A Ca^{2+}-sensitive actin regulatory protein from smooth muscle

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Using a procedure involving DNase I affinity chromatography and Sephadex G-200 gel filtration, we partially purified a Ca^{2+}-sensitive actin regulatory 90-kDa protein from bovine aorta. The 90-kDa protein existed in the form of a complex with actin on a DNase I column even in the presence of 5 mM EGTA, indicating that the 90-kDa protein binds tightly to actin in a Ca^{2+}-insensitive manner. The isolation procedure described above indicates that the 90-kDa protein is present in smooth muscles including aorta, uterus and bladder, but not in skeletal and heart muscles. When added to G-actin before polymerization, the 90-kDa protein increases the initial rate of actin polymerization and lowers the final viscosity at Ca^{2+} concentrations greater than 10^{-7}M. This decrease in viscosity is due to the generation of filaments which cannot be readily sedimented.

Actin polymerization *Actin binding* *Gelsolin* *Ca^{2+}* *Bovine aorta* *Muscle*

1. INTRODUCTION

A class of actin-associated proteins, which are called 'severing and capping proteins' [1], share the ability to nucleate actin polymerization and restricting filament length. Over the past 5 years, Ca^{2+}-dependent severing proteins have been identified in a number of non-muscle cells and tissues, including macrophage [2], platelets [3], blood plasma [4], microvillus [5], thyroid [6], *Physarum* [7,8], and *Dictyostelium* [9]; however, Ca^{2+}-dependent severing and capping proteins have not been isolated from skeletal muscle.

It is well known that 'crude actins' extracted from the acetone powder of smooth muscle polymerize poorly and variably [10], but the poor polymerizability of smooth muscle crude actin has not yet been satisfactorily explained [11,12]. Such observations suggested that there are factors in these preparations which affect the ability of actin to form filaments. We therefore decided to isolate these factors, using as an assay their ability to inhibit the polymerization of actin. This paper describes the partial purification from bovine aorta of a Ca^{2+}-sensitive actin regulatory 90-kDa protein.

Abbreviations: DNase I, pancreatic deoxyribonuclease I (EC 3.1.4.5); PMSF, phenylmethyl sulfonyl fluoride

2. MATERIALS AND METHODS

Actin was prepared from rabbit skeletal muscle acetone powder by the method of Spudich and Watt [13] and further purified by gel filtration on Sephadex G-100.

At each purification step, the activity of the 90-kDa protein was examined in the following way. Samples to be tested were dialyzed against buffer G (2 mM Tris-HCl, pH 7.5/0.2 mM $CaCl_2$/0.5 mM ATP/0.2 mM dithiothreitol/0.01% NaN_3) and assayed in a Cannon-Manning semimicroviscometer (size 100) by their effect on the salt-induced polymerization of skeletal muscle actin. Polymerization was initiated by adding 0.205 mg G-actin to buffer G containing 0.1 M KCl and 1 mM $MgCl_2$ in a total volume of 0.7 ml. The mixture was incubated at 30°C for 1 h, and the final

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equilibrium viscosity was measured in a Cannon-Manning semimicroviscometer. Percent inhibition is defined as: $100 \times (\eta_c - \eta_i)/\eta_c$, where η_c and η_i are the specific viscosities for actin without and with inhibitor, respectively.

Free calcium ion concentrations were estimated by using the computer program of Goldstein [14]. SDS-PAGE was performed as in [15]. Protein concentrations were determined by the method of Bradford [16], using bovine serum albumin as a standard. Pancreatic DNase I (Sigma) covalently linked to Sepharose (Pharmacia) was prepared as in [17].

3. RESULTS

3.1. *Purification of the 90-kDa protein*

The muscle from frozen bovine aorta was homogenized at 4°C in a Waring Blendor with 5 vols of 10 mM Tris-HCl (pH 7.5) containing 0.5 mM PMSF. The suspension was centrifuged for 20 min at 8000 rpm, and the pellet was dehydrated by washing several times with acetone and air-dried overnight. The acetone-dried muscle powder was extracted for 30 min with 20 vols of buffer G followed by centrifugation for 20 min at 8000 rpm, after which the pellet was reextracted with buffer G; 1 M Tris-HCl (pH 7.5) was added to make the combined supernatant 10 mM with respect to Tris-HCl. This crude extract from bovine aorta was fractionated by addition of solid ammonium sulfate at 4°C. The fraction precipitating at 30–60% saturation was redissolved in buffer H (2 mM potassium phosphate, pH 6.8/7 M urea/15 mM β-mercaptoethanol/10% glycerol) and dialyzed against the same buffer for 12 h to denature a large excess of endogenous actin. After denaturation by urea, the 90-kDa protein was renatured without any loss of inhibitory activity. The dialysate was extensively dialyzed against buffer DN (10 mM Tris-HCl, pH 7.5/0.6 M KCl/5 mM $CaCl_2$/0.5 mM ATP) and ultracentrifuged at 105000 × g for 1 h. The resulting supernatant was followed by addition of 2 mg skeletal muscle G-actin/100 g aorta, stirred for 30 min at room temperature, and applied to a DNase I-Sepharose column (2 × 10 cm) equilibrated with buffer DN.

If native G-actin was not added to the supernatant, most of the inhibitory activity did not bind to the DNase I-Sepharose column. The 90-kDa protein was not eluted with buffer containing 5 mM EGTA and 1 M guanidine hydrochloride. Both the 90-kDa protein and actin could be recovered by the 3 M guanidine hydrochloride buffer known to remove actin from the DNase I column [17]. After dialysis against buffer G, this fraction was concentrated by precipitation with ammonium sulfate at 30–60% saturation. The precipitated material was redissolved in, and dialyzed against buffer H containing 1 mM EGTA and 0.01 mM leupeptin for 12 h. The dialysate was extensively dialyzed against buffer S (20 mM Tris-HCl, pH 7.5/1 M KCl/15 mM β-mercaptoethanol/0.2 mM EGTA), applied to a Sephadex G-200 column (1.7 × 90 cm) and eluted with the same buffer. Fractions containing the 90-kDa protein activity were pooled and dialyzed against buffer G. Table 1 summarizes a typical purification of the 90-kDa protein from bovine aorta. SDS-PAGE of active samples after each purification step (fig.1A) demonstrated that increases in specific activity correlated strongly with the enrichment of a 90-kDa polypeptide. The 90-kDa protein and actin of the Sephadex G-200 active fraction were very tightly bound together and resistant to most methods of protein separation.

The isolation procedure of the 90-kDa protein as described here has been applied to other muscle tissues. The uterus and bladder, as shown in fig.1B, were rich sources of the 90-kDa protein. A high activity of the 90-kDa protein was found in chicken gizzard, but it has not been established whether a component of about 85 kDa is a fragmentation product of the 90-kDa protein (fig.1B, lane 4). The skeletal and heart muscle contained no or little activity of the 90-kDa protein. By a densitometry scanning of the SDS gel pattern of fig.1B, we estimated that the yield of the 90-kDa protein from 100 g bovine aorta, uterus and bladder is 2.3 mg, 2.4 mg and 1.5 mg, respectively.

3.2. *Characterization of the 90-kDa protein*

When actin in buffer G containing 0.2 mM Ca^{2+} polymerizes in KCl, there is a long lag phase before the viscosity increases to its equilibrium value (fig.2). The presence of the 90-kDa protein has two effects: (i) the lag phase is abolished; i.e., polymerization is accelerated; but (ii) the final equilibrium viscosity is decreased. There is a pro-

Table 1

Purification of the 90-kDa protein from bovine aorta[a]

Step	Total protein (mg)	Total activity (units)[b]	Specific activity (units/mg)	Recovery (%)	Purification (-fold)
Crude extract	2060	38100	18.5	100	1
$(NH_4)_2SO_4$, 30–60%/ 105000 × *g* supernatant	910	36400	40	96	2.2
DNase I-Sepharose	27	13700	507	36	27
Sephadex G-200	4.5	3800	844	10	46

[a] This preparation was made from 400 g bovine aorta

[b] One unit is defined as the amount of 90-kDa protein that reduces the final specific viscosity by 0.1 from that achieved without inhibitor. The specific viscosity for actin without inhibitor was about 0.35

gressive lowering of the final viscosity with increasing concentrations of the 90-kDa protein. These effects are strongly dependent on free Ca^{2+} concentration. Fig.3 shows the activity of the 90-kDa protein to inhibit actin polymerization as a function of the free Ca^{2+} concentration. Half-maximal activation was at a calculated free Ca^{2+} concentration of 6.8×10^{-8} M. To investigate the mechanism of the Ca^{2+}-sensitive decrease in actin viscosity, we used high-speed centrifugation (fig.4). Actin alone

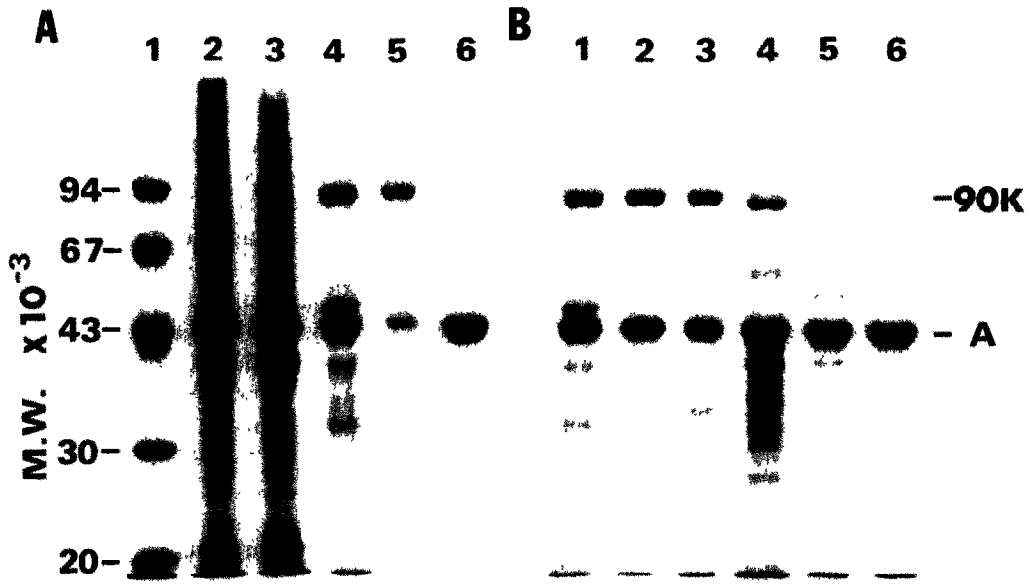

Fig.1. (A) 10% SDS-PAGE of active fractions obtained at various purification steps. The proteins were visualized by staining with Coomassie blue. Lane 1, molecular mass markers; lane 2, crude extract from acetone powder of bovine aorta; lane 3, 105000 × *g* supernatant; lane 4, DNase I-Sepharose fraction; lane 5, Sephadex G-200 fraction; lane 6, purified skeletal muscle actin. (B) Levels of the 90-kDa protein in DNase I-Sepharose fractions of various muscle tissues. Several muscle tissues were fractionated according to the procedure used for bovine aorta; 3 mM guanidine hydrochloride eluates from the DNase I-Sepharose column were analyzed on 10% SDS-PAGE. 90K, 90-kDa protein; A, actin. Lane 1, bovine aorta; lane 2, bovine uterus; lane 3, bovine bladder; lane 4, chicken gizzard; lane 5, canine heart; lane 6, rabbit skeletal muscle.

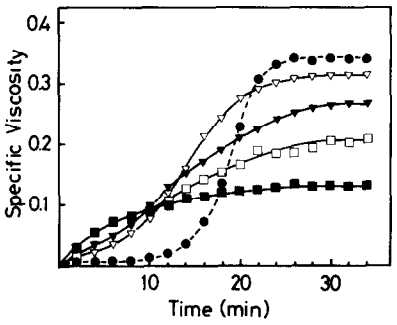

Fig.2. Effect of the 90-kDa protein on actin polymerization. G-actin (250 μg/0.7 ml) was mixed with various amounts of the 90-kDa protein and polymerized at 30°C with 30 mM KCl in buffer G. As a control, actin was polymerized in the absence of the 90-kDa protein. Control (●), 0.63 μg (▽), 1.3 μg (▼), 2.5 μg (□), and 5 μg (■) of the 90-kDa protein.

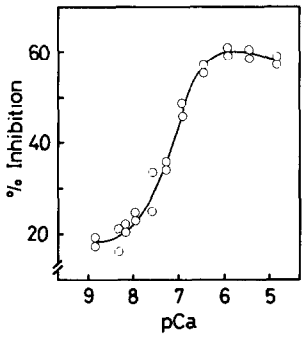

Fig.3. Ca^{2+}-dependence of the 90-kDa protein. The 90-kDa protein (4.1 μg/0.7 ml) was incubated at 30°C for 1 h with G-actin (205 μg/0.7 ml) in buffer G containing 0.1 M KCl and 0.5 mM $MgCl_2$. The free Ca^{2+} concentration was varied by the use of Ca^{2+}/EGTA buffers containing 1 mM EGTA.

polymerized and could be pelleted under these standard conditions, whereas the 90-kDa protein alone could not be sedimented. However, there was significantly less sedimentable actin when the 90-kDa protein and actin were mixed in the presence of Ca^{2+}. It is not clear whether this is simply a reflection of the decreased polymer length or whether there is actually less polymer formed under these conditions.

4. DISCUSSION

The procedures generally used to isolate muscle actin involve preparation of acetone-dried muscle

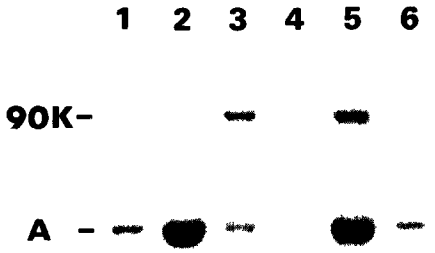

Fig.4. Effect of the 90-kDa protein on the sedimentability of F-actin. G-actin (50 μg/175 μl) was polymerized at 30°C in the presence or absence of the 90-kDa protein (16 μg/175 μl) in buffer G containing 0.1 M KCl and 1 mM $MgCl_2$. The solutions were then centrifuged at 122000 × g for 30 min and the supernatants and washed pellets were subjected to 10% SDS-PAGE. The supernatants (lanes 1,3,5) and the pellets (lanes 2,4,6) from the following incubations are shown in the indicated lanes: lanes 1 and 2, actin alone; lanes 3 and 4, the 90-kDa protein alone; lanes 5 and 6, actin with the 90-kDa protein. 90K, 90-kDa protein; A, actin.

powder from which monomeric actin is extracted and purified by reversible polymerization combined with ultracentrifugation. Earlier observations indicated that polymerizable actin cannot be obtained from vertebrate smooth muscles if the acetone-dried powder is prepared by the method of Straub for skeletal muscle actin [18]. The reason for these observations has not yet been satisfactorily explained [11,12]. Here, we have described experiments to isolate Ca^{2+}-dependent actin regulatory protein from smooth muscle. The 90-kDa protein inhibits actin polymerization and produces a nonsedimentable form of actin in the presence of Ca^{2+}. Using the procedure for the isolation of the 90-kDa protein from bovine aorta, we investigated the distribution of the 90-kDa protein in vertebrate skeletal, heart, and smooth muscle. The smooth muscle including aorta, uterus and bladder, contained the highest levels of 90-kDa protein, whereas the skeletal and heart muscle con-

tained no or little 90-kDa protein. Therefore, the poor polymerizability of smooth muscle crude actin seems to be due to their contamination with the 90-kDa protein which is effective in trace amounts.

As an initial purification step for actin regulatory factor from smooth muscle, we used DNase I-Sepharose affinity chromatography which had been introduced for the purification of villin from chicken intestinal brush borders [5]. The 90-kDa protein retained on the DNase I column was eluted with buffer containing 3 M guanidine hydrochloride, but not with 5 mM EGTA or 5 mM EGTA/1 M guanidine hydrochloride, indicating that the 90-kDa protein binds tightly to actin in a Ca^{2+}-insensitive manner. For the separation of this tightly bound complex, we tried several different chromatographic procedures in the presence of 7 M urea, including DEAE-cellulose chromatography, hydroxylapatite chromatography and gel filtration. However, the 90-kDa protein–actin complex was not separated by these procedures. Several proteins with similar molecular masses and activities have been isolated from a variety of cells and tissues; i.e., gelsolin from rabbit macrophages [2], actin-depolymerizing factor (ADF) or brevin from blood plasma [4,19], a 90-kDa protein from platelets [3]. An accurate comparative study relating these proteins to smooth muscle 90-kDa protein remains to be done. More recently, Hinssen et al. [19] reported the isolation of a Ca^{2+}-dependent actin-severing protein from pig stomach smooth muscle. They showed that the actin modulator has a molecular mass of approx. 85 kDa in the SDS-electrophoresis system and binds to actin in a Ca^{2+}-sensitive manner. This report, together with the present findings, strongly supports the notion that Ca^{2+}-dependent actin regulatory proteins, which inhibit the formation of sedimentable actin filaments, are present in smooth muscles, but not in skeletal and heart muscles. Although we have no ready explanation for possible physiological functions of the 90-kDa protein, its abundance in smooth muscle suggests that the 90-kDa protein may play an important physiological role, perhaps related to regulate contraction-relaxation in smooth muscle.

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