GELSOLIN-RELATED 45 K $M_r$ ACTIN-BINDING PROTEIN FROM BOVINE AORTA

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ABSTRACT
A 45 K $M_r$ G-actin-binding protein was isolated from bovine aorta. This protein had an amino acid composition very similar to that of 84 K $M_r$ gelsolin and cross-reacted with anti-gelsolin antibodies. The 45 K $M_r$ protein consisted of two isoforms, I and II, as revealed by isoelectric focusing. Isoform I was slightly more acidic than isoform II and more abundant. Isoform II was not distinguishable from a chymotryptic peptide, CT47, of gelsolin in chain weight and isoelectric point values. The 45 K $M_r$ protein is shown to bind to G-actin by chemical cross-linking. It considerably lowered the initial rate of actin polymerization, but the inhibition became much less marked at a steady state, suggesting that the binding of this protein to actin was not so tight as CT47. Both isoforms of the 45 K $M_r$ protein were detected in a guanidine-HCl extract of freshly obtained bovine aorta. It is suggested that the 45 K $M_r$ protein derived from 84 K $M_r$ gelsolin exists in vivo.

A number of actin-binding proteins regulate dynamic state of actin filaments that are involved in cell motility and shape formation (for reviews, see 13, 14). In vertebrate smooth muscle (bovine aorta), Nonomura and his collaborators have isolated gelsolin-like 84 K $M_r$ protein that $Ca^{2+}$-dependently severs actin filaments (5). This protein called ‘actin length determinator’ has been shown to control the interactions between myosin and actin in the presence of ATP (4).

During the course of preparing the 84 K $M_r$ protein from bovine aorta according to Ebisawa et al. (5), a 45 K $M_r$ actin-binding protein was isolated in the present study. However, this peptide turned out to be a proteolytic product of the 84 K $M_r$ protein. In view of the natural occurrence of this 45 K $M_r$ peptide in bovine aorta, its possible relationship to the mother protein is discussed in this article.

MATERIALS AND METHODS

Materials
Bovine aorta obtained from a local slaughterhouse was used as material.

Purification of Proteins
84 K $M_r$ protein The method of Ebisawa et al. (4) was adopted. The purification procedure of 45 K $M_r$ protein as a by-product of the 84 K $M_r$ protein preparation is described in Results. Protein concentration was estimated by the method of Lowry et al. (10), using bovine serum albumin as standard.

Actin Preparation of actin from rabbit skeletal muscle and the method of determining G-F transformation using pyrenyl actin were described elsewhere (11).
Extraction of Bovine Aorta with Guanidine-HCl

In order to extract proteins with minimal proteolysis, the method of Hirabayashi et al. (6) was adopted. A piece of bovine aorta was cut in 6 M guanidine-HCl neutralized to pH 7.0 with Tris, and thoroughly extracted. The extract was dialyzed against 6 M urea (for two-dimensional electrophoresis) or 50 mM Tris-HCl (pH 6.7) and 5% 2-mercaptoethanol (for SDS-polyacrylamide gel electrophoresis). For the latter, an SDS solution was added to make the final concentration of 1%, and then heated for 2 min at 90°C.

Gel Electrophoresis and Immunoblots

Molecular weight was estimated from mobilities in SDS-gel electrophoresis in the Weber-Osborn system (17), using bovine serum albumin, ovalbumin, a-chymotrypsinogen and cytochrome c as markers. In some cases, polyacrylamide gel electrophoresis was performed by the method of Laemmli (9). Two-dimensional electrophoresis was carried out according to O'Farrel (12). Proteins from polyacrylamide gels were electrophoretically transferred to nitrocellulose paper (16). The nitrocellulose sheet was treated with anti-gelsolin serum against bovine aorta 84 K Mr gelsolin diluted 500-1,000 times with 0.5 M NaCl and 20 mM Tris-HCl (pH 7.5). The antibodies against bovine aorta 84 K Mr protein was raised in a rabbit (Ebisawa K., to be published). Antibodies reacted with proteins on the nitrocellulose sheet were detected according to the method of De Blas et al. (3).

Amino Acid Composition, Peptide Mapping and Cross-linking

Salt-free protein samples were hydrolyzed in 6 N HCl for 24 h at 110°C, and the amino acid composition was determined by a Hitachi 835 amino acid analyzer.

Peptide mapping was performed using a-chymotrypsin by the method of Cleveland et al. (2).

Chemical cross-linking of G-actin and 45 K Mr protein was performed using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) (15).

Fig. 1 Purification of 45 K Mr protein from bovine aorta. SDS-gel electrophoresis was carried out using 10% polyacrylamide gels (9). A, crude extract. B, an EGTA elute from DNase I affinity column (4). * indicates 45 K Mr protein. C, 45 K Mr protein separated by hydroxylapatite and DEAE-cellulose column chromatography, as described in Results. D, 45 K Mr protein purified by HPLC gel filtration

RESULTS

Isolation of 45 K Mr Protein from Bovine Aorta

Minced aorta was extracted with 3 vol of a solution containing 20 mM Tris-HCl, pH 7.5, 5 mM EDTA, 10 μM DFP, 0.1 mM PMSF, and 0.02% NaNO₃ (4). The supernatant after ultracentrifugation for 1 h at 100,000 g was applied onto DNase I affinity chromatography (4). A crude gelsolin fraction eluted by 20 mM Tris-HCl, pH 7.5, 50 mM KCl, 1 mM MgCl₂, 2 mM EGTA, 1 mM CaCl₂, 10 μM DFP, 0.1 mM PMSF and 0.02% NaN₃, contained three major protein bands in addition to 84 K Mr gelsolin on SDS-gel electrophoresis (Fig. 1B). The three bands had apparent molecular weights of 45 K Mr, 42 K Mr (actin) and 30 K Mr, respectively.

The crude gelsolin fraction was subjected to hydroxylapatite chromatography equilibrated with buffer A (20 mM Tris-HCl, pH 7.5, 50 mM KCl, 2 mM EGTA, 10 μM DFP, 0.1 mM PMSF and 0.01% NaNO₃). When eluted with potassium phosphate, pH 7.0, in a gradient from 0 to 100 mM, a sharp 45 K Mr protein-rich fraction was obtained at around 20 mM. This fraction was further applied onto
DEAE-cellulose column equilibrated with buffer A, and eluted with KCl in a linear gradient from 50 to 150 mM. The fraction eluted at around 70 mM KCl consisted mainly of 45 K $M_r$, protein (Fig. 1C). When necessary, this fraction was concentrated by Centricon-30 (YM-30 membrane, Amicon) and subjected to an HPLC column gel chromatography (TSK 3000SW, Tosoh) using a solution containing 0.3 M potassium phosphate buffer, pH 7.0, 10 $\mu$M DFP and 0.01% NaN$_3$, 15°C. Upper: Mixture of serum albumin (67 K), ovalbumin (45 K) and $\alpha$-chymotrypsinogen (25 K). Lower: 45 K $M_r$ protein. Inset: Estimation of molecular weight from the retention time that the 45 K $M_r$ protein has an asymmetrically elongated shape in its native form.

As seen in Fig. 4 the amino acid composition of the 45 K $M_r$ protein was strikingly similar to that of 84 K $M_r$ gelsolin (4). Significant differences were observed only in the glycine and lysine contents: 12 residues less and 17 residues more per 1,000 residues in 45 K $M_r$ protein than 84 K $M_r$ gelsolin, respectively.

**Characterization of 45 K $M_r$ Protein**

The molecular weight of 45 K $M_r$ protein was estimated to be 45,000 from the same mobility as ovalbumin in SDS-gel electrophoresis (Fig. 2). On the other hand, gel filtration method suggested the molecular weight of native 45 K $M_r$ protein was less than that of $\alpha$-chymotrypsinogen ($M_r$ 25,000), as shown in Fig. 3. From the retention time, the molecular weight was estimated to be as low as 20,000. It is assumed

**Relationship of 45 K $M_r$ Protein to 84 K $M_r$ Gelsolin**

Since the amino acid composition of the 45 K $M_r$ protein closely resembled that of 84 K $M_r$ gelsolin, the possibility that the former was a degradation product of the latter was experimentally examined.

Immunoblot tests clearly showed that both proteins were reactive to anti-84 K $M_r$ gelsolin antibodies (cf. Fig. 8). Next, bovine aorta 84 K
45kDa Protein

Gelsolin

Actin

Fig. 4 Comparison of the amino acid composition of 45 K $M_r$ protein, gelsolin and actin. All the proteins, hydrolyzed for 24 h in 6 N HCl at 110°C, were simultaneously subjected to amino acid determination. The relative values are expressed in star diagrams.

$M_r$ gelsolin was purified and subjected to $\alpha$-chymotrypsin hydrolysis according to Kwiatkowski et al. (8). The 84 K $M_r$ gelsolin was split into two peptides which corresponded to CT47 and CT45 of macrophage or plasma gelsolins (8). Two-dimensional electrophoresis showed that 1) 45 K $M_r$ protein consisted of two forms, larger acidic (I) and smaller alkaline (II) spots (Fig. 5b), 2) the alkaline spot (II), but not the main acidic one (I), corresponded to the CT47 spot (Fig. 5a), and 3) the two isoforms of 45 K $M_r$, protein (Fig. 5d) and both CT47 and CT45 (Fig. 5c) cross-reacted with anti-84 K $M_r$ gelsolin antibodies. To confirm the relationship of the 45 K $M_r$ protein isoforms and CT47 gelsolin peptide, the mixture of both preparations were co-electrophoresed. As is clearly shown in Fig. 6, the less abundant isoform, II, of the 45 K $M_r$ protein comigrated together with CT47. Furthermore, peptide mapping of 45 K $M_r$ protein (mixture of isoforms I and II) and CT47 gelsolin showed that both were not distinguishable from each other (Fig. 7).

Thus, the 45 K $M_r$ protein was shown to be closely related to the proteolytic product

Fig. 5 Two-dimensional gel electrophoresis of 45 K $M_r$ protein and $\alpha$-chymotryptic peptide of 84 K $M_r$ gelsolin and their immunoblots. Bovine aorta 84 K $M_r$ gelsolin (1 mg/ml) was hydrolyzed in 10 mM imidazole buffer, pH 7.0, for 30 min at 20°C in the presence of 1/1,000 $\alpha$-chymotrypsin (TLCK-treated). The reaction was stopped by 0.1 mM DFP. After two-dimensional electrophoresis (12), the proteins were electrophoretically transferred from polyacrylamide gels to nitrocellulose sheets. The sheets were treated with 1,000-fold diluted anti-gelsolin antiserum. a: Coomassie Brilliant Blue (CBB) stain of chymotryptic peptides of gelsolin. b: CBB stain of 45 K $M_r$ protein. c: Immunoblot of a. d: Immunoblot of b (CT47) of 84 K $M_r$ gelsolin, if not identical.

Presence of 45 K $M_r$ Protein in Bovine Aorta

The above-mentioned observations suggested that the 45 K $M_r$ protein might be an artifact originated from the mother molecule, 84 K $M_r$ gelsolin, during preparation procedures. Therefore, freshly excised bovine aorta was treated with 6 M guanidine-HCl solution at the slaughterhouse, and after electrophoresis, immunoblot tests were carried out. One-dimensional electrophoresis showed the presence of 45 K $M_r$ protein, although its amount appeared to be much smaller than that of the
Fig. 6 Two-dimensional gel electrophoresis of 45 K $M_r$ protein and $\alpha$-chymotryptic peptides of 84 K $M_r$ gelsolin. Conditions as in Fig. 5. a and b: $\alpha$-Chymotryptic peptides of gelsolin. b: 45 K $M_r$ protein. c: Mixture of a and b

Fig. 7 Peptide mapping of 45 K $M_r$ protein and CT47 fragment of gelsolin. Peptide mapping was performed by the method of Cleveland et al. (2) using $\alpha$-chymotrypsin (1 $\mu$g). Proteins were electrophoresed in 15% polyacrylamide gels according to Laemmli (9). a, 45 K $M_r$ protein; b, peptide mapping of a; c, CT47 fragment of gelsolin; d, peptide mapping of c.

Fig. 8 Anti-gelsolin-treated immunoblots of a direct guanidine-HCl extract of freshly obtained bovine aorta. A piece of bovine aorta obtained immediately after the sacrifice was directly extracted with 6 M guanidine-HCl (6). After SDS-gel electrophoresis using 10% polyacrylamide gels (9), proteins were transferred to a nitrocellulose sheet, and this was treated with 500-fold diluted anti-gelsolin antiserum. Purified 84 K $M_r$ gelsolin and 45 K $M_r$ protein were simultaneously tested. A: 1, amido black stain of the total SDS extract; 2, its immunoblot; 3, gelsolin; 4, 45 K $M_r$ protein. B: The total guanidine-HCl extract was subjected to two-dimensional electrophoresis as in Fig. 5, and the transferred sheet was treated with anti-gelsolin. Upper spots corresponded to gelsolin and lower two spots indicated by arrowheads corresponded to isoforms, 1 and II, of 45 K $M_r$ protein.

mother molecule (Fig. 8A).

Two-dimensional electrophoresis provided further evidence that the 45 K $M_r$ protein, both I and II isoforms, also existed in the guanidine-HCl extract of freshly obtained bovine aorta (Fig. 8B).

To test the formation of 45 K $M_r$ protein from gelsolin, two-dimensional electrophore-
Fig. 9 Changes in 45 K $M_r$ protein isoforms in bovine aorta during storage at 0°C. Two-dimensional electrophoresis of guanidine-HCl extract from bovine aorta was performed according to O'Farrell (12). The proteins electrophoresed were transferred to nitrocellulose sheets and they were treated with anti-gelsolin antiserum. a: Freshly obtained bovine aorta. b: Stored for 6 h at 0°C. c: Stored for 18 h at 0°C. d: Stored for 24 h at 0°C.

Fig. 10 Effects of 45 K $M_r$ protein on the polymerization of G-actin. Rabbit skeletal muscle G-actin labeled with N-(1-pyrenyl)iodoacetamide, 0.3 mg/ml, was polymerized in 0.1 M KCl, 1 mM MgCl$_2$, 0.2 mM ATP and 0.01% NaN$_3$ at 25°C. Polymerization was monitored by fluorescence assay (excitation, 365 nm, emission, 407 nm) (cf. 11). Various amounts of 45 K $M_r$ protein, indicated in molar ratio to G-actin, were added before the start of polymerization. a: With 0.1 mM CaCl$_2$. b: With 0.1 mM EGTA.

Action of the 45 K $M_r$ Protein on Actin Polymerization

The 45 K $M_r$ protein considerably retarded the polymerization process provided that calcium ions were present (Fig. 10a). In the absence of Ca$^{2+}$, no effect was observed at all (Fig. 10b).

In the presence of Ca$^{2+}$, and of 45 K $M_r$ protein addition in the molar ratio of 1/10 to actin, some 30% decrease in F-actin formation occurred about 20 min after the initiation of polymerization (Fig. 10a). However, on prolonged incubation the decrease in the extent of polymerization became less marked and finally almost negligible 24 h after the onset of polymerization, reaching a steady state.

In order to test any capping action of the 45 K $M_r$ protein, the effects on actin elongation of short F-actin fragments were investigated in the presence of 45 K $M_r$ protein. As seen in Fig. 11a, there was a small but significant decrease in the rate of elongation of actin filaments by 45 K $M_r$ protein during an initial period, but again the effect became less remarked after prolonged incubation. These effects were not observed in the absence of Ca$^{2+}$ (Fig. 11b). It can be concluded that the 45 K $M_r$ protein did not have any F-actin capping activity. This protein did not inhibit
Fig. 11 Effects of 45 K $M_t$ protein on the seed-induced polymerization of G-actin. Conditions as in Fig. 8, except that 50 mM KCl was added to start polymerization. Sonicated F-actin fragments (1/10 of G-actin by weight) was added. a: With 0.1 mM CaCl$_2$. b: With 0.1 mM EGTA

reannealing of F-actin fragmented by sonication. There was no sign of its binding to F-actin by ultracentrifugation assay (data not shown). On the other hand, the binding of the 45 K $M_t$ protein to G-actin was demonstrated by a chemical cross-linking experiment (Fig. 12). In the presence of Ca$^{2+}$, the 45 K $M_t$ protein band disappeared and a new band corresponding to approximately 90 K $M_t$ appeared (Fig. 12d). In the absence of Ca$^{2+}$, such a band was not formed (Fig. 12b).

Fig. 12 Chemical cross-linking of G-actin and 45 K $M_t$ protein. Rabbit skeletal muscle G-actin, 0.2 mg/ml, and 45 K $M_t$ protein, 0.04 mg/ml, were incubated with 5 mM 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) in 10 mM imidazole, pH 7.0, and 0.1 mM CaCl$_2$ or 0.1 mM EGTA. An excess amount of DTT was added to terminate the reaction. The mixture was subjected to SDS-gel electrophoresis using 8% polyacrylamide gels (9). -Ca$^{2+}$, 0.1 mM EGTA; a, b: before and after the treatment with EDC. +Ca$^{2+}$, 0.1 mM CaCl$_2$; c, d: before and after the treatment with EDC

CT47, an $a$-chymotryptic product of gelsolin (7) in two-dimensional electrophoresis: namely, both chain weights and isoelectric points are almost the same. There was a possibility that isoform II, originated from the mother molecule, is converted to slightly acidic isoform I by such a modification of the amino acid residues as phosphorylation.

It should be emphasized that both isoforms of the 45 K $M_t$ protein are detected together with 84 K $M_t$ gelsolin in a direct 6 M guanidine-HCl extract of freshly obtained bovine aorta. This suggests that the 45 K $M_t$ protein exists in vivo, though it is possible that the protein is derived from 84 K $M_t$ gelsolin by proteolytic degradation and possibly by further modification, e.g. phosphorylation. It is to be mentioned that another proteolytic product of gelsolin, CT45, was not detected at all in the total 6 M guanidine-HCl extract of bovine aorta. It is of interest to note that the acidic isoform I of 45 K $M_t$ protein corresponding to the chymotryptic fragment of gelsolin (CT47) increased when bovine aorta was kept for 6-18 h at 0°C, and the alkaline isoform II of 45 K $M_t$ protein appeared to increase in amount after 24 h of storage (Fig. 11d). The other frag-
ment CT45-like spot appeared only faintly after 24 h of storage. Very probably, the fragment might have been further degraded.

It is well established that gelsolin severs an actin filament in the presence of Ca\(^{2+}\), and caps the barbed ends of the resulting short actin filaments (cf. 15). According to Yin and her coworkers (8), plasma gelsolin and cytoplasmic gelsolin consist of 756 (1–756) and 732 (24–756) amino acids, respectively. \(\alpha\)-Chymotrypsin splits gelsolin into two peptides, N-terminal CT45 (1–406) and C-terminal CT47 (407–756) (7, 8). The CT45 possesses Ca\(^{2+}\)-insensitive F-actin-severing domain, and the CT47 has Ca\(^{2+}\)-sensitive G-actin-binding domain.

The present study showed the 45 K M, protein Ca\(^{2+}\)-dependently slowed down the initial rate of polymerization of G-actin suggesting that the action on actin is similar to that of CT47 (7). This is in good agreement with the results of the two-dimensional electrophoresis followed by immunoblot tests discussed above. However, CT47 is reported to lower the extent of actin polymerization in a steady state in proportion to the one-to-one (CT47-G-actin) complex formed (7). Therefore, it appears that the binding of 45 K M, protein to G-actin is a little weaker than that of CT47. The similarity in the amino acid composition between the 45 K M, protein and 84 K M, gelsolin is understandable, because gelsolin molecule consists of two closely related amino acid sequences (8).

In conclusion, it appears that the 45 K M, protein, a proteolytic product of 84 K M, gelsolin exists in bovine aorta in situ.

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