant, p: pellet.

the pellet. Under the conditions employed (10.6 μM of actin, 3.9 μM of p34K and 2.6 μM of tropomyosin), 77-80 % of p34K was sedimented with F-actin in the presence and absence of tropomyosin (Table I). From these results, the binding stoichiometry of p34K to F-actin and F-actin-tropomyosin is calculated as one molecule of p34K per 3-4 actin monomers. This value was independent of Ca^{2+} concentrations (1.8×10^{-8} M - 3.2×10^{-4} M), but the amount of p34K bound to F-actin was decreased by increasing the KCl concentration (100 mM to 500 mM). Even in the presence of 500 mM KCl, p34K was sedimented with F-actin in a molar ratio of one p34K per 13-14 actin monomers.

The p34K bound to the calmodulin-coupled Sepharose 4B in a Ca^{2+}-dependent manner (Fig. 3, A). In the presence of Ca^{2+}, the protein bound to the column was not eluted with 6 M urea. The binding complex of p34K and calmodulin was demonstrated by analytical gel chromatography (Fig. 3, B). In the presence of Ca^{2+}, p34K was complexed with calmodulin and eluted at a position corresponding to an approximate molecular weight of 190000. Estimated mass ratio of p34K and calmodulin in the peak a fraction (Fig. 3, B) was 1 : 1.2, indicating that two or more molecule of calmodulin bind per molecule of p34K. When Ca^{2+} was chelated with 1 mM EGTA, both p34K and calmodulin were eluted at a position corresponding to the Stokes radius about 27 Å. In this buffer condition, calmodulin alone was eluted at the same position as p34K.

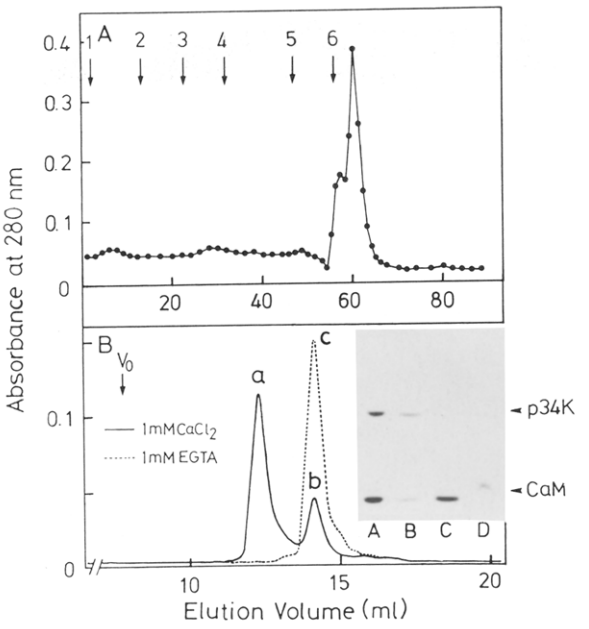

Fig. 3. Ca^{2+}-dependent interaction of p34K with calmodulin.
A: Calmodulin-Sepharose affinity chromatography. Vertical arrows indicate the sample (4 mg of p34K) application (1) and buffer changes (2-6). 2-4: Imidazole-HCl buffer (1 mM $CaCl_2$) containing 0.3 M KCl (2), 6 M urea (3) and 0.1 M KCl (4). 5 and 6: the same buffer (1 mM EGTA) containing 0.1 M KCl (5) and 0.2 M KCl (6).

B: Calmodulin (1.5 mg/ml) and p34K (0.75 mg/ml) were dialized against 20 mM Imidazole-HCl buffer (pH 7.2) containing 0.1 M KCl, 0.2 mM DTT, 2 mM $MgCl_2$ and 0.1 mM EGTA. Calmodulin (200 µl) and p34K (150 µl) were mixed and adjusted the final concentration of $CaCl_2$ or EGTA to 1 mM. The mixture was applied to the column pre-equilibrated with the dializing buffer containing 1 mM $CaCl_2$ or 1 mM EGTA and monitored by FPLC system. Each peak was analyzed by SDS-PAGE (12.5 % acrylamide): lane A, applied sample; lane B, peak a; lane C, peak b; lane D, peak c. CaM: calmodulin.

The possibility that the p34K is a degradative product of well-known calmodulin- and actin-binding proteins such as caldesmon and myosin light chain kinase in the gizzard smooth muscle seems unlikely because antibodies to caldesmon, myosin light chain kinase and p34K were not mutually cross-reactive in immunoblot analysis (data not shown). Furthermore, the p34K in our preparation seems to be different from Mr. 35000 or 33000 calcimedin, a set of Ca^{2+}-dependent hydrophobic binding proteins isolated by Moore and Dedman (17) from chicken gizzard extract. Calcimedin is acidic and does not bind to calmodulin-Sepharose affinity column (18, 19).

In summary, gizzard p34K described here is a protein unrecognized previously in the smooth muscle. The p34K is present in the gizzard smooth muscle in equimolar amount of tropomyosin. The binding of p34K to F-actin and

F-actin-tropomyosin suggests that this protein is one of the major structural components of the smooth muscle, especially of thin filament-related structures. Since the protein binds to calmodulin in a Ca^{2+}-dependent fashion, it probably modulates the Ca^{2+}-regulated events mediated by calmodulin.

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