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A novel 60-kDa smooth muscle protein that binds filamin-actin filament complex

Shohei Maekawa and Hikoichi Sakai

Department of Biophysics and Biochemistry, Faculty of Science, University of Tokyo, Hongo, Tokyo 113, Japan

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From the low salt-extracted debris of bovine stomach smooth muscle, a protein having a molecular mass of 60 kDa in SDS-PAGE was newly isolated. Co-sedimentation assay with actin filaments and several actin binding proteins such as filamin, α-actinin, caldesmon and fodrin showed that this protein co-sediments with actin only in the presence of filamin. Falling ball viscometric assay showed that this protein increases the viscosity of actin-filamin solution in a dose-dependent manner. Immunoblotting analysis showed specific localization of this protein in smooth and striated muscles.

Microfilament; Filamin; Insoluble fraction; 60-kDa protein; (smooth muscle)

1. INTRODUCTION

To understand the structure and function of the cell, it is important to know how microfilaments are organized into bundles or networks or how they are anchored to membranes or subcellular structures such as dense plaques, dense bodies, adherens junctions and z-lines. So far, many actin regulatory proteins have been found and characterized [1,2]. Geiger et al. [3] showed that talin localizes at the focal contact and dense plaques of smooth muscle, that the 135 kDa protein is found at the intracellular junctions and that vinculin is present in all of these structures. However, at present, information is still too limited to understand the organization of microfilaments. Smooth muscle seems to be suitable for such biochemical research as it contains not only abundant microfilaments but also many microfilament-related structures such as dense bodies and dense plaques [4].

In this paper, we show the purification of a new protein having a molecular mass of 60 kDa in SDS-PAGE (60-kDa protein) from bovine stomach smooth muscle. This protein was extracted using high-salt solution from the low salt-extracted debris and was shown to co-sediment with actin filament in the presence of filamin but not to co-sediment in the presence of other actin cross-linking proteins such as α-actinin, caldesmon or fodrin.

Correspondence address: S. Maekawa, Department of Biophysics and Biochemistry, Faculty of Science, University of Tokyo, Hongo, Tokyo 113, Japan

Abbreviations: SDS-PAGE, SDS-polyacrylamide gel electrophoresis; DTT, dithiothreitol; solution A, 10 mM Mes-KOH, 0.2 mM EGTA, 100 mM NaCl, 0.1 mM DTT (pH 6.8); PMSF, phenylmethylsulfonyl fluoride

2. MATERIALS AND METHODS

2.1. *Preparation of proteins*

Actin was prepared according to the method of Spuduch and Watt [5] and further purified using a Sephadex G-100 column as described [6]. Filamin and α-actinin were prepared by the method of Feramisco and Burridge [7]. Caldesmon was prepared according to Bretscher [8]. Fodrin was a

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gift from M. Ishikawa of our laboratory which was prepared as described in [9].

2.2. *Sedimentation assay*

Binding of proteins to actin filaments was assayed by centrifugation following SDS-PAGE according to Laemmli [10]. Actin (0.15 mg/ml) was mixed with sample solutions using a standard assay medium (solution A) containing 2 mM $MgCl_2$. An aliquot of this mixture was centrifuged at 100000 × *g* for 40 min after incubation at 20°C for 30 min and the supernatant and the pellet fractions were electrophoresed. Gels were stained with Coomassie brilliant blue and, if necessary, the intensity of the protein band was determined by scanning gels with a densitometer.

2.3. *Preparation of affinity purified anti-60-kDa protein antibody*

Two rabbits were immunized by injecting purified 60-kDa protein (0.1 mg protein in each time) using complete (first) and incomplete (second and following injections) Freund's adjuvant. After 2 months, blood was obtained and the resultant serum was further purified using nitrocellulose sheets on which purified 60-kDa protein was transferred electrophoretically [11].

2.4. *Others*

Falling ball viscometry was done as described [6]. Transfer of proteins to a nitrocellulose sheet after SDS-PAGE was performed following the method of Towbin et al. [12] in the presence of 0.1% SDS. Tissue extracts were prepared by homogenizing tissues in 10 vols (w/v) of phosphate buffered saline containing 1 mM PMSF. The homogenate was then boiled in the presence of 2% SDS for 5 min. Following centrifugation at 20000 × *g* for 20 min, supernatants were saved as tissue extracts. Protein concentration was determined according to Lowry et al. [13].

3. RESULTS

3.1. *Identification of actin filament co-sedimentable proteins in the muscle extract*

To observe actin binding proteins of the tissue, a low ionic extract was prepared from smooth muscle. After two washes with ice-cold deionized water containing 1 mM PMSF, the tissue was suspended in 10 vols of a solution containing 2 mM Tris-HCl, 1 mM EGTA, 1 mM PMSF (pH 9.0) and incubated at 37°C for 30 min with gentle mixing. After centrifugation at 10000 × *g* for 30 min at 4°C, the supernatant was saved. To this fraction, exogenous actin was added and co-sedimentable proteins were assayed by SDS-PAGE. As shown in fig.1, many protein bands were co-sedimented. From mobilities in SDS-PAGE and the amount in the extract, some proteins such as filamin (a), caldesmon (b) and α-actinin (c) were identified. In this study, we noticed a protein band having a molecular mass of 60 kDa. A large portion of this protein came to the pellet fraction by the co-sedimentation assay, suggesting that the protein has strong affinity to actin

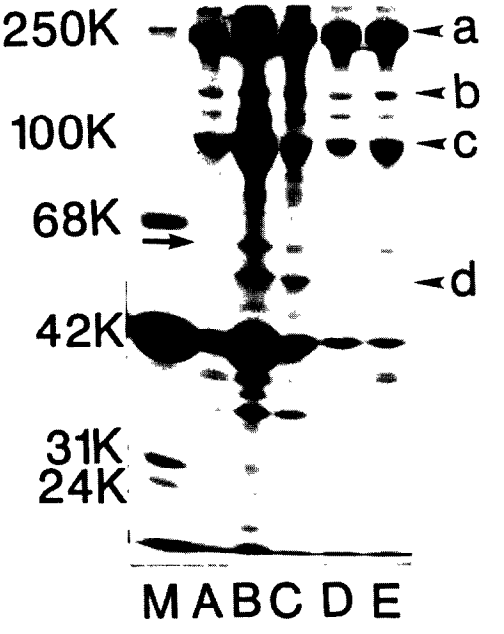

Fig.1. Co-sedimentation assay of smooth muscle extract. After dialysis and centrifugation, low-salt extracts (E) were incubated with (A,B) or without (C,D) exogenous actin (0.15 mg/ml), at 20°C for 30 min, followed by centrifugation to obtain pellets (B,C) and supernatants (A,D) in solution A containing 2 mM $MgCl_2$. Lane M shows the mobility of molecular mass marker proteins such as filamin (250 kDa), α-actinin (100 kDa), BSA (68 kDa), actin (42 kDa), carbonic anhydrase (31 kDa) and chymotrypsinogen (24 kDa). An arrow shows the band of the 60-kDa protein. Protein bands corresponding to filamin (a), caldesmon (b), α-actinin (c) and desmin (d) are shown in lane E.

filaments. Except for some actin-bundling proteins which have molecular masses of 53–58 kDa, little actin binding protein of this molecular size is known [1,2].

3.2. *Purification of the 60-kDa protein*

During trials to purify this protein, we noticed that this protein is much more abundant in the salt-insoluble fraction rather than low salt-soluble fraction in which the protein was first noticed, as described above. Therefore, the residue fraction after the low-salt extraction was used as the starting material. This fraction was incubated in 3 vols of a solution containing 20 mM Tris-HCl, 1 mM EGTA, 1 mM PMSF, 0.1 mM DTT, 0.6 M NaCl (pH 7.5), for 20 min at 0°C. After centrifugation at 10000 × *g* for 30 min, the supernatant was recovered and brought to 30% saturation with respect to ammonium sulfate. Precipitates were collected by centrifugation and dissolved and dialysed against solution A containing 0.6 M NaCL and applied to a Sephacryl S-300 column (5 × 90 cm), which was preequilibrated with the above solution. As shown in fig.2C, the protein was eluted in the middle region of the second peak. The collected fractions were applied to a hydroxyapatite column (2.2 × 20 cm) and proteins were eluted with a linear gradient of potassium phosphate from 60 to 120 mM (fig.2D). Collected fractions were dialysed against a solution containing 10 mM Tris-HCl, 0.1 mM DTT (pH 8.1) and applied to a Whatman DE 52 column (1.5 × 10 cm) and eluted with a linear NaCl gradient from 20 to 100 mM (fig.2E). The final fraction was dialysed against solution A and stored on ice until use. As shown in fig.2 (slot E in SDS-PAGE pattern), the final sample showed a single band having an estimated molecular mass of 60 kDa. About 2 mg protein was recovered from 300 g of wet tissue.

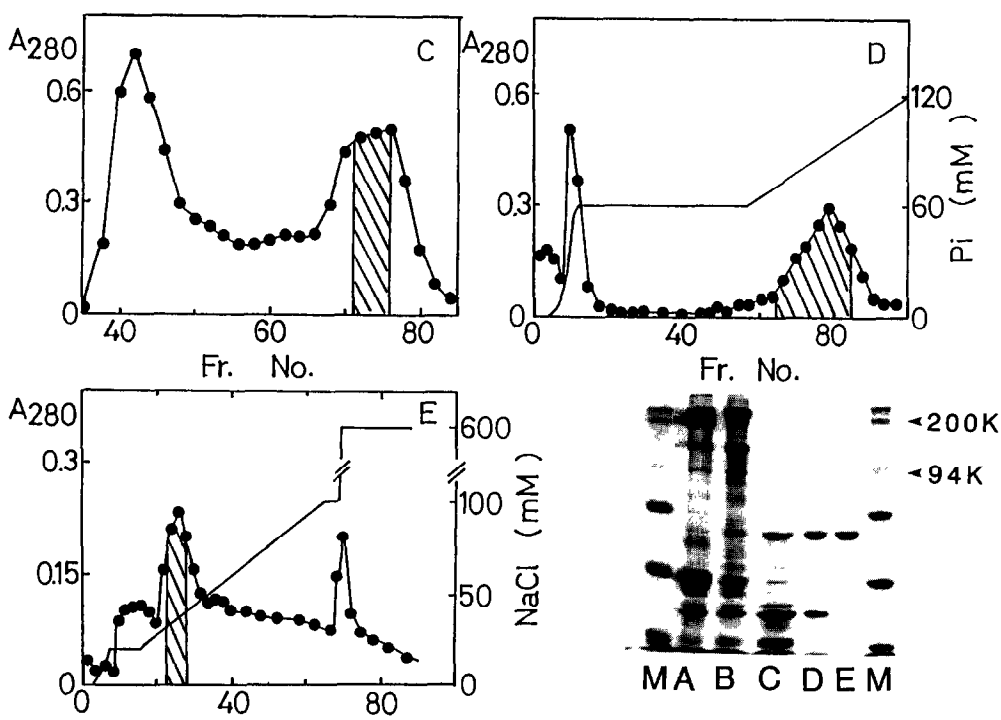

Fig.2. Elution profiles of the 60-kDa protein from Sephacryl S-300 (C), hydroxyapatite (D) and Whatman DE 52 (E) columns and the SDS-PAGE pattern of each fraction, in addition to 0.6 M NaCl extract (A), fraction for Sephacryl column procedure (B), and marker proteins (M) using 10% polyacrylamide gel. In lanes M, myosin (200 kDa) and phosphorylase *b* (94 kDa) were added in addition to the proteins described in fig.1. Fractions showed by shaded areas were collected for the next procedure.

3.3. *The co-sedimentation assay of the 60-kDa protein with the filamin-actin complex*

The co-sedimentation assay of the 60-kDa protein showed that this protein, by itself, has no ability to bind to actin filaments (fig.3, cf. lanes d and e). The presence of filamin, however, caused co-sedimentation of this protein (fig.3, lane b). This effect was not observed in the case of other actin binding proteins such as α-actinin, caldesmon or fodrin. We, therefore, concluded that the co-sedimentation of the 60-kDa protein with actin filaments described in fig.1 resulted from the presence of filamin in the extract. The binding activity of this protein to filamin-actin complex showed no Ca^{2+}-sensitivity either in the presence of absence of calmodulin. Furthermore, changes of pH of the assay condition in a range from 6.4 to 8.2 showed no effect on the activity (not shown). Falling ball viscometry of the actin-filamin complex showed that addition of this protein to the complex causes the viscosity of the solution to increase (fig.4A) in a dose-dependent manner (fig.4B).

The distribution of this protein in tissues studied by immunoblotting showed that this protein is detected in smooth and striated muscles but not in extracts of other tissues such as liver, adrenal or heart (fig.5). In addition, extracts of esophagus, stomach, intestine, artery and uterus of rat contained detectable amounts of this protein (not shown). Immunoblotting of various subfractions of smooth muscle also showed that this protein is hardly extracted by the low-salt alkaline treatment

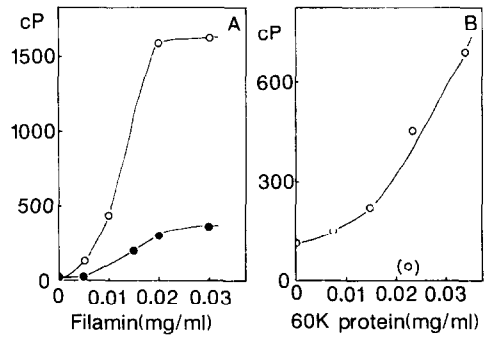

Fig.4. Effect of 60-kDa protein on the falling ball viscosity of the filamin-actin complex solution. (A) Actin (0.12 mg/ml) was incubated with various amounts of filamin in the presence (0.2 mg/ml, ○) or absence (●) of the 60-kDa protein. (B) Actin (0.15 mg/ml) was incubated with filamin (0.01 mg/ml) in the presence of various amounts of the 60-kDa protein. The point in parentheses shows the viscosity of a solution which contains no filamin.

Fig.3. Co-sedimentation of the 60-kDa protein with the filamin-actin complex. Actin (0.15 mg/ml) was polymerized in the presence of filamin (0.15 mg/ml) and 60-kDa protein (0.01 mg/ml) during incubation at 20°C for 30 min in solution A containing 2 mM $MgCl_2$, followed by centrifugation to obtain supernatant (B) and pellet (b). Mixtures containing no actin (A,a), no 60-kDa protein (C,c), no filamin (D,d) or 60-kDa protein alone (E,e) were also treated in the same way as controls. Arrowheads show the bands of filamin (a), 60-kDa protein (b) and actin (c).

Fig.5. Immunoblotting analysis of various tissue extracts. Proteins in extracts from liver (A), heart (B), striated muscle (D) and testis (E) of rat and stomach smooth muscle (C), adrenal medulla (F) and cortex (G) of calf, were transferred to a nitrocellulose sheet after SDS-PAGE, followed by immunoblotting using affinity-purified anti-60-kDa protein antibody. 30 μg proteins were electrophoresed using 8% polyacrylamide gel.

which is usually used to solubilize other actin regulatory proteins such as filamin, α-actinin and vinculin [7] (not shown).

4. DISCUSSION

In this paper, we reported the purification of a newly identified protein which binds to filamin-actin complex but not to actin filament alone. Assuming that the 60-kDa protein binds to filamin, binding studies by Scatchard plot analysis showed that the binding ratio of this protein to filamin dimer was 0.6 with an association constant of 2×10^6, yet the precise binding nature of this protein to actin-filamin complex is not known. Binding of this protein to filamin in the absence of actin filament, if any, seems to be not so strong, since the gel filtration method did not detect association between 60-kDa protein and filamin (not shown).

The immunoblotting analysis showed that the 60-kDa protein is not the breakdown product of a protein with a much larger molecular mass such as filamin, and the specific distribution of this protein in smooth and striated muscle suggests its specialized function in these tissues. Its low solubility in the low ionic strength solution suggests that this protein could anchor actin filaments to intracellular structures such as dense bodies or dense plaques in the presence of filamin. Immunocytochemical studies are now in progress to determine the intracellular localization of this protein.

REFERENCES

[1] Stossel, T.P., Chaponnier, C., Ezzell, R.M., Hartig, J.H., Janney, P.A., Kwiatkowski, D.J., Lind, S.E., Smith, D.B., Southwick, F.S., Yin, H.L. and Zanner, K.S. (1985) Annu. Rev. Cell Biol. 1, 353–402.
[2] Pollard, T.P. and Cooper, J.A. (1986) Annu. Rev. Biochem. 55, 987–1035.
[3] Geiger, B., Volk, T. and Volberg, T. (1985) J. Cell Biol. 101, 1523–1531.
[4] Tsukita, S., Tsukita, S. and Ishikawa, H. (1983) Cell Tissue Res. 229, 233–242.
[5] Spudich, I.A. and Watt, S. (1971) J. Biol. Chem. 246, 4866–4871.
[6] Maekawa, S., Endo, S. and Sakai, H. (1983) J. Biochem. 94, 1329–1337.
[7] Feramisco, J.R. and Burridge, K. (1980) J. Biol. Chem. 255, 1194–1199.
[8] Bretscher, A. (1984) J. Biol. Chem. 259, 12773–12880.
[9] Ishikawa, M., Murofushi, H. and Sakai, H. (1983) J. Biochem. 94, 1209–1217.
[10] Laemmli, U.K. (1970) Nature 227, 680–685.
[11] Maekawa, S. and Sakai, H. (1987) Exp. Cell Res., in press.
[12] Towbin, H., Staehlin, T. and Gordon, J. (1979) Proc. Natl. Acad. Sci. USA 76, 4350–4354.
[13] Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275.