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CAP $Z_{(36/32)}$ IS A CONTAMINANT AND THE MAJOR INHIBITOR OF ACTIN NETWORK FORMATION IN CONVENTIONAL ACTIN PREPARATIONS

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SUMMARY: Previous studies have demonstrated that conventional actin preparations contain a potent factor which reduces the low shear viscosity of actin filaments (1). In this paper we demonstrate that Cap $Z_{(36/32)}$, a recently described protein from skeletal muscle that caps the barbed end of actin filaments and localizes to the Z-line of skeletal muscle (2,3), is the major factor affecting the low shear viscosity of actin prepared from muscle as described by Spudich and Watt (4). © 1987 Academic Press, Inc.

In 1980, MacLean-Fletcher and Pollard reported that conventional actin preparations contain a factor that inhibits actin filament self-associations (1). Based on sensitivity to trypsin and heat, it was felt that this factor was most likely proteinaceous. The identity of the protein responsible for this activity could not be established, however, due to the small quantity of the inhibitor in actin preparations. Since this factor can be separated from actin by gel-filtration, this procedure is now commonly used in the purification of actin.

We have recently isolated a protein from skeletal muscle, now referred to as Cap $Z_{(36/32)}$, which binds with high affinity to the barbed end of actin filaments, and markedly reduces the low shear viscosity of actin filaments (2). Although Cap $Z_{(36/32)}$ is best extracted from muscle under chaotropic conditions (0.6 M KI), we considered the possibility that small quantities of this protein might be liberated under the low ionic strength conditions which remove actin from muscle residues. If so, Cap $Z_{(36/32)}$ would be a likely candidate to represent the inhibitor described by MacLean-Fletcher and Pollard. In this paper, we describe immunological studies using affinity-purified antibodies against Cap $Z_{(36/32)}$ which indicate that this protein is present in actin prepared as described by Spudich and Watt (4), and that immunoprecipitation of this protein restores low shear viscosity to actin preparations which have not been gel-filtered.

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METHODS

Preparation of actin: Actin was purified as described by Spudich and Watt (4) using modifications described by Pardee and Spudich (5) and MacLean-Fletcher and Pollard (1). Actin was extracted from acetone powder of chicken pectoralis muscle using buffer A (0.2 mM ATP, 0.2 mM $CaCl_2$, 0.5 mM 2-mercaptoethanol, 5 mM Tris-HCl, pH 8.0), 20 ml/g of acetone powder. After clarification by centrifugation at 20,000 x g for 1 h, the actin was polymerized for 2 h at 4°C in the presence of 1 mM ATP, exposed to 0.6 M KCl for 30 min, and harvested by centrifugation at 150,000 x g for 100 min. After dialysis against buffer A for 3 days, the actin was clarified by centrifugation at 150,000 x g for 100 min. The resulting actin (~6 mg/ml) was then gel-filtered on a Sephacryl S-200 (Pharmacia) column equilibrated with buffer A.

Measurements of actin polymerization: Low shear viscosity was measured essentially as described by MacLean-Fletcher and Pollard (1). Actin, 7 µM, was polymerized in the presence of 100-120 mM KCl, 2 mM $MgCl_2$ in buffer A and the indicated concentration of Cap $Z_{(36/32)}$ (Fig. 1), or an aliquot of the column fraction indicated in Fig. 2a (1/10 of the total sample volume). Immediately after the addition of salt, the sample was drawn into 100 ul capillary tubes (Corning), which were then sealed at the bottom with clay. After a 1 h incubation, the time required for a 0.025 inch steel ball to move 3 cm through the tube when held at an angle of 60° was measured. Inhibition of actin polymerization induced by erythrocyte spectrin-band 4.1-actin complex was measured as previously described (2), using pyrene-actin fluorescence as a measurement of polymerization. Actin, 11.9 µM (5% pyrene actin), was polymerized in 0.4 mM $MgCl_2$, 22.5 µg/ml of erythrocyte complex in buffer A in the presence of a one-fourth volume of the column fraction indicated in Fig. 2a, or an equal volume of buffer A as a control. Results are expressed as the percent inhibition of polymerization 35 min after initiation of polymerization as compared to control samples.

Purification of Cap $Z_{(36/32)}$ and preparation of antibodies: Cap $Z_{(36/32)}$ was purified as previously described (2). Antibodies were prepared by injection of NZW rabbits with 100 µg of soluble purified Cap $Z_{(36/32)}$ in Freund's complete adjuvant, followed by injection of the same material in Freund's incomplete adjuvant every 2-3 weeks for 3-5 injections. Affinity-purified antibodies were prepared by absorption of the antisera on an affinity column prepared using cyanogen bromide-activated Sepharose 4B (Pharmacia) and 0.6 mg of Cap $Z_{(36/32)}$. Washing of the column and elution of the antibodies were carried out as described by Bennett and Davis (6).

Immunoprecipitation of the inhibitor using the affinity-purified antibodies against Cap $Z_{(36/32)}$: Fifty µl of pre-column actin diluted 1:4 (v/v) with post-column actin (final concentration 65 µM) was exposed to the indicated concentration of affinity-purified anti-Cap $Z_{(36/32)}$ antibody or pre-immune IgG in 100 µl of phosphate buffered saline. After incubation overnight at 4°C, 3 µl of Protein A-Sepharose (Pharmacia) (IgG binding capacity 60 µg) was added to the solution. After another hour of incubation at the same temperature, the IgG-Protein A-Sepharose complexes were removed by centrifugation at 13,000 x g for 10 min. Falling ball times on the supernatant actin solutions were then determined as described above, using a final concentration of 14.6 µM actin in each sample.

Electrophoresis and Immunoblots: Electrophoresis and immunoblotting experiments were carried out as previously described (2) using 5-20% linear gradient SDS-polyacrylamide separating gels and 3% stacking gels.

RESULTS

As shown in Fig. 1, nanomolar concentrations of purified Cap $Z_{(36/32)}$ significantly reduced the low shear viscosity of micromolar concentrations

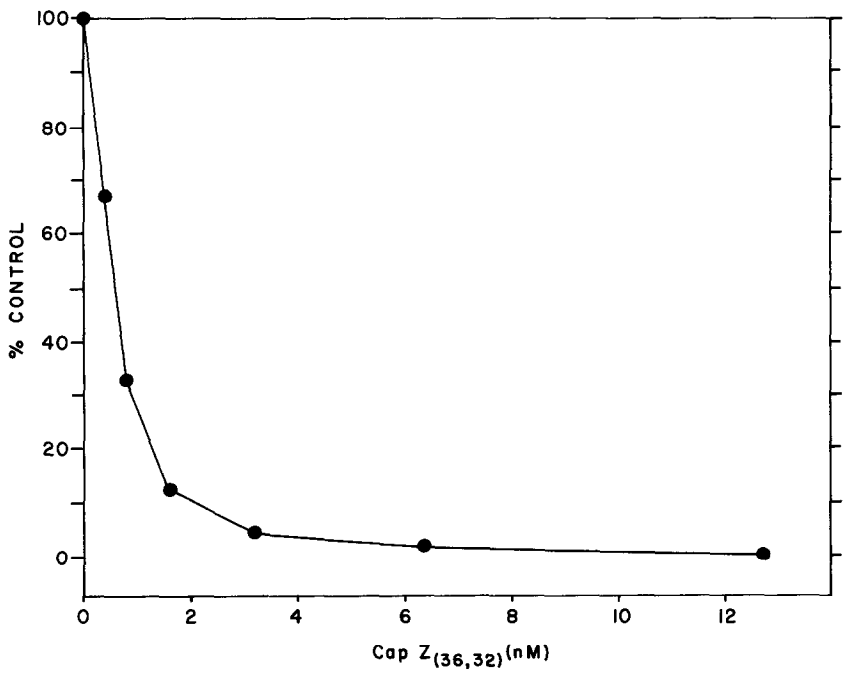

Figure 1. Reduction of the low shear viscosity of actin by Cap $Z_{(36/32)}$. Actin, 11.9 μM, prepared and gel-filtered as described in METHODS, was polymerized in the presence of 116.7 mM KCl, 2 mM $MgCl_2$ and the indicated concentration of Cap $Z_{(36/32)}$ in buffer A plus 3.33% sucrose and 1.67 mM K_2HPO_4. After 2 h and 15 min, the viscosity of the solution was measured in the falling ball viscometer as described in METHODS. Results are expressed as the ratio of experimental to control times.

of actin. A 90% reduction of low shear viscosity was seen at concentrations of approximately 2 nM (0.13 μg/ml) Cap $Z_{(36/32)}$ in 11.9 μM (500 μg/ml) actin. This contrasts with the 7 μg/ml of inhibitor required to see a 90% reduction of low shear viscosity of 500 μg/ml actin in MacLean-Fletcher and Pollard's experiments, suggesting that the specific activity of Cap $Z_{(36/32)}$ is at least 50 times higher than the active material identified in their studies.

To attempt to identify Cap $Z_{(36/32)}$ in actin preparations, pre- and post-column fractions were examined for 1) the ability to reduce the low shear viscosity of actin in the falling ball assay, 2) inhibition of erythrocyte spectrin-band 4.1-actin complex-induced polymerization (the assay originally used to detect Cap $Z_{(36/32)}$ in muscle extracts (2)), and 3) reactivity with the anti-Cap $Z_{(36/32)}$ antibodies on immunoblots. As shown in Fig. 2, the peaks of activity in the falling ball assay and the complex-induced polymerization assays co-localized, and were the same fractions that contained immunoreactive protein bands at the molecular weight of the two subunits of Cap $Z_{(36/32)}$ on immunoblots.

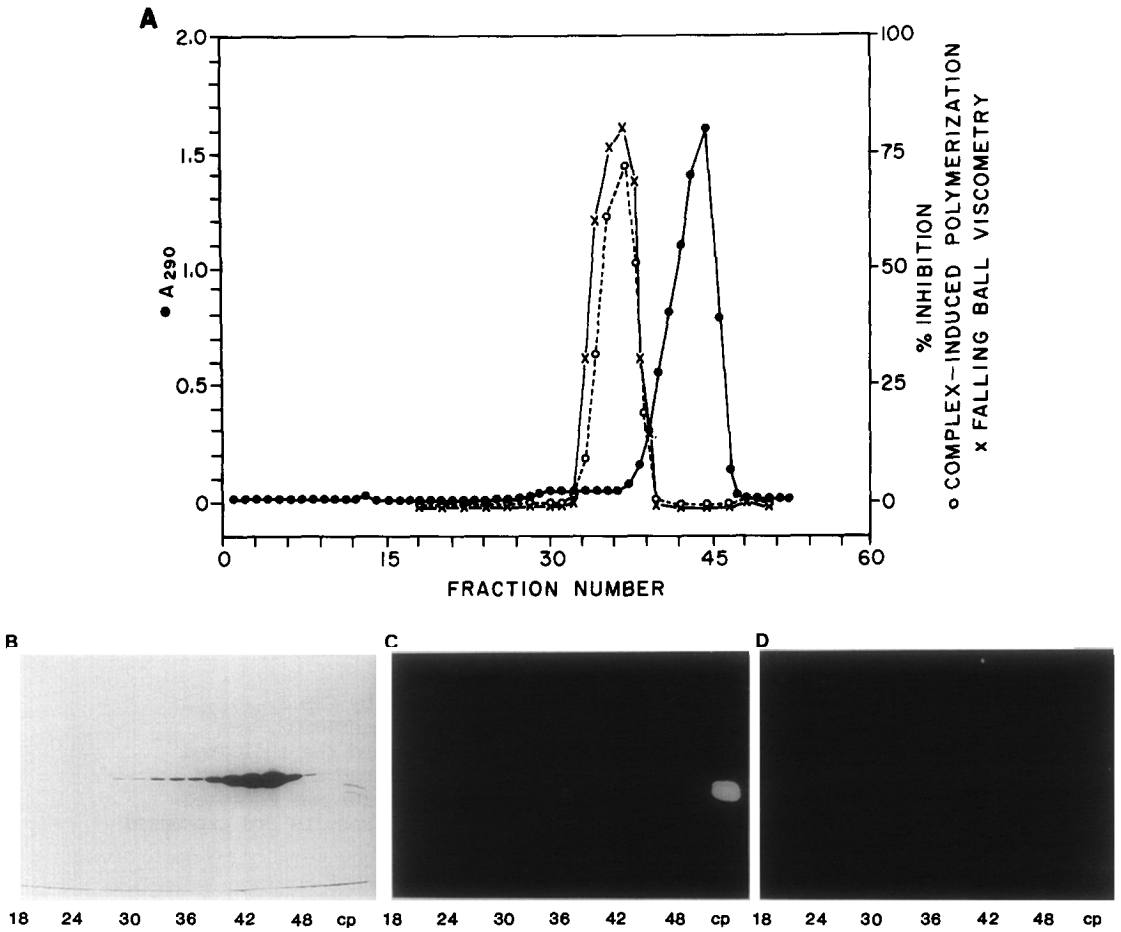

Figure 2. Co-elution of Cap $Z_{(36/32)}$ and the inhibitor described by MacLean-Fletcher and Pollard after gel-filtration of conventional actin preparations. Fifty mg of actin in an 8 ml volume was applied to a 2.6 x 90 cm Sephacryl S-200 (Pharmacia) column equilibrated with buffer A, eluted at 20 ml/hr, and collected in 5 ml fractions. Fractions were assayed for absorbance at 290 nm (●), ability to reduce the low shear viscosity of actin in the falling ball assay (using 100 mM KCl, 2 mM $MgCl_2$)(X), and inhibition of erythrocyte complex-induced polymerization (O) as described in METHODS (A). The same fractions were also subjected to SDS-polyacrylamide gel electrophoresis and immunoblotting using affinity-purified antibodies against Cap $Z_{(36/32)}$. Coomassie Blue stained gel (B). Immunoblots using immune (C) and pre-immune (D) antibodies.

These findings strongly suggested that Cap $Z_{(36/32)}$ could be responsible for the activity of the preparation described by MacLean-Fletcher and Pollard. To test this hypothesis, the ability of the anti-Cap $Z_{(36/32)}$ antibodies to precipitate the activity of the inhibitor from pre-column actin was evaluated. As shown in Fig. 3, 80-90% of the activity of the inhibitor as measured by the falling ball assay was removed at the highest concentrations of anti-Cap $Z_{(36/32)}$ antibodies.

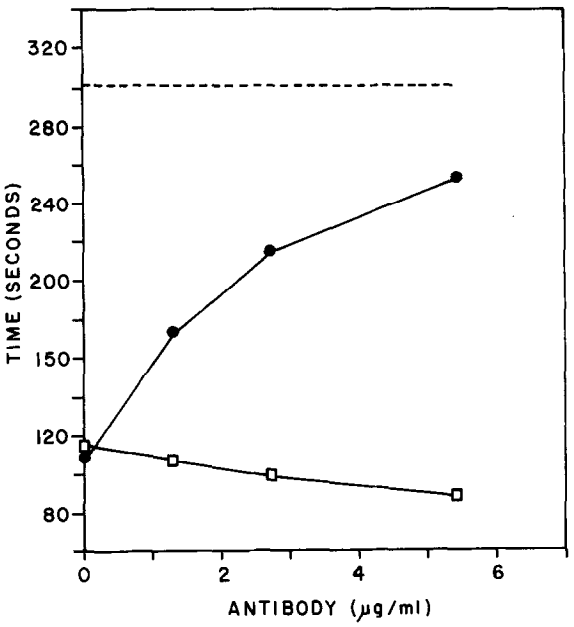

Figure 3. Immunoprecipitation of the factor that reduces the low shear viscosity of actin using affinity-purified antibodies against Cap $Z_{(36/32)}$. Falling ball times were determined after immunoprecipitation of pre-column actin samples using affinity-purified anti-Cap $Z_{(36/32)}$ antibodies (●) or pre-immune IgG (□) as described in METHODS. The falling ball time for the same concentration of post-column actin is shown by the dashed line.

DISCUSSION

These studies demonstrate that conventional actin preparations contain significant quantities of Cap $Z_{(36/32)}$, a recently described actin-capping protein from muscle which binds to the barbed end of actin filaments. Like the inhibitor of actin filament self-association from conventional actin preparations described by MacLean-Fletcher and Pollard, Cap $Z_{(36/32)}$ is easily removed from actin preparations by gel-filtration, and has the same elution characteristics. This, and the fact that the inhibitory activity can be removed by immunoprecipitation with antibodies affinity-purified against Cap $Z_{(36/32)}$, suggest that Cap $Z_{(36/32)}$ is the protein responsible for the activity previously described by MacLean-Fletcher and Pollard.

It is not suprising that Cap $Z_{(36/32)}$ has not been identified in actin preparations in previous studies, since it represents such a small fraction of the protein on Coomassie Blue stained polyacrylamide gels of actin. In our studies, Cap $Z_{(36/32)}$ was detected only by immunological techniques. The low concentrations of Cap $Z_{(36/32)}$ necessary to produce a significant reduction of the low shear viscosity of actin illustrates the difficulty that can be encountered in assessing the effects of protein preparations on actin. Since Cap $Z_{(36/32)}$ has now been detected in low (buffer A) and high (0.6 M KI) salt extracts of muscle, it is likely that this protein

may contaminate other muscle protein preparations. This paper should serve to alert others to this possibility.

NOTE ADDED IN PROOF

In Figure 2 (B-D), the position of a sample containing partially purified Cap Z (36/32) is indicated by the letters cp.

ACKNOWLEDGEMENTS

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