

# Secretory Cell Actin-binding Proteins: Identification of a Gelsolin-like Protein in Chromaffin Cells

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**Abstract.** Chromaffin cells, secretory cells of the adrenal medulla, have been shown to contain actin and other contractile proteins, which might be involved in the secretory process.

Actin and  $\text{Ca}^{++}$ -sensitive actin-binding proteins were purified from bovine adrenal medulla on affinity columns using DNase-I as a ligand. Buffers that contained decreasing  $\text{Ca}^{++}$  concentrations were used to elute three major proteins of 93, 91, and 85 kD. The bulk of the actin was eluted with guanidine-HCl buffer plus some 93- and 91-kD proteins. These  $\text{Ca}^{++}$ -sensitive regulatory proteins were shown to inhibit the gelation of actin using the low-shear falling ball viscometer and by electron microscopy. Actin filaments were found to be shortened by fragmentation.

Using antibody raised against rabbit lung macrophage gelsolin, proteolytic digestion with Staphylococcus V8 protease and two-dimensional gel electrophoresis, the 91-kD actin-binding protein was shown to be a gelsolin-like protein. The 93-kD actin-binding protein also showed cross-reactivity with anti-gelsolin antibody, similar peptide maps, and a basic-shift in  $\text{pH}_i$  indicating that this 93-kD protein is a brevin-like pro-

tein, derived from blood present abundantly in adrenal medulla. Purification from isolated chromaffin cells demonstrated the presence of 91- and 85-kD proteins, whereas the 93-kD protein was hardly detectable.

The 85-kD protein is not a breakdown product of brevin-like or gelsolin-like proteins. It did not cross-react with anti-gelsolin antibody and showed a very different peptide map after mild digestion with V8 protease. Antibodies were raised against the 93- and 91-kD actin-binding proteins and the 85-kD actin-binding protein. Antibody against the 85-kD protein did not cross-react with 93- and 91-kD proteins and vice versa.

In vivo, the cytoskeleton organization of chromaffin secretory cells is not known, but appears to be under the control of the intracellular concentration of free calcium. The ability of calcium to activate the gelsolin-like protein, and as shown elsewhere to alter fodrin localization, provides a mechanism for gel-sol transition that might be essential for granule movement and membrane-membrane interactions involved in the secretory process.

**C**ONTRACTILE proteins have been detected in a variety of non-muscle cells and it is generally assumed that these proteins are involved in many important cellular functions that require movement. Secretion by exocytosis is a process that shares many features with the process of excitation-contraction described in the muscle (50, 51). Thus, it has been proposed that the movement of secretory vesicles and the subsequent fusion of the vesicle membrane with the plasma membrane may be mediated by contractile elements.

For several experimental reasons, adrenal medullary chromaffin cells provide an excellent system to study the molecular mechanisms underlying transport and release of secretory products (33, 34, 52). Chromaffin granules have been extensively studied because they are easy to obtain as a homogeneous population (reference 58 for review). Moreover, chromaffin cells can be maintained in primary culture, and they represent a convenient model to study the functional aspect

of secretion because influences on catecholamine release can be rigidly controlled (1, 2, 34, 53). Biochemical and immunological techniques have demonstrated the presence of actin and myosin in chromaffin cells (3, 20, 21, 29, 30, 54). Moreover, the specific association of actin filaments with the secretory granule membrane (6, 9, 54) suggests that contractile elements may play an active role in the cellular transport of secretory granules and in an as yet unidentified step involved in the secretory process. Chromaffin granule membrane is also able to promote G-actin polymerization. The involvement of a membrane-associated alpha-actinin-like protein in the stabilization of actin nuclei for the assembly of actin filaments has recently been demonstrated (6). In addition, chromaffin granule membrane is able to cross-link F-actin filaments and to increase the viscosity of an actin solution; the binding of F-actin to granule membrane is calcium-dependent (13) and is due to the presence of a spectrin-like

(fodrin) protein associated with the granule membrane (4).

The consistency of the cytoplasm in intact cells is able to be reversibly transformed from a low viscosity liquid (sol) to a rigid gel. An attractive hypothesis is that the movement of cellular organelles may be regulated by the reversible gel-sol transition. Many actin-binding proteins that could regulate this process have recently been described (see references 12, 32, and 47 for review). The activity of many of these proteins is calcium-dependent, and this property may confer the calcium sensitivity of the gel-sol transformation in the intact cell. In secretory cells, it is possible that the increase of intracellular calcium levels preceding exocytosis (reference 33 for review) may regulate the interactions of actin filaments with actin-binding proteins resulting in changes of the cytoplasmic viscosity. Interestingly, fodrin, a calcium-dependent actin-binding protein, has recently been described to be redistributed when secretory cells are stimulated with nicotinic agonists or calcium ionophores (41).

In chromaffin cells, fodrin appears to be preferentially localized in the subcortical cytoplasm, a region characterized by a dense network of interacting filaments. In resting conditions for which the calcium concentration is  $10^{-8}$  M (37), fodrin may induce the formation of a rigid gel near the plasma membrane, which acts as a physical barrier preventing granules from undergoing exocytosis. The present paper deals with the characterization of additional calcium-dependent actin-binding proteins from the soluble cytosol of chromaffin cells. These proteins are actin-fragmenting proteins, and they decrease the viscosity of actin filaments when micromolar levels of calcium are attained. The presence of these proteins in secretory cells raises the possibility that they could modulate the gel-sol transition during stimulation, thus facilitating the movement of secretory granules towards and their juxtaposition on the cell membrane.

## Materials and Methods

### Preparation of DNase I-Sepharose

DNase I-Sepharose was prepared by incubating 5 g of cyanogen bromide-activated Sepharose (CNBr-Sepharose, Pharmacia, Bois d'Arcy, France) with 100 mg of DNase I from bovine pancreas (Serva Feinbiochemica, Heidelberg, FRG.; 2,000 KU/mg). Coupling was carried out at 4°C for 12 h in 0.1 M  $\text{NaHCO}_3$ , 0.5 M NaCl at pH 8.3. The gel was then extensively washed with coupling buffer, and the excess cyanogen bromide was blocked by incubation for 16 h in 100 ml coupling buffer containing 0.2 M glycine. The gel was subsequently washed three times with 500 ml coupling buffer and 500 ml acetate buffer (0.1 M Na-acetate, 0.5 M NaCl, pH 4.0). Finally the gel was washed with 1 liter of coupling buffer, and 1 liter of 5 mM Tris-HCl (pH 7.5), 0.5 mM  $\text{CaCl}_2$ , and poured onto the column. The DNase-column was equilibrated with buffer A (20 mM imidazole, pH 7.5, 1 mM dithiothreitol [DTT]<sup>1</sup>, 0.25 mM phenylmethylsulfonyl fluoride [PMSF], 1 mM  $\text{CaCl}_2$ , 0.5 mM Na-ATP).

### Purification of Actin-binding Proteins from Adrenal Medulla

Bovine or pig adrenal glands obtained from the local slaughterhouse were kept on ice and the medullae were dissected from the cortices. 20–30 g of bovine medullae or 10 g of pig medullae were usually obtained. Adrenal medullae were homogenized in 0.3 M sucrose, 20 mM imidazole (pH 7.5), 5 mM DTT, 1 mM PMSF, 0.75 mM  $\text{NaN}_3$ , 5 mM N-ethylmaleimide, 1 mM EGTA, 1 mM Na-ATP (1 g of medulla in 4 ml of solution), using a motor-driven Potter-Elvehjem homogenizer. The homogenate was centrifuged at 800 g for 15 min. The supernatant was filtered on cheese cloth and centrifuged at 20,000 g for 20 min. The resulting supernatant was then centrifuged at 100,000 g for 60 min and dialyzed against 20 mM imidazole (pH 7.5), 1 mM DTT, 0.25 mM PMSF, 1 mM  $\text{CaCl}_2$ . After the dialysis step,  $\text{CaCl}_2$  and Na-ATP were added to

obtain a final concentration of 2 mM and 0.5 mM, respectively. The solution was then passed through the DNase-I-Sepharose column (0.9 × 15 cm) that had been pre-equilibrated with buffer A. The column was then washed extensively with buffer A containing 0.6 M NaCl. The calcium-activated proteins were eluted with buffer B (20 mM imidazole, pH 7.5, 1 mM DTT, 0.25 mM PMSF, 10 mM EGTA, 0.5 mM Na-ATP). When optical density at 280 nm had returned to the basal value, the column was washed with the guanidine-HCl buffer (3 M guanidine-HCl, 0.5 M Na-acetate, 30% glycerol, 1 mM  $\text{CaCl}_2$ , 0.5 mM Na-ATP, pH 6.5). Finally, the column was regenerated by washing with buffer A. Proteins eluted with EGTA and with guanidine-HCl were dialyzed against 20 mM imidazole pH 7.5, 0.25 mM PMSF, 1 mM EGTA, 0.75 mM  $\text{NaN}_3$ , 0.5 mM DTT, concentrated under high pressure, and analyzed by SDS polyacrylamide gel electrophoresis.

### Purification of Actin-binding Proteins from Isolated Chromaffin Cells

Nine bovine adrenal glands were perfused for 30 min (37°C) with calcium-free Krebs (154 mM NaCl; 5.6 mM KCl; 3.6 mM  $\text{NaHCO}_3$ ; 5 mM glucose; 5 mM Hepes, pH 7.4) solution. Perfusion was continued for 60 min with fresh Krebs solution to which 0.1% *Clostridium histolyticum* collagenase (Boehringer GmBH, Mannheim, FRG) and 0.5% bovine serum albumin (BSA) had been added. After perfusion, the medullae were carefully dissected and further dissociation of chromaffin cells was achieved by passing the cells through a nylon sieve (120  $\mu\text{m}$  pore size). Cells were collected in calcium-free Krebs solution and purified by centrifugation on Percoll (Pharmacia) gradients (5). Chromaffin cells were then washed by low speed centrifugation in calcium-free Krebs solution. Finally chromaffin cells ( $6 \times 10^7$  cells per gland) were collected by centrifugation and homogenized in 5 ml of 0.3 M sucrose containing 20 mM imidazole (pH 7.5), 5 mM DTT, 1 mM PMSF, 0.75 mM  $\text{NaN}_3$ , 5 mM N-ethylmaleimide, 1 mM EGTA, 1 mM Na-ATP. The homogenate was immediately centrifuged for 60 min at 100,000 g and the resulting supernatant dialyzed against buffer A. The solution was applied to the DNase-I-Sepharose column. After washing with buffer A containing 0.6 M NaCl, the column was eluted with 10 ml guanidine-HCl buffer. Eluted proteins were dialyzed as described, lyophilized, and analyzed by SDS gel electrophoresis.

### Measurement of Viscosity

Apparent viscosity was estimated using the low-shear falling ball viscometer (44). Rabbit skeletal muscle G-actin (46) was polymerized in 0.1 M KCl and 2 mM Mg-ATP for 60 min at room temperature. F-actin at a final concentration of 0.8 mg/ml was then mixed with the concentrated actin-binding proteins at a defined concentration in an assay buffer (40 mM Pipes, pH 6.8, 20 mM KCl, 1.2 mM  $\text{MgCl}_2$ ) containing EGTA and calcium to give the indicated final free-calcium concentration. Samples were drawn into 700- $\mu\text{l}$  capillary tubes, sealed with plasticine at one end, and kept at 25°C for 120 min. Experimental conditions were as described (4).

### Electron Microscopy

Actin filaments were obtained by polymerization of 0.1 mg/ml G-actin in the presence of 0.1 M KCl and 2 mM Mg-ATP for 60 min at 25°C. The filaments formed were incubated for 120 min at 25°C with actin-binding proteins eluted from the DNase-I-Sepharose column with buffer B. The final actin-binding protein concentration was 9  $\mu\text{g}/\text{ml}$ , and the molar ratio to actin was ~1:20. The composition of the buffer solution was 40 mM Pipes (pH 6.8), 20 mM KCl, 1 mM  $\text{MgCl}_2$  either with 5 mM EGTA ( $10^{-8}$  M free calcium concentration) or with EGTA, and calcium to give a final free calcium concentration of  $10^{-5}$  M. Samples were mounted on carbon-coated Formvar grids, negatively stained with 1% aqueous uranyl acetate, and viewed at 80 kV with a Philips EM 420 electron microscope. Filament lengths were estimated from twenty random fields, printed at a final magnification of 120,000 diameters. They were then sub-classed at 0.1- $\mu\text{m}$  intervals to produce a distribution histogram.

### Peptide Mapping

Peptide mapping was performed after limited proteolysis with Staphylococcus V8 protease (Worthington Biochemical Corp., Freehold, NJ) as described by Cleveland et al. (10) with some modifications. Proteins were first separated electrophoretically on 1.5-mm thick, 10% acrylamide gel. Gels were stained for 2 h in 0.1% Coomassie Brilliant Blue, 50% methanol, and 10% acetic acid, and destained for 4 h in 5% methanol and 10% acetic acid. The gel was then soaked in water, and the bands of interest were cut out and trimmed to 7-mm wide. The bands were soaked for 30 min in 10 ml Tris-HCl (125 mM), pH 6.8, 0.1% SDS, 1 mM EDTA, and then boiled 2 min in 50  $\mu\text{l}$  Tris-HCl (62.5 mM),

<sup>1</sup> Abbreviations used in this paper: DTT, dithiothreitol; PMSF, phenylmethylsulfonyl fluoride.

pH 6.8, 0.1% SDS, 1 mM EDTA, and 10% glycerol. Finally the gels were placed in sample wells of a second SDS polyacrylamide gel and each slice was overlaid with 10–100 ng of protease. A 10% polyacrylamide gel (1.5-mm thick) with a long stacking gel (5 cm) was used as the second gel. The stacking gel solutions contained 1 mM EDTA, but EDTA was absent from running gel solutions. After a 30-min incubation at room temperature, electrophoresis was performed at 30 V until the bromophenol blue reached the end of the stacking gel. The current was then turned off for 60 min, and electrophoresis was run at 80 V overnight. Low molecular weight proteolytic fragments were detected by the silver staining technique (38).

### Antisera to Bovine Chromaffin Cell Actin-binding Proteins

1.5 mg of actin-binding proteins obtained by chromatography on DNase-I-Sepharose were separated by electrophoresis on 10% polyacrylamide gel. After electrophoresis, the gel was briefly stained with Coomassie Brilliant Blue and scanned at 633 nm. The 91 and 85-kD protein bands represented ~300  $\mu$ g and 350  $\mu$ g of proteins. These bands were cut off the gel, homogenized in complete Freund's adjuvant (BD Mérieux, Lyon, France), and injected subcutaneously into rabbits. A similar injection was repeated 2 wk later except that the bands were homogenized in incomplete Freund's adjuvant (BD Mérieux). Rabbits were bled from the ear vein 40 d after the first injection. Immunoglobulins were not further purified.

### Macrophage Gelsolin and Gelsolin Antibodies

Rabbit macrophage gelsolin and anti-gelsolin antibodies were a generous gift from Dr. Helen L. Yin (Massachusetts General Hospital, Boston, MA). The purification of gelsolin from rabbit lung macrophages and the characterization of anti-gelsolin antibodies have been reported (56, 57, 59). The antibodies cross-react with gelsolin from pig, human, and rat but do not recognize bovine gelsolin.

### Electrophoresis

Monodimensional polyacrylamide gel electrophoresis was performed using 8–20% gradient gels with either Tris-acetate electrophoresis buffer composed of

40 mM Tris (pH 7.4), 20 mM Na-acetate, 2 mM EDTA, and 0.2% SDS, or with Tris-glycine electrophoresis buffer containing 25 mM Tris (pH 8.3), 192 mM glycine, and 0.1% SDS. Protein samples were solubilized in 10 mM Tris (pH 8.0), 1 mM EDTA, 3% SDS, 10% beta-mercaptoethanol, and 10% glycerol. Usually electrophoresis was run at 100 V for 18 h. The conditions for separation of proteins by electrophoresis on two-dimensional gels have been described elsewhere (6).

### Electrophoretic Blotting and Immunological Detection

Proteins resolved on SDS polyacrylamide gels were transferred electrophoretically to nitrocellulose sheets (0.45- $\mu$ m pore size, HA filter type; Millipore SA, Molsheim, France) for 3 h at 500 mA (6, 49). Normal goat serum (10%) was included in all the incubation steps when rabbit antisera raised against bovine chromaffin cell actin-binding proteins were used. Incubations with macrophage gelsolin goat antibodies were performed in the presence of 10% fetal calf serum. The nitrocellulose blots were incubated overnight at 4°C with rabbit antisera (anti-91-kD or anti-85-kD proteins) at a final dilution of 1:1,000 or with goat anti-gelsolin antibodies with a 1:800 dilution. After washing, the blots were incubated 2 h with a 1:1,000 dilution of horseradish peroxidase-labeled goat anti-rabbit (Nordic Immunological Laboratories, Tilburg, The Netherlands) or rabbit anti-goat (Institut Pasteur Production, Paris, France) immunoglobulins. Immunoreactive bands were visualized by incubation for 10 min in 25 mM phosphate buffer (pH 7.5) containing 125 mM NaCl, 0.18 mg/ml 2-chloronaphthol, and 0.01% H<sub>2</sub>O<sub>2</sub>.

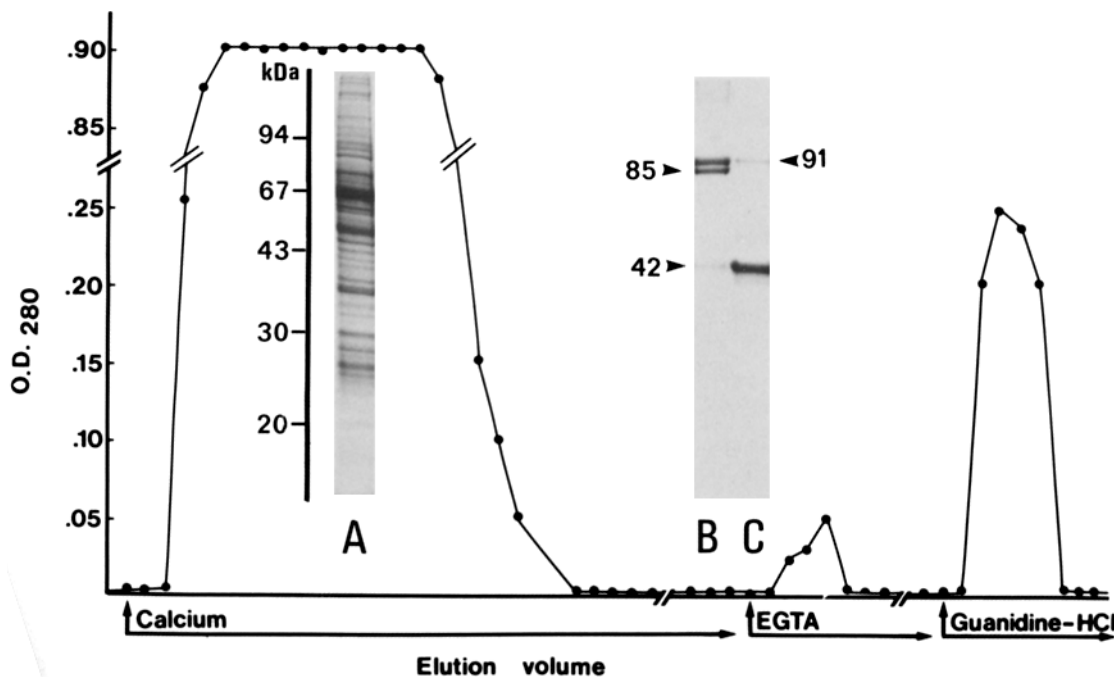
### Others

Free calcium concentrations in calcium-EGTA buffers were calculated according to Katz et al. (24). Proteins were estimated according to Lowry et al. (35) with BSA (Sigma Chemical Co., St Louis, MO) as standard.

### Results

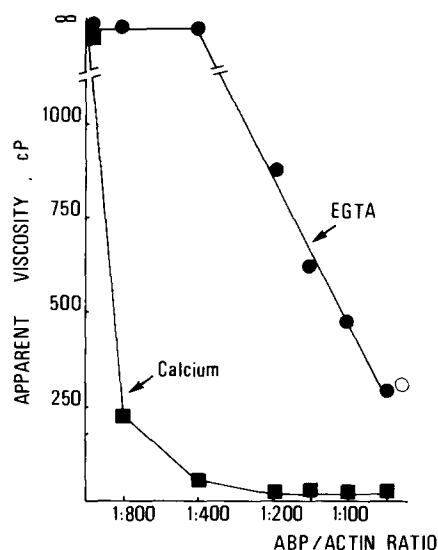
#### Isolation of Calcium-sensitive Actin-binding Proteins

Lee et al. (29) have reported that actin can be purified from chromaffin cell cytosol by affinity chromatography on DNase-



**Figure 1.** Isolation of actin-binding proteins from adrenal medulla cytosol. The cytosol fraction (100,000 *g* supernatant) was prepared from bovine adrenal medulla and dialyzed against ATP-free buffer A. The dialyzed fraction to which Na-ATP was added was passed through the DNase-I-Sepharose column. The optical density of the eluted material was monitored at 280 nm, and the peaks were collected. The major portion of the cytosol proteins was not retained by the column. Lane A is the electrophoretic profile of proteins not retained on DNase-I-Sepharose. The column was washed overnight with buffer A containing 0.6 M NaCl, and calcium-dependent actin-bound proteins were eluted with EGTA-containing buffer. Finally, the column was washed with guanidine-HCl buffer. Lanes B and C are, respectively, the electrophoretic profiles of the proteins eluted with EGTA and with guanidine-HCl. Electrophoretic protein markers have molecular weights of 94, 67, 43, 30, and 20 kD.

I. We considered that the cytosol of chromaffin cells might contain actin-regulating proteins that would interact differently according to the free calcium concentration. Therefore the possibility of isolating actin with associated proteins by chromatography on DNase-I-Sepharose with decreasing calcium concentration was tested. Fig. 1 gives the result of such a typical experiment. A cytosolic soluble fraction (100,000 g supernatant) was prepared from bovine adrenal medulla and applied to the DNase-I-Sepharose affinity column in the presence of calcium. The major portion of the soluble proteins was not retained on the column (Fig. 1, first peak, lane A). Elution with the EGTA-containing buffer produced a small peak (Fig. 1, second peak) that represented only 0.5% of the total applied protein. Subsequent elution with guanidine-HCl buffer released further protein (Fig. 1, third peak) which represented ~2.5% of the total applied protein. Two major proteins of 91 and 85 kD and traces of actin were eluted when calcium was complexed with EGTA (Fig. 1, lane B). The bulk of actin (42 kD) plus some 91-kD protein and a minor component of 58 kD were eluted by washing the column with guanidine-HCl buffer (Fig. 1, lane C). The 85-kD protein was never detected in guanidine-HCl-eluted fractions, indicating



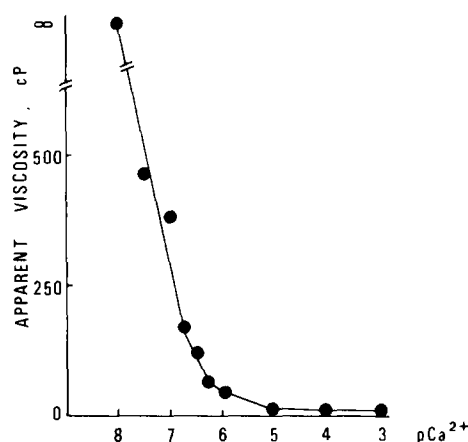
**Figure 2.** Effect of various concentrations of purified actin-binding-proteins on the apparent viscosity of F-actin solution. The concentration of actin was fixed at 0.8 mg/ml. G-actin (5–10 mg/ml in 2 mM Tris-HCl, pH 8.0, 0.2 mM Na-ATP, 0.5 mM  $\beta$ -mercaptoethanol, 0.2 mM  $\text{CaCl}_2$ ) was polymerized by addition of 100 mM KCl and 2 mM Mg-ATP. Various concentrations of freshly prepared actin-binding proteins were then mixed with F-actin and incubated in a buffer composed of 40 mM Pipes, 20 mM KCl, 1 mM  $\text{MgCl}_2$  with 5 mM EGTA (free  $\text{Ca}^{++}$  concentration was  $10^{-8}$  M; ●) or without EGTA (free  $\text{Ca}^{++}$  concentration was  $10^{-5}$  M; ■). Actin-binding protein/actin ratio is the molar ratio of the 91-kD protein (which represented 50% of total actin-binding proteins) to actin. Samples were drawn into capillary tubes, and the tubes were mounted at an angle of 45°. The time taken for a stainless steel ball to fall to the bottom of the tube was measured and apparent viscosities were calculated (4).  $\infty$  indicates that the ball was trapped by the gelled solution and did not sediment. Each point is the mean of four determinations using the same actin-binding protein preparation. At  $10^{-8}$  M  $\text{Ca}^{++}$ , a decrease of viscosity was observed which was due to the imidazole buffer. ○, indicates the viscosity of actin solution mixed with the actin-binding protein-free buffer at the same concentration as that corresponding to the 1:50 actin-binding protein/actin ratio.

that EGTA completely dissociated the 85-kD protein-actin complexes bound to the DNase-I Sepharose.

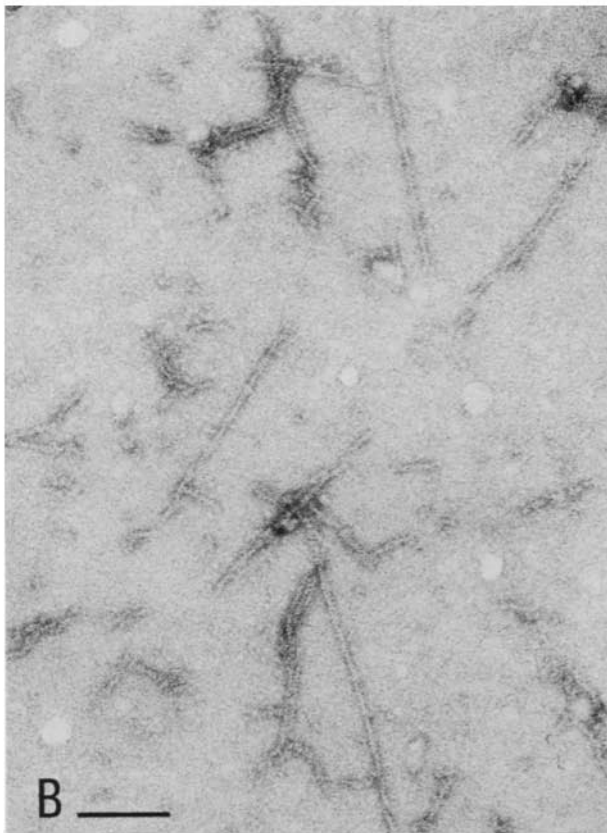
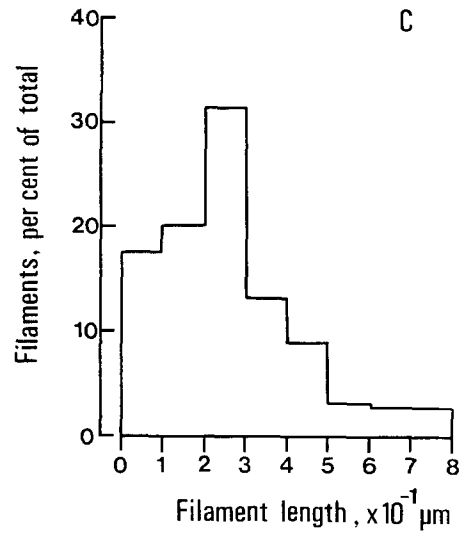
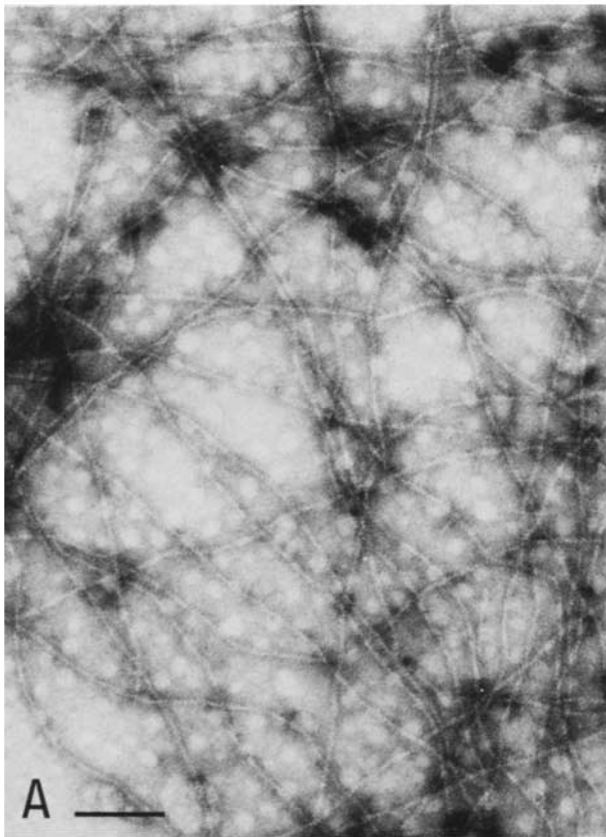
### Effect of EGTA-eluted Proteins on the Viscosity of F-Actin Solution

Fig. 2 shows the effect of various concentrations of the EGTA-eluted actin-binding proteins on the viscosity of preformed actin filaments. A dramatic decrease in the apparent viscosity was observed when F-actin (0.8 mg/ml) was incubated with actin-binding proteins in the presence of calcium. The addition of 4.2  $\mu\text{g}/\text{ml}$  actin-binding protein to the F-actin solution (actin-binding protein/actin molar ratio 1:400) did not modify the apparent viscosity of the solution when calcium was absent, but the viscosity decreased rapidly from ~1,400 cP to 6 cP when calcium was present at  $10^{-5}$  M. Higher actin-binding protein concentrations (actin-binding protein/actin molar ratio over 1:100) induced a dramatic decrease of viscosity to close to 1 cP in the presence of calcium, but appeared also to affect the viscosity in the presence of EGTA (Fig. 2). This effect was attributed to the imidazole buffer itself which affects the apparent viscosity of F-actin. The capacity of altering the viscosity of actin solution was lost when actin-binding protein fractions were frozen and kept at  $-20^\circ\text{C}$ . Changes in viscosity could be measured only with fresh actin-binding protein preparations or with fractions kept at  $+4^\circ\text{C}$ . However, once F-actin and actin-binding proteins were mixed, the calcium-dependent inhibition of actin gelation could be measured for up to 48 h at room temperature.

The effect of a fixed concentration of actin-binding proteins on the viscosity of actin filaments was examined as a function of the free calcium concentration in the medium (Fig. 3). The viscosity of F-actin alone was not sensitive to changes in the calcium concentration within the range examined. Actin-binding protein produced a rapid decrease in the apparent



**Figure 3.** Effect of actin-binding proteins on apparent viscosity of F-actin at different  $\text{Ca}^{++}$  concentrations. F-actin (0.8 mg/ml) was incubated in the presence of 4.2  $\mu\text{g}/\text{ml}$  of actin-binding proteins (actin-binding protein/actin molar ratio 1:400) and various concentrations of free  $\text{Ca}^{++}$ . Indicated free calcium concentrations were obtained using  $\text{CaCl}_2$ -EGTA buffers. The incubation buffer was composed of 40 mM Pipes (pH 6.8), 20 mM KCl, 1 mM  $\text{MgCl}_2$ , 5 mM EGTA, and various EGTA/ $\text{CaCl}_2$  ratios. pCa was calculated using the binding constant for Ca-EGTA at pH 6.8 of  $4.4 \times 10^5$  (24). Apparent viscosity (cP) was measured with the falling ball viscometer. The presence of actin-binding proteins did not modify the apparent viscosity of F-actin solution at a free calcium concentration of  $10^{-8}$  M.



**Figure 4.** Electron micrographs of negatively stained actin filament preparations after addition of actin-binding proteins (actin-binding protein/actin molar ratio 1:20) in the presence or absence of calcium. (*A*) At free calcium concentration  $\sim 10^{-8}$  M. Note the exclusive presence of long filaments forming an interconnecting network typical of actin gels. This appearance is identical to polymerized actin alone. Bar, 0.1  $\mu\text{m}$ . (*B*) At free calcium concentration  $\sim 10^{-5}$  M. Note the dramatic difference compared with *A*; only short filaments are present. Bar, 0.1  $\mu\text{m}$ . (*C*) Distribution of filament length. At free calcium concentration  $\sim 10^{-5}$  M (sample shown in *B*), the lengths of 115 filaments were measured taken from 20 randomly selected fields.

viscosity when the free calcium concentration was raised from  $10^{-8}$  M to  $10^{-5}$  M. For an actin concentration of 0.8 mg/ml, 4.2  $\mu$ g/ml actin-binding proteins (actin binding protein/actin molar ratio 1:400) produced 72% reduction of viscosity in presence of  $2.5 \times 10^{-7}$  M free calcium.

As judged from SDS polyacrylamide gel electrophoresis, the skeletal muscle actin used in viscosity experiments was pure. Therefore, the calcium-dependent effect of actin-binding proteins was mainly due to a direct effect on actin filaments rather than on a possible cross-linking protein present in actin preparation. The decrease of viscosity could result from a reduction of either the total number of actin filaments or the average length of individual filaments. Electron microscopy on negatively stained preparations was used to study the effect of actin-binding proteins on preformed actin filaments in the presence and absence of calcium. Actin alone, in the presence of  $\text{CaCl}_2$  or EGTA, polymerized into extremely long filaments; such filaments proved too long to permit them to be followed (and thus accurately measured) for their entire length. It was, however, estimated that their minimum length was not less than 5–10  $\mu$ m. In the presence of EGTA, the addition of actin-binding proteins (actin binding protein/actin molar ratio 1:20) did not change the assembly of the long actin filaments (Fig. 4A). Short filaments were never observed in these preparations. In contrast, only short filaments were visible in samples containing actin-binding proteins when the free calcium concentration was  $10^{-5}$  M (Fig. 4B). There was found to be a large variation in filament length, but the average length was dramatically reduced ( $0.26 \mu\text{m} \pm 0.17 \mu\text{m}$ , Fig. 4C) by comparison with the control. Thus, the solation of actin gels observed in the presence of actin-binding proteins appears to be due to a fragmentation process and not to depolymerization or bundling of filaments.

### Presence of a Gelsolin-like Protein

Actin-binding proteins isolated from chromaffin cell cytosol interact with actin in a calcium-dependent manner. Similar effects have been observed with gelsolin, a 91-kD actin-binding protein originally purified from lung macrophages (61). In the presence of micromolar calcium concentrations, gelsolin shortens actin filaments and decreases the viscosity of actin. These properties led us to look for the presence of a gelsolin-like protein in the chromaffin cell actin-binding proteins.

Gelsolin isolated from rabbit lung macrophages migrated as a single polypeptide with an apparent molecular weight of 91 kD by one-dimensional gel electrophoresis (Fig. 5C, lane 1). By two-dimensional gel electrophoresis, the protein was resolved into several well-defined spots with  $\text{pH}_i$  ranging from 6.15 to 6.40 (Fig. 5B). The actin-binding proteins from bovine adrenal medulla were also analyzed by two-dimensional electrophoresis as shown on Fig. 5A. The 85-kD protein resolved into several spots with different  $\text{pH}_i$ , from 6.60 to 6.40. Surprisingly, the 91-kD protein was separated into two different components with close molecular weights, each of them showing multiple spotting. The arrows indicate the component that had the same migration as macrophage gelsolin. The second component migrated as more basic spots with  $\text{pH}_i$  ranging from 6.65 to 6.45. Thus, two different polypeptides of similar molecular weight but different isoelectric point co-migrated as a unique component on one-dimensional polyacrylamide gels (see Fig. 1, lane B). It is possible to resolve

on one-dimensional polyacrylamide gel proteins of close molecular weight but differing in  $\text{pH}_i$  by changing the  $\text{pH}$  of the electrophoresis buffer. Fig. 5C shows the migration of purified macrophage gelsolin (lane 1), EGTA-eluted proteins (lane 2), and guanidine-HCl eluted proteins (lane 3) when electrophoresis was carried out using the Tris-glycine ( $\text{pH}$  8.3) buffer instead of the Tris-acetate ( $\text{pH}$  7.4) buffer. Under these conditions, two polypeptides of molecular weight 91 and 93 kD were separated. They were found with the 85-kD protein and some actin (42 kD) in the EGTA eluate fraction (see also lane B, Fig. 1). Moreover, 91- and 93-kD proteins were also present in the fraction eluted with guanidine-HCl. Macrophage gelsolin co-migrated with the 91-kD band in the Tris-glycine buffer system. Since the two proteins had very similar isoelectric points on two-dimensional gel electrophoresis, macrophage gelsolin and the 91-kD protein could be related proteins.

To test this hypothesis, mono-dimensional peptide maps of macrophage gelsolin, the 91- and the 93-kD proteins, were compared after limited proteolysis with Staphylococcus V8 protease. Peptide mapping showed very close similarity between the three proteins (Fig. 6). Macrophage gelsolin (Fig. 6, lane A) was digested into two major peptides of molecular weight 65 kD, 35 kD, and a group of peptides of low molecular

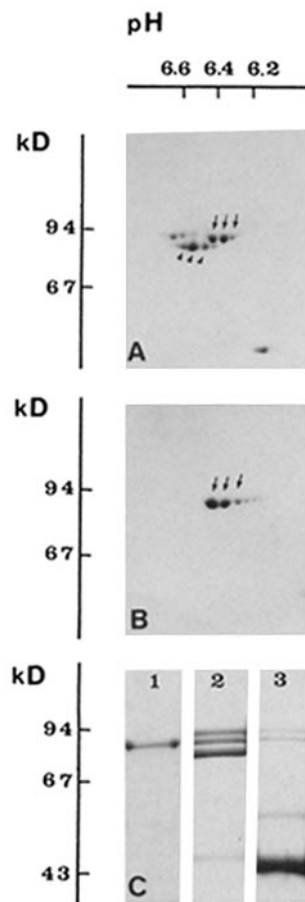
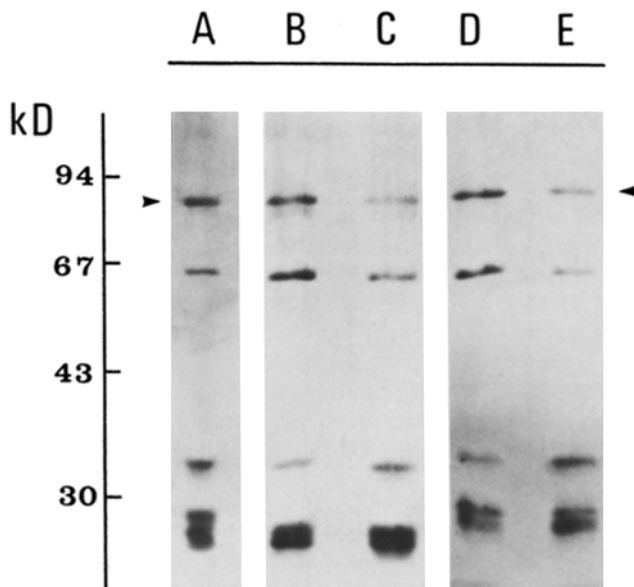
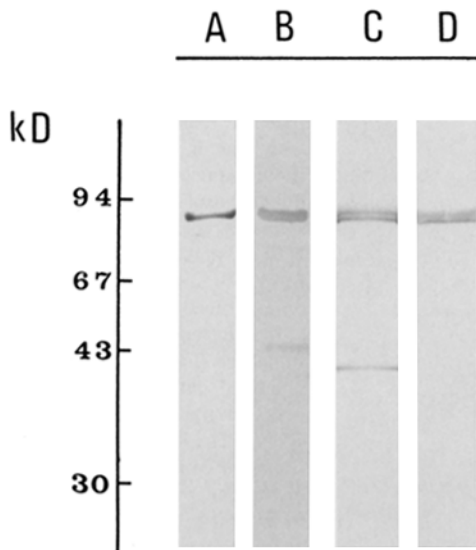


Figure 5. One- and two-dimensional gel electrophoretograms of purified macrophage gelsolin and of adrenal medulla actin-binding proteins. Two-dimensional gel electrophoretogram of bovine adrenal medulla actin-binding proteins is shown in A and macrophage gelsolin in B. The two gels were run in parallel in order to compare the migration of gelsolin with that of the actin-binding proteins present in adrenal medulla cytosol. Macrophage gelsolin was resolved into several defined spots with  $\text{pH}_i$  ranging from 6.40 to 6.15. The two adrenal protein bands (91 and 85 kD) detected on one-dimensional gels (Fig. 1, lane D) were separated into three components in the two-dimensional system. The 85-kD component consisted of several spots (arrowheads) with  $\text{pH}_i$  ranging from 6.60 to 6.40. The 91-kD band on one-dimensional gels was resolved into two components each showing multiple spotting. Arrows indicate spots co-migrating with spots of macrophage gelsolin. In C, one-dimensional gel electrophoretogram of macrophage gelsolin (1), EGTA-eluted proteins (2), and guanidine-HCl-eluted proteins (3) was performed in the Tris-glycine buffer. In this buffer, the 91-kD protein obtained when electrophoresis was carried out in Tris-acetate buffer was resolved into two bands of 93 and 91 kD (lanes 2 and 3). Macrophage gelsolin (lane 1) was found to co-migrate with the 91-kD band when electrophoresis was run in Tris-glycine.



**Figure 6.** One-dimensional peptide map of peptides generated by proteolysis with *Staphylococcus* V8 protease of macrophage gelsolin (A), 91-kD protein (B and C), and 93-kD protein (D and E). Protein band was excised from a gel, cleaved with either 20 ng (A, B, and D) or with 50 ng (C and E) of *Staphylococcus* V8 protease, and analyzed on a 10% polyacrylamide gel. Note after limited proteolysis two major peptides of molecular weight 65 and 35 kD and a group of peptides of molecular weight 25–28 kD are formed. Arrowheads indicate native undigested proteins. The structural similarities show that the 93- and 91-kD proteins are related to macrophage gelsolin.



**Figure 7.** Immunoblots illustrating the presence of gelsolin-like protein as one of chromaffin cell actin-binding proteins. Macrophage gelsolin (A) and pig actin-binding proteins (C) were separated by one-dimensional gel electrophoresis using Tris-glycine buffer. The proteins were transferred to nitrocellulose sheets and the blots were subsequently incubated with anti-gelsolin antibody at a 1:800 dilution. Lane B shows the reactivity of the antibody with macrophage gelsolin. The same antibody cross-reacted with the 91- and 93-kD proteins present in pig actin-binding protein (lane D). The 85-kD protein that was detected in some pig actin-binding protein preparations was not recognized by the anti-gelsolin antibody. Thus, actin-binding proteins purified from adrenal medulla contain two gelsolin-like proteins of 91 and 93 kD.

weight 25–28 kD. The major peptide fragments of the 91- (Fig. 6, lanes B and C) and 93-kD proteins (Fig. 6, lanes D and E) were identical, with apparent molecular weights of 65, 35, and 28 kD. These results suggest that macrophage gelsolin and the chromaffin cell actin-binding proteins of molecular weight 91 and 93 kD are related proteins.

Finally, anti-macrophage gelsolin antibody was used to test the immunological homology that might exist between the three proteins. Since anti-gelsolin antibody did not recognize bovine samples, actin-binding proteins were prepared from pig adrenal medulla by chromatography on DNase-I-Sepharose. The electrophoretic pattern of proteins obtained when the DNase-I-Sepharose column was eluted with EGTA-containing buffer is shown in Fig. 7 (lane C). The electrophoresis was performed in Tris-glycine buffer to clearly separate the 91-kD from the 93-kD protein (Fig. 7, lane C). In contrast with bovine adrenal protein, the amount of the 85-kD protein band varied in an unpredictable way from one preparation to the other when pig adrenal medulla was used as starting material. In some experiments, the 85-kD band was almost absent as shown in Fig. 7, lane C.

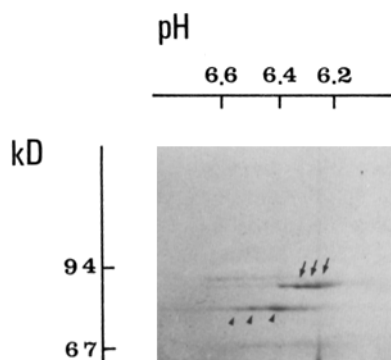
Using the immunoblotting technique, anti-gelsolin antibody cross-reacted with pig adrenal medulla 91- and 93-kD proteins (Fig. 7, lane D). However, no cross-reaction was detected with the 85-kD protein even when gels were overloaded (data not shown). Therefore, on the basis of similarities in molecular weight and also of immunological cross-reactivity of the 91- and 93-kD polypeptides with anti-macrophage gelsolin antibody, it can be concluded that gelsolin-like proteins are present as the major actin-binding proteins purified by DNase-I-Sepharose affinity chromatography from cytosolic fractions of adrenal medulla.

#### *Presence of a Brevin-like Protein*

A protein with gelsolin-like activity exists in high concentration in plasma (16, 39). This protein, known as brevin (17), reacts immunologically with anti-macrophage gelsolin antibody (48, 62). Since actin-binding proteins were isolated from intact adrenal medullary tissue, the 91- and 93-kD gelsolin-like proteins might have been purified from the plasma present in the tissue rather than the chromaffin cells themselves. Therefore, actin-binding proteins were purified from chromaffin cells isolated on Percoll gradients. From  $3 \times 10^8$  cells, 30 mg of soluble cytosolic proteins could be extracted. Since actin-binding proteins prepared by chromatography represent only 0.5% of the total cytosolic proteins, proteins absorbed to the DNase-I were eluted in only one step with the guanidine-HCl buffer. This fraction contained the bulk of actin together with actin-binding proteins. Fig. 8 shows the two-dimensional electrophoretic profile of the actin-binding proteins. The 91- and 85-kD proteins were present in this fraction, but the 93-kD protein could hardly be detected (compare Fig. 8 with Fig. 5A), indicating that the 91- and 85-kD actin-binding proteins are true components of the chromaffin cell cytosol and that the 93-kD protein originates mainly from a nonchromaffin fraction. Since numerous blood vessels irrigate the adrenal medulla, it is highly probable that the 93-kD actin-binding protein isolated from the gland is a brevin-like protein rather than a gelsolin-like protein.

#### *The 85-kD Actin-binding Protein*

In contrast with the 91- and 93-kD proteins, the 85-kD

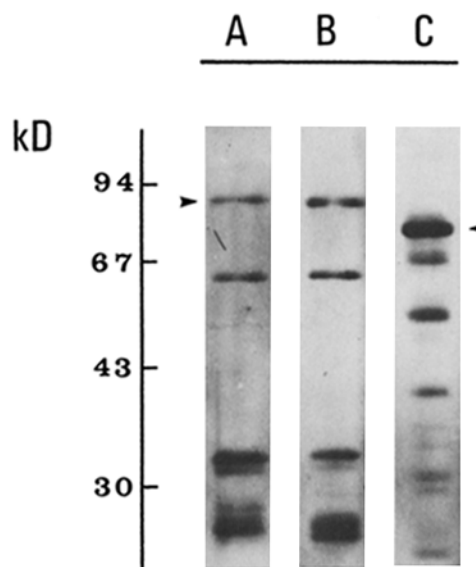


**Figure 8.** Two-dimensional electrophoresis of actin-binding proteins prepared from chromaffin cells purified on Percoll gradients. Only the area where actin-binding proteins were found to migrate is shown. The 85-kD (arrowheads) and 91-kD (arrows) proteins are present on the electrophoretogram indicating that these two actin-binding proteins are components of chromaffin cell cytosol. The 93-kD protein is hardly detectable, suggesting that this protein mainly originates from a nonchromaffin source when intact adrenal medullae are used. Thus, chromaffin cells possess two cytosolic actin-binding proteins, a gelsolin-like protein (91 kD) and an 85-kD protein.

protein, which was present in EGTA eluates obtained by chromatography on DNase-I-Sepharose, was never detected in guanidine-HCl eluates. In other words, the 85-kD protein-actin complexes were completely dissociated by reducing the calcium concentration, whereas some gelsolin-like protein remained bound to actin even in the absence of calcium. This observation accentuates certain functional differences between the 85-kD protein and the 91- and 93-kD gelsolin-like proteins and it renders unlikely the possibility that the 85-kD protein could be a breakdown product of the gelsolin-like protein. Moreover, the ratio between the three actin-binding proteins was never modified when additional protease inhibitors (diisopropylfluorophosphate, pepstatin A, and leupeptin) were included in the buffer used to homogenize bovine adrenal medulla. Nevertheless, the structural relation between the three proteins was investigated by comparing one-dimensional peptide maps generated after limited proteolysis with *Staphylococcus* V8 protease. Fig. 9 shows that while the 93-kD protein (lane A) and the 91-kD (lane B) protein were degraded into similar peptide fragments, the 85-kD protein (lane C) generated a completely different peptide profile. As described above, the two gelsolin-like proteins were cleaved into three major peptides of 65, 35, and 28 kD. The 85-kD protein was cleaved into three major peptides of 73, 55, and 40 kD, and none of them co-migrated with the cleavage products of either the 91- or the 93-kD proteins. This clearcut difference in cleavage products shows that the 85-kD protein is not related to gelsolin-like protein.

#### **Antibodies against Adrenal Medulla Actin-binding Proteins**

Antibodies were prepared against the three actin-binding proteins. Proteins were separated by electrophoresis in the Tris-acetate buffer, and the bands of interest (91 and 85 kD) were cut off the gel and injected into rabbits. The specificity of the antisera was tested by the immunoblotting technique (Fig. 10). The anti-91-kD protein antibody cross-reacted with the 91-kD and the 93-kD proteins separated by electrophoresis in the Tris-glycine buffer (Fig. 10, lanes A and B). This was



**Figure 9.** One-dimensional peptide maps generated after limited proteolysis by *Staphylococcus* V8 protease. Bovine adrenal medullary actin-binding proteins were separated on an SDS polyacrylamide gel. The 93-kD (A), 91-kD (B), and 85-kD bands (C) were cut from the gel, cleaved with 50 ng protease, and analyzed on a 10% acrylamide gel. Arrowheads point to native undigested proteins. Note that the 91- and 93-kD proteins showed similar peptide fragments, while the 85-kD protein generated completely different peptides. This difference in cleavage products strongly suggests that the 85-kD protein is not related to the two other actin-binding proteins.

expected since the 91-kD band used to prepare the antibody contained the two proteins. However, the anti-91-kD antibody did not recognize the 85-kD protein. Similarly the anti-85-kD protein antibody specifically detected the 85-kD band but did not cross-react with the 91 and 93-kD proteins (Fig. 10, lane C).

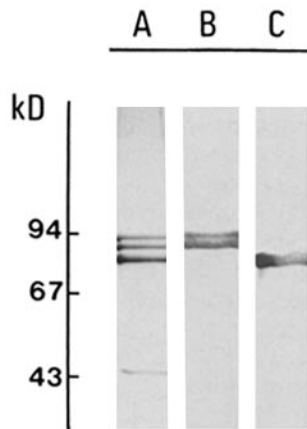
Therefore, it may be concluded that the 85-kD protein is structurally and functionally different from the gelsolin-like proteins found as the major actin-binding proteins of the adrenal medulla. The activity towards actin of the 85-kD protein remains to be characterized.

#### **Discussion**

Soluble  $\text{Ca}^{++}$ -dependent actin-binding proteins are present in the chromaffin secretory cells of adrenal medulla. These proteins were purified by affinity chromatography on DNase-I, using a technique similar to that used for the purification of actin-associated calcium-dependent proteins from platelet extracts (56). The technique yields three major soluble proteins that were eluted from the column in low calcium-containing buffers. These proteins have molecular weights of 93, 91, and 85 kD and almost similar  $\text{pH}_i$ . Addition of these proteins to muscle F-actin had different effects depending on the free calcium concentration. Micromolar free calcium concentrations provoked a dramatic decrease in apparent viscosity of F-actin solutions. Electron microscopic studies revealed a shortening of actin filaments, showing that adrenal medullary actin-binding proteins are calcium-dependent actin-fragmenting proteins.

This activity suggested the presence of gelsolin-like proteins. Gelsolin is a calcium-binding protein originally extracted





**Figure 10.** Immunoblots illustrating the specificity of the antibodies raised against bovine chromaffin cell actin-binding proteins. Actin-binding proteins obtained by DNase-I-Sepharose chromatography were separated by electrophoresis in Tris-acetate buffer. The 91- and 85-kD bands were excised from the gel, homogenized, and injected into rabbits. The specificity of each antibody was tested by immunoblotting. Actin-binding proteins separated by elec-

trophoresis in Tris-glycine buffer in order to resolve the 93- and 91-kD proteins (lane A), were subsequently transferred to nitrocellulose sheets. The blots were incubated with anti-85-kD antibodies (lane C) or anti-91-kD antibodies (lane B) at a 1:1,000 dilution. The anti-85-kD antibody specifically cross-reacted with the 85-kD band and did not cross-react with 91- or 93-kD proteins or with actin (42 kD). Similarly, the anti-91-kD antibody specifically recognized the 91- and 93-kD proteins and showed no cross-reactivity with the 85-kD protein or with actin.

from lung macrophages (60) that confers calcium sensitivity on the regulation of actin networks. In the presence of calcium at micromolar concentration, gelsolin shortens F-actin filaments leading to a decrease in the viscosity of F-actin (61). Calcium-activated gelsolin is also able to bind G-actin and to form a complex that can nucleate actin polymerization. Therefore, the number of individual filaments increases and the average length of each filament decreases when gelsolin- $\text{Ca}^{2+}$  is added to G-actin before polymerization (63). The net result of such an action is the dramatic decrease of viscosity of F-actin solutions. In the present study, the 91-kD actin-binding protein is shown to be structurally and immunologically related to macrophage gelsolin. The identity between the two proteins was deduced from the following experiments: (a) macrophage gelsolin and the 91-kD protein co-migrated on one- and two-dimensional gel electrophoretograms with an apparent molecular weight of 91 kD and a pI, ranging from 6.15 to 6.40; (b) peptide maps of macrophage gelsolin and the 91-kD chromaffin cell protein generated after limited proteolysis by *Staphylococcus* V8 protease were similar; and (c) antibodies raised against macrophage gelsolin cross-reacted with the chromaffin cell 91-kD protein. Furthermore, the two proteins shorten actin filaments, though it is not yet known whether the chromaffin cell 91-kD protein is able to enhance G-actin nucleation, thus affecting the rate of actin assembly. A protein of 90 kD has been purified from bovine adrenal medulla (15) that is able to increase the initial rate of actin assembly. This protein promotes the nucleation of actin, while it inhibits gelation of F-actin *in vitro*. It seems probable that this protein is similar to the 91-kD protein described here; therefore, the adrenal medullary gelsolin-like protein could also affect actin nucleation.

Recently, using antibodies against macrophage gelsolin, Yin et al. detected gelsolin in a wide variety of cells, and they suggested that the calcium-dependent mechanism for the regulation of actin cytoskeletal structures first characterized in macrophages may be applicable to other cells (62). Several

groups have found that platelets contain a gelsolin-like 91-kD protein that modulates actin assembly (27, 36, 40). Chromaffin cells are neurosecretory cells derived from the neural crest, and they are structurally, functionally, and embryologically related to neurones (14). The present data showing that gelsolin is present in chromaffin cells is the first evidence for the localization of gelsolin in cells of neuronal origin. A 90-kD protein has been purified from brain and spinal cord extracts by DNase-I chromatography (23, 42), and this protein might be a gelsolin-like protein although this remains to be shown. However, whole brain was used as starting material, and it is difficult to discriminate between neuronal and nonneuronal proteins. The observation that the 91-kD gelsolin-like protein can be purified from chromaffin cells isolated on Percoll gradients clearly indicates that gelsolin is a true component of secretory cells and therefore of paraneuronal cells.

In addition to the 91-kD gelsolin-like protein, a second actin-binding protein was purified from bovine adrenal medulla with a molecular weight of 93 kD. Experimental data support the idea that the 93-kD protein could be the plasma protein brevin. Brevin and gelsolin are two related proteins since brevin is immunologically reactive with polyclonal antibodies against macrophage gelsolin (48, 62). In a recent report (64), Yin et al. showed that hepatocyte cytoplasmic gelsolin and plasma brevin are structurally similar but not identical, since brevin differs from gelsolin by the presence of 25 additional amino acids at its N-terminal which explains its higher apparent molecular weight and the difference in pI. In the presence of calcium, brevin promotes G-actin nucleation and shortens F-actin filaments into fragments (17, 48). Thus, brevin is also functionally similar to gelsolin. The isoelectric point of the 93-kD actin-binding protein from adrenal medulla was slightly more basic than that of macrophage gelsolin. The 93-kD protein also reacted with antibodies against macrophage gelsolin. In addition, gelsolin and the 93-kD protein generated identical peptide maps after mild proteolysis with *Staphylococcus* V8 protease, which is in agreement with the peptide maps of brevin and gelsolin (64). All these observations strongly suggest that the 93-kD protein described here is brevin, derived from contaminating plasma and not from chromaffin cells.

Recently, Bryan and Kurth described the presence of two actin-binding sites on each gelsolin molecule (8). They observed that in the presence of calcium, gelsolin forms an EGTA-stable binary complex with one molecule of actin. One molecule of calcium is trapped within the binary complex and cannot be removed by EGTA. A ternary complex can further be generated from monomeric actin and the binary complex, and this interaction is reversible and depends on calcium concentration (8). Similar calcium-independent complexes have also been described between actin and plasma brevin (18). The 91- and 93-kD proteins were always detected in the fraction eluted from DNase-I-Sepharose with guanidine-HCl, and it has never been possible to completely remove these proteins with EGTA-containing buffers. Actin was also detected in the EGTA eluates. The presence of such calcium-induced binary and ternary complexes in the adrenal cytosolic fraction can explain these results. Washing the column with the EGTA-containing buffer released an actin-gelsolin complex from the ternary complexes, since the binding of the second actin is reversible in the absence of calcium, while the EGTA-stable binary complexes remained bound to the col-

umn and could only be eluted with guanidine. In their elegant study, Kurth and Bryan demonstrated that platelet activation triggers the formation of binary complexes (28). Inactivated platelets primarily contain free gelsolin, while gelsolin becomes tightly complexed to actin in activated platelets. The transient increase in cytoplasmic calcium levels during platelet activation could be the signal for triggering the formation of gelsolin-actin complexes. The question of whether stimulation of chromaffin cells provokes the formation of gelsolin-actin complexes is currently under investigation.

In addition to gelsolin-like proteins, the chromaffin cell cytosol contains a third calcium-dependent, actin-binding protein of 85 kD. This polypeptide is not a proteolytic breakdown product of the 91- or the 93-kD proteins because: (a) unlike gelsolin and brevin, the 85-kD protein was never detected in the guanidine-eluted fraction, suggesting that the actin-85-kD interaction is completely reversible when the calcium concentration is decreased; (b) peptide maps of gelsolin and 85-kD protein show clear differences in cleavage products; (c) antibodies raised against chromaffin cell gelsolin do not cross-react with the 85-kD protein; and (d) antibodies raised against this 85-kD protein do not recognize gelsolin or brevin from the adrenal medulla. Thus, the 85-kD actin-binding protein is structurally different from gelsolin and brevin. A complete purification must be achieved to determine the role of this 85-kD protein on the regulation of actin. It is of interest to note that an 85-kD calcium-sensitive gelation factor that co-purified with a gelsolin-like 90-kD protein has been described in *Acanthamoeba* extracts (43). The gelation activity of this 85-kD protein could only be detected once complete purification was achieved. An 85-kD actin-binding protein was also purified from platelets (31) and also from brain extracts, though the brain 85-kD protein was isolated with a presumed gelsolin-like protein (42, 55). The presence of an 85-kD protein in cells and tissues in which gelsolin is also present could indicate a functional relationship between these two proteins regarding the regulation of actin filaments during cell activity.

The many different proteins that modify actin polymerization and actin filament structures can be divided into three functional classes (12): (a) actin gelation proteins which cross-link actin filaments into networks and therefore increase the apparent viscosity of F-actin; (b) actin filament-length regulators which sever preformed actin filaments and nucleate actin polymerization: they lower the viscosity of F-actin solutions by reducing the length of filaments; and (c) actin-polymerizing inhibitors which bind to monomeric actin in such a way that the complex cannot serve as a nucleus for addition of free monomers and polymerization. It is noteworthy that proteins of two different groups are often described to be present together in the cytoplasm of the same cell. For instance, platelets possess an actin filament-length regulator, gelsolin, and an actin-gelation protein, alpha-actinin (45). The cytoplasm of macrophages contains gelsolin and an actin-crosslinking protein (19). An actin-fragmenting protein, severin, and a gelation protein have been isolated from *Dictyostelium discoideum* (7, 11). It is likely that the two types of protein coordinate their activities, thus giving to the cell a double control of the cytoplasmic structure. Since fodrin, a calcium-dependent actin cross-linking protein, has been detected in chromaffin cells (41), a protein acting as its counterpart was expected to be present in the cytoplasm. What may

be the physiological significance of calcium-dependent actin-regulating proteins in neurosecretory cells? Biochemical evidence suggests a close relationship between actin filaments and neurosecretory granules (4, 6, 13, 57). Moreover, using stereotransmission electron microscopy, Kondo et al. observed the three-dimensional lattice of microfilaments contiguous to the surface of secretory granules and to the plasma membrane in chromaffin cells (26). They suggested that these filaments are implicated in granule movement as well as in secretion of stored material from granules. In nerve cells, Hirokawa described the presence of cross-connections between neurofilaments, microtubules, microfilaments, and membranous organelles in the axon (22). Thus, the following model can be reasonably proposed. In resting cells, the calcium concentration is close to  $10^{-8}$  M and granules may be trapped in a filamentous network. Fodrin, mainly localized in the subplasmalemmal region of the cell (41), cross-links actin filaments and forms a physical barrier preventing granules from closely approaching the plasma membrane. On stimulation, the intracellular calcium level increases, and fodrin becomes inactive. Gelsolin, in the presence of calcium, is activated and shortens actin filaments, thereby inducing solation of the cytoplasm and permitting granules to move and undergo exocytosis. Perrin and Aunis described fodrin movements in stimulated chromaffin cells (41) giving the appearance of free spaces in the subplasmalemmal actin network, which appear to be necessary for granule movement and perhaps for the fusion of secretory granules with the plasma membrane.

In conclusion, there is circumstantial evidence for a role of calcium-dependent actin-binding proteins in the secretory process. The next step will be to determine how these proteins regulate actin structures in living cells and how movements of intracellular organelles are controlled. Microinjection of specific antibodies into functional chromaffin cells (25) will give further clues on the role of these proteins in the process of stimulus-secretion coupling.

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## References

1. Almazan, G., D. Aunis, A. G. Garcia, C. Montiel, G. P. Nicolas, and P. Sanchez-Garcia. 1984. Effects of collagenase on the release of [ $^3$ H]-noradrenaline from bovine cultured adrenal chromaffin cells. *Brit. J. Pharmacol.* 81:599-610.
2. Amy, C., and N. Kirshner. 1982. Na<sup>+</sup>-uptake and catecholamine secretion by primary cultures of adrenal medulla cells. *J. Neurochem.* 39:132-142.
3. Aunis, D., B. Guérold, M. F. Bader, and J. Ciesielski-Treska. 1980. Immunocytochemical and biochemical demonstration of contractile proteins in chromaffin cells in culture. *Neuroscience.* 5:2261-2277.
4. Aunis, D., and D. Perrin. 1984. Chromaffin granule membrane-F-actin interactions and spectrin-like protein of subcellular organelles: a possible relationship. *J. Neurochem.* 42:1558-1569.
5. Bader, M. F., J. Ciesielski-Treska, D. Thiersé, J. E. Hesketh, and D. Aunis. 1981. Immunocytochemical study of microtubules in chromaffin cells in culture and evidence that tubulin is not an integral protein of the chromaffin granule membrane. *J. Neurochem.* 37:917-933.
6. Bader, M. F., and D. Aunis. 1983. The 97 kD-alpha-actinin-like protein in chromaffin granule membranes from adrenal medulla: evidence for localiza-

- tion on the cytoplasmic surface and for binding to actin filaments. *Neuroscience*. 8:165-181.
7. Brown, S. S., K. Yamamoto, and J. A. Spudich. 1982. A 40,000-dalton protein from *Dictyostelium discoideum* affects assembly properties of actin in a calcium-dependent manner. *J. Cell Biol.* 93:205-210.
  8. Bryan, J., and M. C. Kurth. 1984. Actin-gelsolin interactions. Evidence for two actin-binding sites. *J. Biol. Chem.* 259:7480-7487.
  9. Burrige, K., and J. H. Phillips. 1975. Association of actin and myosin in the secretory granule membranes. *Nature (Lond.)*. 254:526-529.
  10. Cleveland, D. W., S. G. Fischer, M. W. Kirshner, and U. K. Laemmli. 1977. Peptide mapping by limited proteolysis in sodium dodecyl sulfate and analysis by gel electrophoresis. *J. Biol. Chem.* 252:1102-1106.
  11. Condeelis, J., S. Geosits, and M. Vahey. 1982. Isolation of a new actin-binding protein from *Dictyostelium discoideum*. *Cell Motil.* 2:273-285.
  12. Craig, S. W., and T. D. Pollard. 1982. Actin-binding proteins. *Trends Biochem. Sci.* 7:88-92.
  13. Fowler, V. M., and H. B. Pollard. 1982. Chromaffin granule membrane-F-actin interactions are calcium sensitive. *Nature (Lond.)*. 295:336-339.
  14. Fujita, T. 1977. Concept of paraneurones. *Archiv. Histol. Jpn.* 40:1-12.
  15. Grumet, M., and S. Lin. 1981. Purification and characterization of an inhibitor protein with cytochalasin-like activity from bovine adrenal medulla. *Biochim. Biophys. Acta.* 678:381-387.
  16. Harris, H. E., J. R. Bamberg, and A. G. Weeds. 1980. Actin filament disassembly in blood plasma. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 121:175-177.
  17. Harris, H. E., and J. H. Schwartz. 1981. Characterization of brevin, a serum protein that shortens actin filaments. *Proc. Natl. Acad. Sci. USA.* 79:6798-6802.
  18. Harris, H. E., and A. G. Weeds. 1983. Plasma actin depolymerizing factor has both calcium-dependent and calcium-independent effects on actin. *Biochemistry*. 22:2728-2741.
  19. Hartwig, J. H., and T. P. Stossel. 1975. Isolation and properties of actin, myosin and a new actin-binding protein in rabbit alveolar macrophages. *J. Biol. Chem.* 250:5699-5705.
  20. Hesketh, J. E., D. Aunis, P. Mandel, and G. Devilliers. 1978. Biochemical and morphological studies of bovine adrenal medullary myosin. *Biol. Cell.* 33:109-208.
  21. Hesketh, J. E., J. Ciesielski-Treska, and D. Aunis. 1981. A phase contrast and immunofluorescence study of adrenal medullary chromaffin cells in culture: neurite formation, actin and chromaffin granule distribution. *Cell Tissue Res.* 218:331-343.
  22. Hirokawa, N. 1982. Cross-linker system between neurofilaments, microtubules and membranous organelles in frog axons revealed by the quick-freeze, deep-etching method. *J. Cell Biol.* 94:129-142.
  23. Isenberg, G., R. Ohnheiser, and H. Maruta. 1983. Cap 90, a 90-kDa calcium-dependent F-actin-capping protein from vertebrate brain. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 163:225-229.
  24. Katz, A. M., D. I. Repke, J. E. Upshaw, and M. A. Polascik. 1970. Characterization of dog cardiac microsomes. Use of zonal centrifugation to fractionate fragmented sarcoplasmic reticulum, (Na<sup>+</sup>, K<sup>+</sup>)-activated ATPase and mitochondrial fragments. *Biochim. Biophys. Acta.* 205:473-490.
  25. Kenigsberg, R. L., and J. M. Trifaro. 1985. Microinjection of calmodulin antibodies into cultured chromaffin cells blocks catecholamine release in response to stimulation. *Neuroscience*. 14:335-347.
  26. Kondo, H., J. Woloszewicz, and G. D. Pappas. 1982. The microtrabecular lattice of the adrenal medulla revealed by poly ethylene glycol embedding and stereo electron microscopy. *J. Neurosci.* 2:57-65.
  27. Kurth, M. C., L. L. Wang, J. Dingus, and J. Bryan. 1983. Purification and characterization of a gelsolin-actin complex from human platelet. *J. Biol. Chem.* 258:10895-10903.
  28. Kurth, M. C., and J. Bryan. 1984. Platelet activation induces the formation of a stable gelsolin-actin complex from monomeric gelsolin. *J. Biol. Chem.* 259:7473-7479.
  29. Lee, R. W. H., W. E. Mushynski, and J. M. Trifaro. 1979. Two forms of cytosolic actin in adrenal chromaffin cells. *Neuroscience*. 4:843-852.
  30. Lee, R. W. H., and J. M. Trifaro. 1981. Characterization of anti-actin antibodies and their use in immunocytochemical studies on the localization of actin in adrenal chromaffin cells in culture. *Neuroscience*. 10:2087-2108.
  31. Lin, S., D. H. Cribbs, J. A. Wilkins, J. F. Casella, W. W. Magargal, and D. C. Lin. 1982. The capactins, a class of proteins that cap the ends of actin filaments. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* 299:263-273.
  32. Lin, S., J. A. Wilkins, D. H. Cribbs, M. Grumet, and D. C. Lin. 1982. Proteins and complexes that affect actin filament assembly and interactions. *Cold Spring Harbor Symp. Quant. Biol.* 46:625-631.
  33. Livett, B. G. 1984. The secretory process in adrenal medullary cells. In *Cell Biology of the Secretory Process*. M. Cantin, editor. Karger, Basel. 310-358.
  34. Livett, B. G., P. Boksa, D. M. Dean, F. Mizobe, and M. M. Lindenbaum. 1983. Use isolated chromaffin cells to study basic release mechanisms. *J. Auton. Nerv. Syst.* 7:59-86.
  35. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* 193:265-275.
  36. Markey, F., T. Persson, and U. Lindberg. 1982. A 90,000 dalton actin-binding protein from platelets. Comparison with villin and plasma brevin. *Biochim. Biophys. Acta.* 709:122-133.
  37. Marty, A. 1981. Calcium-dependent K<sup>+</sup> channels with large unitary conductance in chromaffin cell membranes. *Nature (Lond.)*. 291:497-500.
  38. Morrissey, J. H. 1981. Silver stain for proteins in polyacrylamide gels: a modified procedure with enhanced uniform sensitivity. *Anal. Biochem.* 117:307-310.
  39. Norberg, R., R. Thorstensson, G. Utter, and A. Fragaues. 1979. F-actin depolymerizing activity of human serum. *Eur. J. Biochem.* 100:575-583.
  40. Olomucki, A., C. Huc, F. Lefebvre, M. Coué. 1984. Isolation and characterization of human blood platelet gelsolin. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 174:80-85.
  41. Perrin, D., and D. Aunis. 1985. Stimulation induces alpha-fodrin reorganization in secretory cells. *Nature (Lond.)*. 315:589-592.
  42. Petrucci, T. C., C. Thomas, and D. Bray. 1983. Isolation of a calcium-dependent actin-fragmenting protein from brain, spinal cord, and cultured neurones. *J. Neurochem.* 40:1507-1516.
  43. Pollard, T. D., J. Levy, G. Isenberg, and U. Aebi. 1980. Two new *Acanthamoeba* proteins involved with regulation of actin filament cross-linking. *J. Cell Biol.* 87 (2, Pt. 2):223a. (Abstr.)
  44. Pollard, T. D. 1982. A falling ball apparatus to measure filament cross-linking. *Methods Cell Biol.* 24:301-311.
  45. Rosenberg, S., A. Stracher, and K. Burrige. 1981. Isolation and characterization of a calcium-sensitive alpha-actinin-like protein from human platelet cytoskeletons. *J. Biol. Chem.* 256:12986-12991.
  46. Spudich, J. A., and S. Watt. 1971. The regulation of rabbit skeletal muscle contraction. I. Biochemical studies of the interaction of the tropomyosin-troponin complex with actin and the proteolytic fragments of myosin. *J. Biol. Chem.* 246:4866-4871.
  47. Taylor, D. L., and M. Fenchheimer. 1982. Cytoplasmic structure and contractility: the solution-contraction coupling hypothesis. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* 299:185-197.
  48. Thorstensson, R., G. Utter, and R. Norberg. 1982. Further characterization of the calcium-dependent F-actin depolymerizing protein of human serum. *Eur. J. Biochem.* 126:11-16.
  49. Towbin, M., T. Staehelin, and J. Gordon. 1979. Electrophoresis transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. USA.* 76:4350-4354.
  50. Trifaro, J. M. 1977. Common mechanisms of hormone secretion. *Annu. Rev. Pharmacol.* 17:27-47.
  51. Trifaro, J. M. 1978. Contractile proteins in tissues originating in the neural crest. *Neuroscience*. 3:1-24.
  52. Trifaro, J. M. 1982. The cultured chromaffin cell: a model for the study of biology and pharmacology of paraneurones. *Trends Pharmacol. Sci.* 3:389-392.
  53. Trifaro, J. M., and R. W. H. Lee. 1980. Morphological characteristics and stimulus-secretion coupling in bovine adrenal chromaffin cells in culture. *Neuroscience*. 5:1533-1546.
  54. Trifaro, J. M., and C. Ulpian. 1976. Isolation and characterization of myosin from the adrenal medulla. *Neuroscience*. 1:483-488.
  55. Verkhovsky, A. B., I. G. Surgucheva, and V. I. Gelfand. 1984. Phalloidin and tropomyosin do not prevent actin filament shortening by the 90 kD protein-actin complex from brain. *Biochem. Biophys. Res. Commun.* 123:596-603.
  56. Wang, L., and J. Bryan. 1981. Isolation of calcium-dependent platelet proteins that interact with actin polymerization. *J. Biol. Chem.* 256:9693-9697.
  57. Wilkins, J. A., and S. Lin. 1981. Association of actin with chromaffin granule membranes and the effect of cytochalasin B on the polarity of actin filament elongation. *Biochim. Biophys. Acta.* 642:55-66.
  58. Winkler, H., and S. W. Carmichael. 1982. The chromaffin granule. In *The Secretory Granule*. A. M. Poisner and J. M. Trifaro, editors. Elsevier Biomedical Press, Amsterdam. 3-79.
  59. Yin, H. L., and T. P. Stossel. 1979. Control of cytoplasmic actin gel-sol transformation, by a calcium dependent regulatory protein. *Nature (Lond.)*. 281:583-585.
  60. Yin, H. L., and T. P. Stossel. 1980. Purification and structural properties of gelsolin, a calcium-activated regulatory protein of macrophages. *J. Biol. Chem.* 255:9490-9493.
  61. Yin, H. L., K. S. Zaner, and T. P. Stossel. 1980. Calcium control of actin gelation. Interaction of gelsolin with actin filaments and regulation of actin gelation. *J. Biol. Chem.* 255:9494-9500.
  62. Yin, H. L., J. H. Albrecht, and A. Fattoum. 1981. Identification of gelsolin, a calcium-dependent regulatory protein of actin gel-sol transformation, and its intracellular distribution in a variety of cells and tissues. *J. Cell Biol.* 91:901-906.
  63. Yin, H. L., J. H. Hartwig, K. Maruyama, and T. P. Stossel. 1981. Calcium control of actin filament length. Effects of macrophage gelsolin on actin polymerization. *J. Biol. Chem.* 256:9693-9697.
  64. Yin, H. L., J. Kwiatkowski, J. E. Mole, and F. S. Cole. 1984. Structure and biosynthesis of cytoplasmic and secreted variants of gelsolin. *J. Biol. Chem.* 259:5271-5276.