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Chromaffin cell scinderin, a novel calcium-dependent actin filament-severing protein

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Scinderin, a novel Ca^{2+}-activated actin filament-severing protein, has been purified to homogeneity from bovine adrenal medulla using a combination of several chromatographic procedures. The protein has an apparent mol. wt of 79 600 ± 450 daltons, three isoforms (pIs 6.0, 6.1 and 6.2) and two Ca^{2+} binding sites (K_d 5.85 × 10^{-7} M, B_{max} 0.81 mol Ca^{2+}/mol protein and K_d 2.85 × 10^{-6} M, B_{max} 1.87 mol Ca^{2+}/mol protein). Scinderin interacts with F-actin in the presence of Ca^{2+} and produces a decrease in the viscosity of actin gels as a result of F-actin filament severing as demonstrated by electron microscopy. Scinderin is a structurally different protein from chromaffin cell gelsolin, another actin filament-severing protein described. Scinderin and gelsolin have different mol. wts, isoelectric points, amino acid composition and yield different peptide maps after limited proteolytic digestion by either *Staphylococcus* V8 protease or chymotrypsin. Moreover, scinderin antibodies do not cross-react with gelsolin and gelsolin antibodies fail to recognize scinderin. Immunofluorescence with anti-scinderin demonstrated that this protein is mainly localized in the subplasmalemma region of the chromaffin cell. Immunoblotting tests with the same antibodies indicated that scinderin is also expressed in brain and anterior as well as posterior pituitary. Presence of scinderin and gelsolin, two Ca^{2+}-dependent actin filament-severing proteins in the same tissue, suggests the possibility of synergistic functions by the two proteins in the control of cellular actin filament networks. Alternatively, the actin filament-severing activity of the two proteins might be under the control of different transduction and modulating influences.

Key words: actin/actin-severing proteins/chromaffin cell/gelsolin/scinderin

Introduction

Chromaffin cell secretion requires the movement of chromaffin vesicles toward the plasma membrane, the fusion of these chromaffin granules with the plasmalemma and extrusion of granular contents to the cell exterior by exocytosis (Viveros, 1975; Trifaró, 1977). It has been postulated that the cytoskeleton and its regulatory proteins may be involved in one or more of the steps leading to the release of granule-packaged material (Trifaró *et al.*, 1982, 1984, 1985a,b, 1988). Actin-binding proteins such as α-actinin and fodrin have been detected in chromaffin granule membranes (Aunis *et al.*, 1980; Trifaró *et al.*, 1982; Aunis and Perrin, 1984). Moreover, a mesh of actin filaments is localized underneath the plasma membrane of chromaffin cells (Lee and Trifaró, 1981; Trifaró *et al.*, 1984, 1988; Cheek and Burgoyne, 1986). It has also been postulated that chromaffin granules, through their actin-binding proteins, contribute to stabilize this actin network (Fowler and Pollard, 1982; Trifaró *et al.*, 1982, 1984). At the same time, the subplasmalemma actin network would oppose the movement of vesicles to releasing sites at the plasma membrane (Trifaró *et al.*, 1982, 1984; Cheek and Burgoyne, 1986, 1987; Sontag *et al.*, 1988). It is also possible that the dynamics of this cell actin network is controlled by Ca^{2+}-dependent proteins such as gelsolin. In our earlier studies on the isolation and characterization of chromaffin cell actin, we observed that another protein was eluted together with actin from DNase I affinity columns (Lee *et al.*, 1979; Trifaró *et al.*, 1985a; Bader *et al.*, 1986). Subsequent work from our laboratory determined that gelsolin was one of the proteins retained by the DNase I affinity columns and eluted by EGTA-containing buffers (Trifaró *et al.*, 1985a; Bader *et al.*, 1986). Our later studies also described the elution by EGTA buffers of another protein of unknown function; this protein was earlier referred to as 85 kd protein (Bader *et al.*, 1986).

The present studies describe the isolation, characterization and functional properties of this protein. We have named this protein scinderin (a name derived from the Latin 'scindere' meaning: 'to cut') because of its actin filament-severing properties. The present studies also clearly demonstrate that gelsolin and scinderin are two distinct and different proteins. Part of these results have been presented elsewhere (Trifaró *et al.*, 1989).

Results

Isolation and purification of scinderin

Chromaffin cell cytosol was treated with ammonium sulphate (65% saturation) and the sediment obtained by centrifugation was dialysed as indicated in Materials and methods. The preparation was sequentially subjected to four chromatographic procedures, DEAE-Sepharose CL-6B, Sephadex G-100, actin-DNase I-Sepharose 4B and DEAE-5PW (Figure 1). The actin-DNase I affinity column step was used on the basis of our previous observations (Bader *et al.*, 1986) on the retention by this column of scinderin together with gelsolin and their subsequent elution by EGTA-containing buffers. Fractions obtained from these columns that tested positive for scinderin in immunoblots were collected, concentrated and applied to the next column (Figure 1). Electrophoretic patterns of these pooled fractions are also shown in Figure 1. This procedure allowed the early separation of as much as 90% of gelsolin (after ammonium

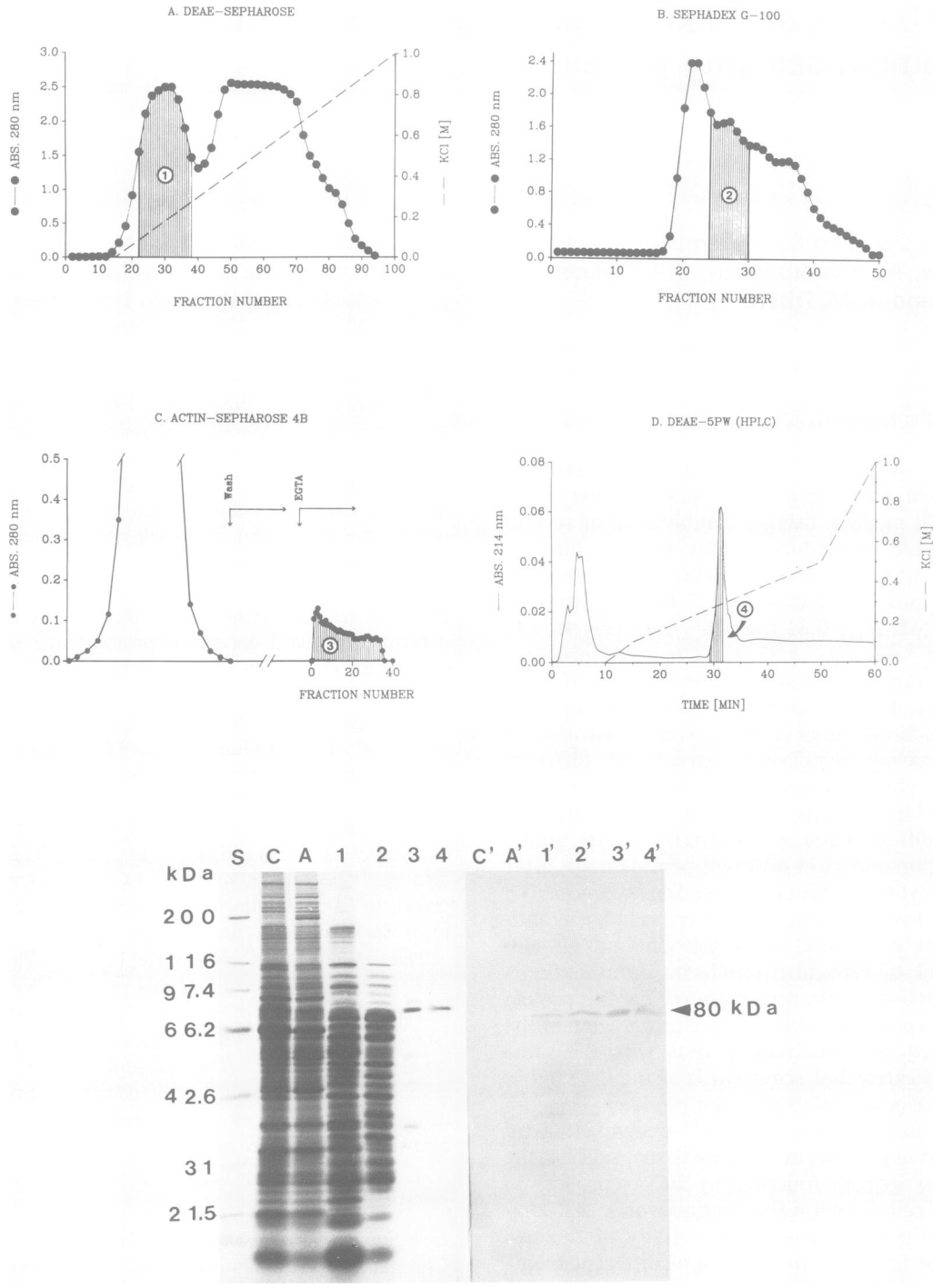

Fig. 1. Purification of scinderin. Adrenal medullae were homogenized, centrifuged and a 65% ammonium sulphate fraction was prepared, dialysed and clarified by centrifugation as described in Materials and methods. The preparation was then subjected to four subsequent chromatographic procedures (**A**–**D**). The elution profile of the first column (DEAE–Sephadex CL6B) is shown in **A**. Fractions 22–38 (shaded area 1) which tested positive for scinderin in immunoblots were pooled, concentrated and filtered through Sephadex G-100 (**B**). Scinderin-positive fractions (24–30; shaded area 2) were combined and applied to an actin–DNase I affinity column (**C**). The fractions collected during elution with EGTA-containing buffer (shaded area 3) were pooled, concentrated and subjected to HPLC (**D**) using a DEAE–5PW column. The shaded area 4 under the peak corresponds to a fraction containing 98–99% pure scinderin. For further details on chromatography, see Materials and methods. At the bottom of the figure, electrophoretic patterns and immunoblots of different fractions are shown. Protein aliquots (150 μg) were separated by electrophoresis (S, C, A, 1–4) and subsequently electroblotted (C′, A′, 1′–4′). The lanes correspond to mol. wt standards (S), cytosol (C), ammonium sulphate sediment (A) and pooled fractions (shaded areas 1–4) from column effluents. Lanes C′, A′, 1′, 2′, 3′ and 4′ are immunoblots of the same fractions. Scinderin was too diluted to be detected by immunoblotting in cytosolic (C′) and ammonium sulfate (A′) fractions.

sulphate precipitation and DEAE–Sepharose chromatography) from scinderin and at the same time provided the evidence for the interaction of scinderin with actin rather than with gelsolin, since the preparation loaded on the actin affinity column was almost devoid of gelsolin; this was not the case in our earlier experiments (Bader *et al.*, 1986). After HPLC on a DEAE–5PW column, the preparation of scinderin obtained was 98% pure and the [^{125}I]protein A

Table I. Purification of adrenal medullary scinderin

Purification step	Total protein (mg)	Scinderin			Recovery (% total activity)	Purification (times)
		Total[a] activity	Protein (mg)	Specific activity		
Cytosol	3440	ND	ND	–	–	–
Ammonium sulphate	686	ND	ND	–	–	–
DEAE–Sepharose CL-6B	290	5136	1.60	17.7	100	1
Sephadex G-100	139	4077	1.27	29.3	79	1.6
Actin–Sepharose 4B	0.94	1926	0.60	2049	37	116
DEAE–5PW	0.41	1316	0.41	3210	26	181

Cytosol was prepared from 25 bovine adrenal medullae.
[a]Scinderin total activity (relative densitometric units) was measured by the [^{125}I]Protein A immunoblotting technique as described in Materials and methods.
ND, scinderin was too diluted to be detected by immunoblotting in cytosolic and ammonium sulphate fractions.

assay indicated that scinderin had been enriched 181 times when compared to the protein preparation eluted from the first column (Table I). The final purification factor is obviously >181 because scinderin was too dilute in cytosol and ammonium sulphate fractions to be detected by immunoblotting followed by the [^{125}I]protein A assay (Table I). Therefore, the ammonium sulphate cut was determined by trial and error and by measuring scinderin activity by immunoblotting only after DEAE–Sepharose chromatography. The property of decreasing actin gel viscosity observed in the cytosol, ammonium sulphate sediments as well as in the eluates from the DEAE–Sepharose column were due, in part, to the presence of gelsolin and consequently, viscosity measurements were not used to monitor the fractions. The apparent mol. wt of scinderin, as determined by SDS–PAGE (Figure 2) and confirmed by immunoblotting was 79 600 ± 450 (n = 11) and this mol. wt was different from that of gelsolin (89 800 ± 480 daltons, n = 4).

Scinderin, a calcium binding protein

Samples of scinderin were run on SDS–PAGE and electrotransferred to nitrocellullose membranes. The membranes containing scinderin were incubated with ^{45}Ca either in the absence or presence of an excess of non-radioactive Ca^{2+}. Figure 3 shows the binding of ^{45}Ca by scinderin and its competition and displacement by non-radioactive Ca^{2+}.

The interaction of scinderin with Ca^{2+} was also studied by equilibrium dialysis. The relationship between Ca^{2+} concentration and its binding to scinderin is shown in Figure 4. From the binding curve, a mean saturation value of 1.8 ± 0.08 (mol Ca^{2+}/mol protein, n = 3) was obtained at Ca^{2+} concentrations of 10^{-4}–10^{-3} M. The insert in Figure 4 shows the Scatchard plot obtained with the binding values. The data indicate the presence in scinderin of two Ca^{2+} binding sites of different affinities, one site with a K_d of 5.85 × 10^{-7} M and a B_{max} of 0.81 mol Ca^{2+}/mol protein and the second site with K_d and B_{max} of 2.85 × 10^{-6} M and 1.87 mol Ca^{2+}/mol protein, respectively.

Effect of scinderin on F-actin gel viscosity and filament length

Mixtures of scinderin and F-actin at different molar ratios were prepared and tested in the absence (presence of 5 mM EGTA) or in the presence of 10^{-5} M free Ca^{2+}. The addition of scinderin did not modify the viscosity of the actin

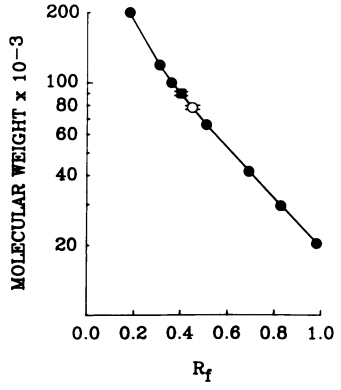

Fig. 2. Apparent mol. wts of scinderin and gelsolin as determined by SDS–PAGE. Plot of the apparent mol. wts of mol. wt standards (●) versus their relative mobilities. The markers used were myosin (200 000), β-galactosidase (116 250), phosphorylase b (97 400), bovine serum albumin (66 200), ovalbumin (42 699), carbonic anhydrase (31 000) and soybean trypsin inhibitor (21 500). The mean ± SE apparent mol. wt values of gelsolin (■) and scinderin (○) calculated from 4 and 11 different electrophoresis runs respectively are indicated in the figure.

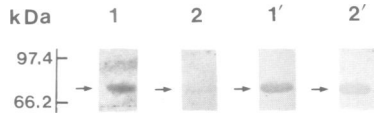

Fig. 3. Overlay test of ^{45}Ca binding by scinderin. Samples (20 μg protein) of purified scinderin were subjected to SDS–PAGE and subsequently transferred to nitrocellulose membranes as described in Materials and methods. The nitrocellulose membranes were incubated with 10^{-6} M ^{45}Ca (μCi/ml) in the absence (**lane 1**) or in the presence (**lane 2**) of 2 × 10^{-3} M $CaCl_2$. After several washes, the nitrocellulose membranes were dried and exposed for autoradiography as indicated in Materials and methods. Autoradiographies shown in **lanes 1** and **2** and **lanes 1′** and **2′** are the immunoblots of the same samples of scinderin performed with scinderin antibody.

solutions when Ca^{2+} was absent. In the presence of Ca^{2+}, a decrease in the viscosity of actin solutions was observed with molar ratios of scinderin to actin >1:3200 (Figure 5A). At the scinderin–actin molar ratio of 1:800, a decrease in viscosity from 480 cP to 10 cP was observed. The effect of Ca^{2+} concentration on scinderin-induced decrease of actin viscosity was also tested at a fixed molar ratio (1:1600). Changes in the viscosity were observed between Ca^{2+} concentrations of 10^{-8} and 10^{-6} M with the maximal fall in viscosity observed at 10^{-6} M Ca^{2+} (Figure 5B). For this

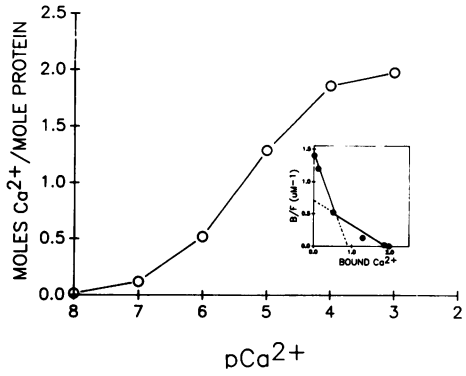

Fig. 4. Ca^{2+} binding to scinderin. Equilibrium dialysis was carried out at free Ca^{2+} concentrations between 10^{-8} and 10^{-3} M as described in Materials and methods. The Ca^{2+} saturation curve and the Scatchard plot (insert) were constructed from data obtained in duplicate from three different preparations of scinderin. Two Ca^{2+} binding sites were observed, a high affinity site with a K_d = 5.85 × 10^{-7} M and B_{max} = 0.81 M Ca^{2+}/mol protein and a low affinity site with a K_d = 2.85 × 10^{-6} M and B_{max} = 1.87 M Ca^{2+}/mol protein.

particular scinderin–actin molar ratio, a 50% reduction in the viscosity of the preparation was observed at 0.4 × 10^{-7} M Ca^{2+}. Since the actin preparation used was pure as judged by SDS–PAGE and because the polymerized actin preparation did not change its viscosity in the presence of Ca^{2+} (Figure 5B), it can be concluded that the changes in viscosity observed were due to scinderin.

The interaction of scinderin with actin was also investigated by electron microscopy. Under conditions suitable for polymerization, actin alone, either in the presence of 10^{-5} M Ca^{2+} or in the absence of Ca^{2+} and in the presence of EGTA and scinderin, formed a network of very long filaments (Figure 6). Short filaments were never observed under these conditions. In contrast, only short filaments were observed when scinderin was added to actin networks in the presence of Ca^{2+} (Figure 6). Under these conditions, it was possible to measure the length of the actin filaments. The filaments observed were shorter than 0.8 μm with >50% of the filaments with lengths of 0.2 μm or shorter (Figure 6). The average filament length observed under these conditions was 0.32 ± 0.04 μm (n = 183). This length corresponds to filaments formed by ~58 actin monomers (Pollard and Cooper, 1986).

Scinderin distribution in cultured chromaffin cells and its expression in other secretory tissues

Immunofluorescence studies with anti-scinderin on cultured chromaffin cells showed a weak and diffuse cytoplasm staining and a strong fluorescence ring pattern at the cortical cytoplasmic region, thus suggesting a preferential subplasmalemma localization for this actin-severing protein (Figure 7A and C). A more diffused cortical cytoplasmic fluorescence pattern was obtained when anti-gelsolin was used (Figure 7D and F). Cortical surface distribution of gelsolin has been previously reported (Yin *et al.*, 1981). Scinderin antibodies were also used to see if scinderin is also expressed in other secretory tissues. Cytosol fractions from bovine brain, anterior and posterior pituitaries were prepared and incubated in the presence of Ca^{2+} with actin–DNase I–Sepharose 4B beads. The beads were extensively washed with the same buffer and then washed one more time with EGTA-containing buffer to elute Ca^{2+}-dependent actin-binding

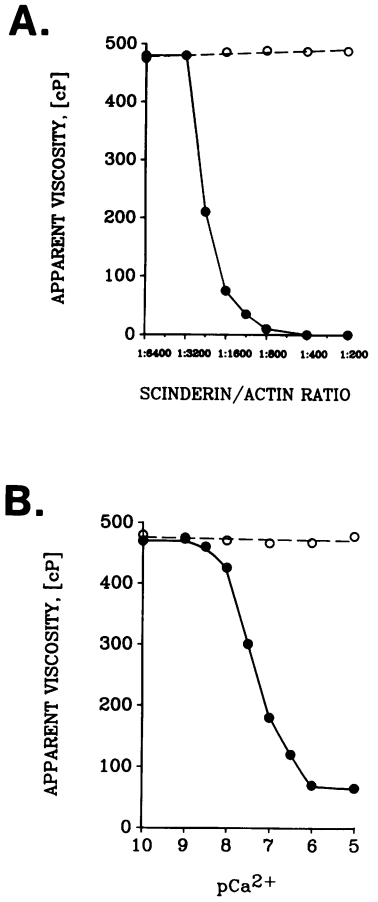

Fig. 5. Effect of scinderin on the apparent viscosity of F-actin. (**A**) Effect of various concentrations of scinderin. G-actin (10 mg/ml) in 2 mM Tris–HCl, pH 8, 0.2 mM ATP, 0.2 mM $CaCl_2$ and 0.005% NaN_3 was polymerized for 2 h at 4°C by addition of 100 mM KCl, 1 mM $MgCl_2$ and 0.8 mM ATP. The F-actin thus obtained was sedimented by centrifuation at 80 000 *g* for 3 h and resuspended in incubation buffer (40 mM PIPES, pH 6.8, 100 mM KCl, 2 mM $MgCl_2$, 2 mM ATP, 5 mM EGTA and 0.05% NaN_3. Apparent viscosity was determined using a falling ball viscometer. F-actin (final concentration 1.91 mg/ml = 44.37 μM) and scinderin (final concentration 17.6–0.56 μg/ml = 0.22–0.007 μM) were mixed together in the presence of either $CaCl_2$–EGTA buffer (10^{-5} M free Ca^{2+}, ●) or 5 mM EGTA (○). The sample (200 μl) was shaken at high speed (Vortex) for 10 s and ~100 μl were drawn into a capillary tube. The tube was sealed with plasticine at one end and incubated in horizontal position for 120 min at 25°C. At the end of the incubation period, the tube was mounted at an angle of 45° and apparent viscosity was measured and calculated as described by MacLean-Fletcher and Pollard (1980). (**B**) Effect of free Ca^{2+} concentration on scinderin activity. F-actin (1.91 mg/ml = 44.37 μM) was incubated in a capillary tube with 2.21 μg scinderin/ml (molar ratio to actin 1:1600) for 120 min at 25°C. The incubation buffer was the same as above containing 5 mM EGTA and different concentrations of $CaCl_2$ to obtain the free Ca^{2+} concentrations indicated in the abscissa. pCa^{2+} values were calculated using the binding constant for Ca-EGTA at pH 6.8 of 2.14 × 10^6 (Caldwell, 1970). Filled circles and solid lines represent incubation of F-actin and scinderin in the presence of 10^{-10}–10^{-5} M free Ca^{2+} and open circles and broken lines represent incubation of F-actin alone in the presence of the same Ca^{2+} concentrations.

proteins. Samples of this last wash were subjected to SDS–PAGE followed by transfer to nitrocellulose membranes. Immunoblots with scinderin antibodies demonstrated the presence of the actin-severing protein in the three secretory tissues tested (Figure 8), thus suggesting that scinderin might be expressed in all secretory tissues.

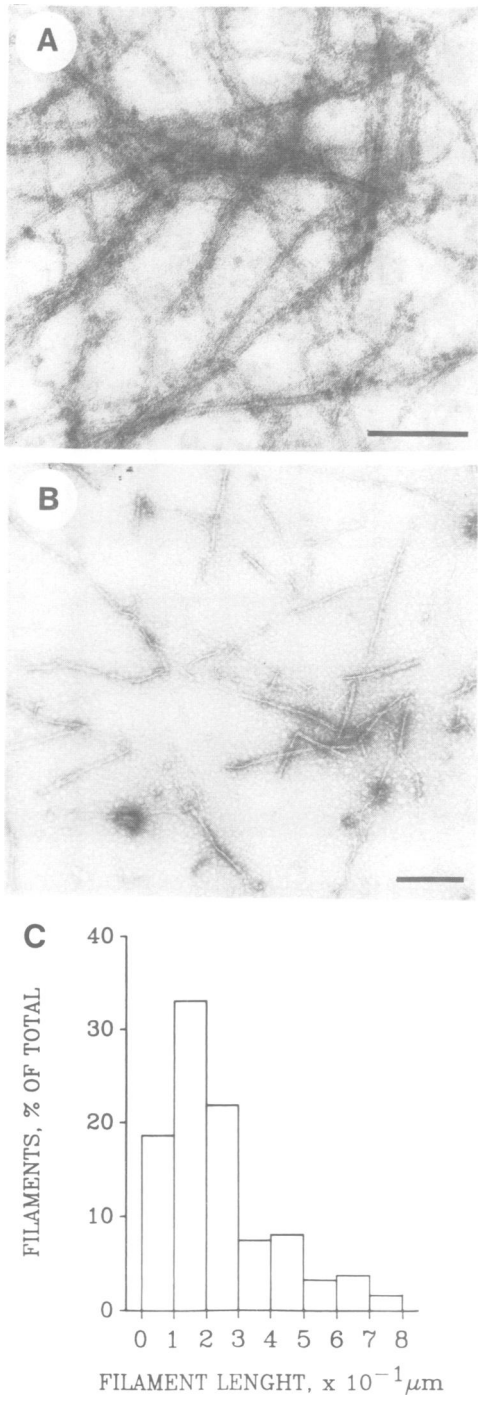

Fig. 6. Electron micrographs of negatively stained mixtures of F-actin filaments and scinderin (scinderin to actin molar ratio of 1:20) in the absence (**A**) or in the presence (**B**) of 10^{-5} M free Ca^{2+}. A mesh of interconnecting filaments can be observed in A. The bar indicates 0.1 μm. In **B**, as a result of the Ca^{2+}-activated effect of scinderin on F-actin, only short filaments are observed. The bar represents 0.1 μm. A histogram with the distribution of the length of 183 short F-actin filaments is shown in **C**.

Comparison between scinderin and gelsolin

A partially purified preparation containing both scinderin and gelsolin was subjected to SDS–PAGE followed by transfer to nitrocellulose membranes. Polyclonal antibodies raised against chromaffin cell gelsolin did not cross-react with scinderin when tested in immunoblots and scinderin polyclonal antibodies did not recognize gelsolin (Figure 9). Not only were the mol. wts (Figure 2) and isoelectric points of gelsolin and scinderin different (Figure 10D), but also the one- and two-dimensional peptide maps obtained after limited proteolytic digestion with either a *Staphylococcus* V8 protease (data not shown) or chymotrypsin showed marked differences in peptide composition (Figure 10A–C). Two-dimensional electrophoresis (Figure 10D) showed the presence of three isoforms of pI 5.8, 5.9 and 6.0 for adrenal medullary gelsolin and three distinct isoforms for scinderin (pI = 6.0, 6.1 and 6.2). The mol. wt of adrenal medullary gelsolin fragments obtained by chymotrypsin digestion were similar to those previously described for human plasma gelsolin (Kwiatkowski *et al.*, 1985). Upon incubation using a protein to enzyme ratio of 400:1, five fragments were obtained from gelsolin (Figure 10A and C). Mol. wts and pI of these fragments were 80 kd (pI 6.0), 50 kd (two isoforms of pI 5.5 and 5.7), 46.5 kd (pI = 5.5), 31 kd (pI = 5.5) and 16 kd (pI = 6.1). Chymotryptic digestion of scinderin under similar experimental conditions gave a quite different peptide pattern (Figure 10B). The polypeptide pattern showed two main proteolytic fragments of mol. wts 40 kd (two isoforms of pI 6.0 and 6.1) and 34 kd (two isoforms of pI 5.7 and 5.9) respectively, and a small 32 kd (pI = 5.8) fragment (Figure 10B and C). The amino acid composition of two different preparations of purified scinderin were also determined. Table II shows these results together with those previously published for gelsolin (Yin and Stossel, 1980; Petrucci *et al.*, 1983). The content of lysine is lower in scinderin compared to that of gelsolin; however, scinderin is a more basic protein than gelsolin (Figure 9D), since the content of acidic residues (aspartic and glutamic) is much greater in the latter protein. Moreover, the content of isoleucine and tyrosine residues is higher in scinderin than gelsolin, thus indicating that scinderin cannot be a breakdown product of gelsolin.

Discussion

Early work from our laboratory demonstrated the presence in chromaffin cells of gelsolin, a Ca^{2+}-activated actin-severing protein (Trifaró *et al.*, 1985a; Bader *et al.*, 1986). In addition to these findings, two other laboratories have described the presence of actin-severing proteins in the adrenal medulla (Grumet and Lin, 1981; Ashino *et al.*, 1987). Although the data from these studies suggested that experiments were performed with proteins which promote nucleation and sever actin, the true identity of these proteins was not determined. Thus, in contrast to our observations (Trifaró *et al.*, 1985a; Bader *et al.*, 1986), no comparisons between macrophage gelsolin and these adrenal medullary actin modulators was made in these studies (Grumet and Lin, 1981; Ashino *et al.*, 1987).

The present studies demonstrate that, in addition to gelsolin, the chromaffin cells contain scinderin, another Ca^{2+}-activated actin filament-severing protein. Scinderin is not a proteolytic breakdown product of gelsolin since (i) the two proteins have different chromatographic behaviour; (ii) scinderin and gelsolin have different apparent mol. wts, isoelectric points and amino acid composition; (iii) equilibrium dialysis studies indicated that scinderin possesses two Ca^{2+} binding sites of different affinity whereas similarly published studies for gelsolin indicated the

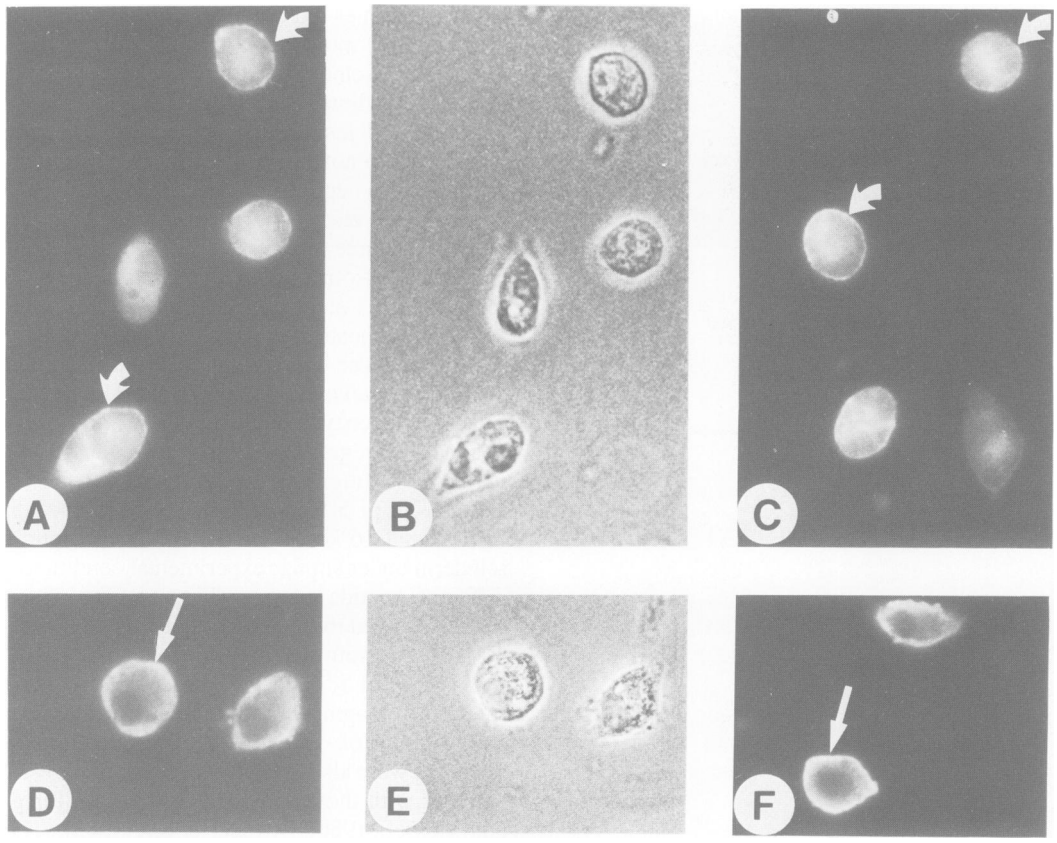

Fig. 7. Chromaffin cells as they appeared after staining by antibodies against scinderin (antiserum 6; **A** and **C**) and gelsolin (**D** and **F**). Chromaffin cells cultured on collagen-coated coverslips were fixed and stained with corresponding antibodies as previously described (Lee and Trifaró, 1981). The preparations were examined by incident light fluorescence (epifluorescence). Strong and well defined cortical cytoplasmic fluorescence is observed with anti-scinderin (**A** and **C**, curved arrows) whereas anti-gelsolin staining shows a strong but more diffused cortical cytoplasmic fluorescence (**D** and **F**, straight arrows). **B** and **E** are phase contrast images of A and D, respectively. Magnifications were ×400.

presence of only one type of Ca^{2+} binding sites on this protein (Yin and Stossel, 1980); (iv) limited proteolytic digestion with either *Staphylococcus* V8 protease or chymotrypsin clearly yielded different peptide maps for these two proteins; (v) the two proteins were immunologically different, since no cross-reactivity was observed with either of the two antibodies. Therefore, the above evidence clearly indicates that gelsolin and scinderin are structurally different proteins. Scinderin, although structurally different from gelsolin, shares all gelsolin functions so far explored. Both are cytosolic proteins which bind actin and sever actin filaments in a Ca^{2+}-dependent manner, producing short F-actin filaments with a consequent solation and decrease in the viscosity of actin solutions. Moreover, the present immunofluorescence studies indicate that in cultured chromaffin cells both scinderin and gelsolin are mainly concentrated in the cortical cytoplasm under the plasma membrane. A similar distribution for gelsolin has been described for other cell types (Yin *et al.*, 1981). This as well as the above observations raised the question of why there are two actin filament-severing proteins present in the same cell. Difference in Ca^{2+} sites and affinities for these two proteins may account for different types of regulation. Furthermore, gelsolin activity is also modulated by products of the phosphatidylinositol pathway (Yin *et al.*, 1988) and it is not known whether scinderin is similarly regulated or whether other transducing mechanisms (e.g. protein kinase C, calmodulin, cyclic AMP, G-proteins, etc.) are also involved in the differential regulation of these two proteins.

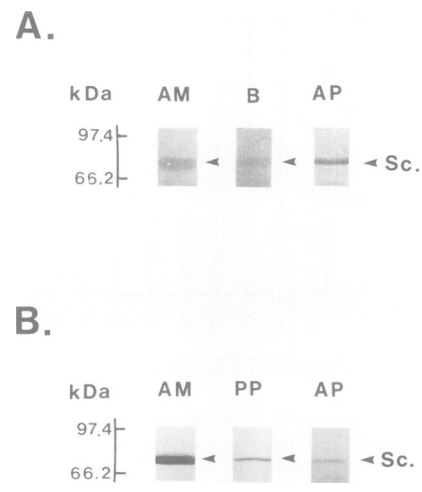

Fig. 8. Expression of scinderin in secretory tissues. Ca^{2+}-dependent actin-binding proteins were purified from bovine adrenal medulla (AM), brain (B), anterior pituitary (AP) and posterior pituitary (PP) using DNase I–actin affinity chromatography as described in Materials and methods. The EGTA eluate was concentrated, transblotted onto nitrocellulose paper and incubated with scinderin antisera. **Panel A** shows results with antiserum 1 (diluted 1:125) and **panel B**—with antiserum 6 (diluted 1:1000). Arrowpoints indicate the position of scinderin (Sc).

In recent years, several types of actin modulators have been isolated and characterized (Stossel *et al.*, 1985; Pollard and Cooper, 1986). According to their main functions, these

proteins can be divided into three groups: (i) actin gelation and cross-linking proteins such as filamin (Wang *et al.*, 1975), α-actinin (Feramisco and Burridge, 1980), macrophage actin-cross-linking protein (Hartwig and Stossel, 1975), *Dictyostelium discoideum* gelation protein (Condeelis *et al.*, 1982) and spectrin or fodrin (Aunis and Perrin, 1984;

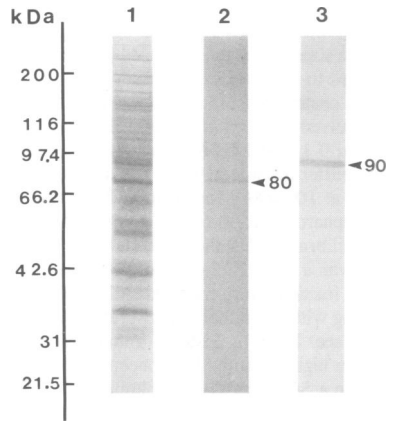

Fig. 9. Specificity of the anti-scinderin and anti-gelsolin polyclonal antibodies. Partially purified preparation of adrenal medullary cytosol (ammonium sulphate sediment) containing both proteins was subjected to SDS−PAGE and subsequently electrotransferred to nitrocellulose membranes. **Lane 1** shows proteins stained with amido black 10B, **lane 2** after incubation with anti-scinderin and **lane 3** after incubation with gelsolin antibody. Antibody dilutions used were 1:125 and 1:1000 for anti-scinderin (antiserum 1) and anti-gelsolin, respectively. The scinderin antibody specifically cross-reacts with a 80 kd band and did not cross-react with gelsolin (90 kd), actin (42 kd) or any other protein present in the preparation. Similarly, gelsolin antibody only recognizes a 90 kd protein and shows no cross-reactivity with scinderin or any other protein present in the ammonium sulphate sediment.

Table II. Comparison between the amino acid composition of scinderin and gelsolin

Amino acid	Scinderin		Gelsolin[a]
	Preparation 1	Preparation 2	
Asp	62	66	85
Glu	87	92	105
Ser	53	51	50
Gly	70	67	69
His	18	17	17
Arg	43	40	41
Thr	42	43	42
Ala	63	60	73
Pro	33	32	49
Val	61	57	58
Met	11	9	17
Ile	41	38	29
Leu	66	65	73
Phe	34	34	34
Lys	33	34	51
Tyr	32	31	24

[a]Data from Yin and Stossel (1980) as modified by Petrucci *et al.* (1983). The data for gelsolin are expressed as number of residues per molecule of protein of mol. wt of 90 000. The mol. wts of preparations 1 and 2 were 81 216 and 80 029, respectively. They were calculated according to the composition of the preparations, assuming that their content in Phe was the same as that found in gelsolin.

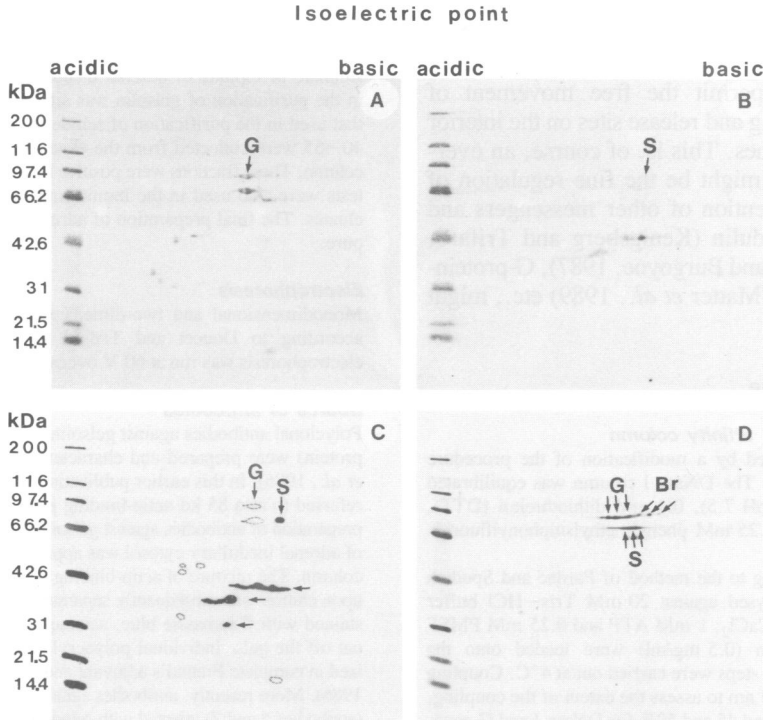

Fig. 10. Chymotrypsin cleavage products of adrenal medullary gelsolin and scinderin. Gelsolin (G) and scinderin (S) were digested with chymotypsin at a protein−enzyme ratio of 400:1 as indicated in Materials and methods. Peptide maps of gelsolin and scinderin are shown in **A** and **B** respectively. In **C**, a compositive diagram of superimposed peptide maps for both gelsolin and scinderin is shown; the arrows (solid spots) indicate cleavage products of scinderin, whereas open spots correspond to cleavage products of gelsolin. A mixture of gelsolin and scinderin prior to digestion is shown in **D**. Three isoforms for each of the two proteins (gelsolin, pI = 5.8, 5.9 and 6.0; scinderin, pI = 6.0, 6.1 and 6.2) were observed. The other three isoforms (Br.) may correspond to brevin as described previously (Bader *et al.*, 1986).

Bennet, 1985); (ii) Ca^{2+}-dependent actin severing proteins such as gelsolin (Yin and Stossel, 1979, 1980), villin (Bretscher and Weber, 1980) and fragmin (Hasegawa *et al.*, 1980); chromaffin cell scinderin should be included in this group; and (iii) proteins that inhibit actin polymerization by binding to monomeric actin such as profilin (Nystrom *et al.*, 1979) and DNase I (Mannherz *et al.*, 1975). Protein complexes formed by either of these two last proteins with actin cannot serve as nucleus for the addition of free actin monomers and polymerization (Pollard and Cooper, 1986). α-Actinin and fodrin, two of the proteins classified among the first group, have been detected in chromaffin cells (Aunis *et al.*, 1980; Trifaró *et al.*, 1982; Aunis and Perrin, 1984). α-Actinin has been found associated with chromaffin granule membranes (Aunis *et al.*, 1980; Trifaró *et al.*, 1982). This protein can stabilize actin nuclei and promote its polymerization (Pollard and Cooper, 1986). Cytochemical evidence has indicated that F-actin is also concentrated in the subplasmalemma region of the chromaffin cells (Lee and Trifaró, 1981; Cheek and Burgoyne, 1986). In these studies, a subplasmalemma fluorescent ring was observed when the resting chromaffin cells were treated with either actin antibodies or rhodamine-labelled phalloidin. Upon chromaffin stimulation by either nicotine (Cheek and Burgoyne, 1986) or acetylcholine (Trifaró *et al.*, 1989), rhodamine phalloidin fluorescence appeared fragmented and even disappeared in some subplasmalemmal areas. This phenomenon was accompanied by a decrease in the amount of chromaffin cell F-actin as measured by the DNase I inhibition assay (Cheek and Burogyne, 1986; Trifaró *et al.*, 1989). One is tempted to speculate that cell stimulation and Ca^{2+} entry activates actin-severing proteins such as gelsolin and scinderin. This activation will produce shortening of actin filaments, with a consequent removal of the actin network at the subplasmalemmal area. This area of decreased cytoplasm viscosity will permit the free movement of secretory granules to docking and release sites on the interior surface of plasma membranes. This is, of course, an oversimplified picture of what might be the fine regulation of exocytosis in which intervention of other messengers and modulators such as calmodulin (Kenigsberg and Trifaró, 1985), cyclic AMP (Cheek and Burgoyne, 1987), G-protein-induced actin ribosylation (Matter *et al.*, 1989) etc., might take place.

Materials and methods

Preparation of actin–DNase I affinity column

DNase I–Sepharose was prepared by a modification of the procedure described by Bader *et al.* (1986). The DNase I column was equilibrated with 20 mM Tris–HCl buffer (pH 7.5), 0.1 mM dithiothreitol (DTT), 2 mM $CaCl_2$, 1 mM ATP and 0.25 mM phenylmethylsulphonylfluoride (PMSF, Sigma Chemical Co.).

G-Actin was prepared according to the method of Pardee and Spudich (1982) and was extensively dialysed against 20 mM Tris–HCl buffer (pH 7.5); 0.1 mM DTT; 2 mM $CaCl_2$; 1 mM ATP and 0.25 mM PMSF overnight. Then, 10 mg G-actin (0.5 mg/ml) were loaded onto the DNase I–Sepharose column. All steps were carried out at 4°C. Coupling procedures were monitored at 280 nm to assess the extent of the coupling, which was between 80 and 90%, and 45 and 50% for DNase I and G-actin, respectively.

Purification of scinderin (80 kd protein)

Bovine adrenal glands obtained from a local slaughterhouse were kept on ice and transported to our laboratory. The adrenal glands were extensively perfused with ice-cold Locke's solution to remove traces of blood and the medullae were dissected from the cortices. Between 70 and 100 g of medullae from 25–30 glands 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 NaN_3, 0.1 mM EGTA, 1 mM ATP, 300 mM KCl, 10 μg/ml soybean trypsin inhibitor, 1 μg/ml of Leupeptin, and 0.1 mM DFP (1 g of medulla in 4 ml of solution) using a motor-driven Potter Elvehjem homogenizer. The homogenate was centrifuged at 1000 *g* for 10 min and the resulting supernatant was centrifuged again at 100 000 *g* for 60 min. To the supernatant thus obtained (cytosol), enough ammonium sulphate was added to reach 65% saturation. This mixture was stirred for 20 min and then centrifuged (15 000 *g* for 20 min). The sediment was collected and dissolved in 80 ml of buffer A (20 mM Tris–HCl (pH 7.5); 0.1 mM DTT and 0.1 mM EGTA) containing 20 mM KCl and dialysed against the same buffer for at least 12 h. The dialysed material was clarifed by centrifugation at 100 000 *g* for 30 min. The sample was then loaded onto a DEAE–Sepharose CL-6B column (2.5 × 35 cm, Pharmacia) which had been pre-equilibrated with the same buffer. The column was eluted at 20 ml × h^{-1} with a linear KCl gradient of 0.02–1 M KCl (540 ml total volume) and 6 ml fractions were collected. All column fractions were tested by immunoblotting with an antibody (antiserum 1) raised against scinderin (80 kd protein; see below). Sixteen immunoreactive fractions were concentrated under high pressure. The concentrated material was separated by gel filtration on a Sephadex G-100 column (2.5 × 90 cm; Pharmacia) pre-equilibrated with buffer A containing 100 mM KCl. The column was perfused at a flow rate of 20 ml × h^{-1} and fractions with the same volume as above were collected. Immunoreactive fractions were combined again and $CaCl_2$ was added to obtain a final concentration of 2 mM. Combined fractions from the previous eluates were loaded onto an actin–DNase I–Sepharose 4B affinity column (0.9 × 10 cm) that had been pre-equilibrated with buffer A containing 100 mM KCl and 2 mM $CaCl_2$. The column was then washed extensively with buffer A containing 0.5 M KCl. Finally, actin-binding proteins were eluted with buffer A containing 20 mM KCl and 10 mM EGTA. The EGTA eluate was concentrated as above. Finally, further purification was carried out on a Waters DEAE–5PW column (0.75 × 7.5 cm, Millipore) using a Waters HPLC system. The volume of injected sample was 1 ml/mg protein. The column was eluted for 10 min at 0.8 ml/min with buffer A containing 20 mM KCl then with linear gradient ranging from 0 to 10% buffer B (1 M KCl in buffer A) over 5 min, from 10 to 50% B for 50 min and finally from 50 to 100% B for 10 min. The eluate was separated in 0.8 ml fractions.

Purification of gelsolin

The starting material for the purification of gelsolin was an 80% ammonium sulphate precipitate of adrenal medullary cytosol. The protocol followed in the purification of gelsolin was similar (four chromatography steps) to that used in the purification of scinderin, except that in this case, fractions 40–55 were collected from the eluate from a DEAE–Sepharose CL-6B column. These fractions were positive for gelsolin in immunoblot tests. These tests were also used in the monitoring of all subsequent chromatography eluates. The final preparation of adrenal medullary gelsolin was 90–95% pure.

Electrophoresis

Monodimensional and two-dimensional electrophoresis were performed according to Doucet and Trifaró (1988). Usually, monodimensional electrophoresis was run at 60 V overnight in a Bio-Rad Protean I apparatus.

Source of antibodies

Polyclonal antibodies against gelsolin (90 kd protein) and scinderin (80 kd protein) were prepared and characterized as previously described (Bader *et al.*, 1986). In this earlier publication from our laboratory, scinderin was referred to as a 85 kd actin-binding protein of unknown function. For the preparation of antibodies against gelsolin and scinderin (antibody 1), a sample of adrenal medullary cytosol was applied to a DNase I–Sepharose affinity column. The mixture of actin-binding proteins (90 kd, 80 kd, etc.) obtained upon elution was subsequently separated by SDS–PAGE. Gels were briefly stained with Coomassie blue, scanned at 633 nm and protein bands were cut off the gels. Individual polyacrylamide peptide bands were homogenized in complete Freund's adjuvant and used for immunization (Bader *et al.*, 1986). More recently, antibodies against scinderin were raised in two rabbits (antibodies 6 and 7) injected with scinderin purified as described in this paper.

Immunoblotting procedures and [^{125}I]protein A method

The protocol for immunoblotting was based on the technique of Towbin *et al.* (1979). Protein samples (20 μg) were subjected to electrophoresis on 10% polyacrylamide gels (Doucet and Trifaró, 1988), transferred to nitrocellulose membranes (Hoefer Scientific Inst.) and processed for immunoblotting as described previously (Fournier and Trifaró, 1988; Fournier *et al.*,

1989). Antibodies were used at the following dilutions: anti-scinderin (antibody 1, 1:125; antibody 6, 1:1000) and anti-gelsolin 1:1000. The second antibody used was alkaline phosphatase-conjugated anti-rabbit IgG (1:3000 dilution).

The procedure for [^{125}I]Protein A immunoblotting was performed essentially as described by Burnette (1981) and Fournier *et al.* (1989). Autoradiograms were scanned and this information was used to calculate total and specific activities of different protein fractions containing scinderin.

Chromaffin cell cultures

Chromaffin cells were isolated from bovine adrenal glands and cultured as described by Trifaró and Lee (1980).

Indirect immunofluorescence of cell cultures

Chromaffin cells grown on collagen-coated coverslips were fixed, permeabilized and processed for immunofluoresence as described by Lee and Trifaró (1981).

Measurement of viscosity

Apparent viscosity was measured by the method of MacLean-Fletcher and Pollard (1980) using a low-shear falling ball viscometer. Rabbit skeletal G-actin, prepared by the method of Pardee and Spudich (1982) was polymerized and then homogenized in assay buffer as described by Bader *et al.* (1986). EGTA-$CaCl_2$ buffers were prepared as described by Caldwell (1970). Other conditions were as described by Bader *et al.* (1986).

Electron microscopy

F-actin (0.2 mg/ml) in assay buffer (40 mM PIPES, 0.1 M KCl, 2 mM $MgCl_2$, 2 mM ATP, 0.05% NaN_3, pH 6.8), containing either 5 mM EGTA or EGTA and $CaCl_2$ to give final free calcium concentration 10^{-5} M, was incubated with scinderin for 120 min at 25°C. The final concentration of the actin-binding protein was 18.6 μg/ml, corresponding to a molar ratio of scinderin to actin of 1:20. The samples were mounted on carbon-coated Formvar grids, negatively stained with 1% aqueous uranyl acetate and examined at × 33 000 magnification with a Philips EM 420 electron microscope. The length of the F-actin filaments treated with scinderin (80 kd protein) was calculated from 20 photographs of randomly chosen fields printed at a final magnification of × 82 500.

^{45}Ca overlay procedure

Purified protein (20 μg) was subjected to SDS–PAGE on 10% polyacrylamide gel followed by transfer to nitrocellulose membranes as described by Doucet and Trifaró (1988). For ^{45}Ca overlay, the procedure described by Hincke (1988) was followed. Membranes were either incubated in overlay buffer containing 10^{-6} M ^{45}Ca (sp. act. = 17 μCi/nmol) or in overlay buffer (Hincke, 1988) containing 10^{-6} M ^{45}Ca and 2×10^{-3} M $CaCl_2$. The membranes were subjected to autoradiography for 7 days as described previously (Fournier *et al.*, 1989).

Peptide mapping

Scinderin and gelsolin, both purified separately, were subjected to limited proteolytic digestion with either α-chymotrypsin (Sigma Chemical Co.) or staphylococcal V8 protease (Boehringer Gm BH Mannheim). Twenty μg of each protein in 20 mM Tris (pH 7.5), 200 mM KCl, 0.1 mM EGTA and 0.1 mM DTT was incubated at 25°C with either 50 ng of α-chymotrypsin (Kwiatkowoski *et al.*, 1985) or 50 ng of staphylococcal V8 protease (Bader *et al.*, 1986). After 15 min incubation, the digestion was stopped by adding enough ice-cold PMSF (100 mM) and di-iso propyl fluorophosphate (DPF, 100 mM) to reach a final concentration of 1 and 0.5 mM respectively. Digested proteins were subjected to two-dimensional electrophoresis according to the method of Doucet and Trifaró (1988) but with the omission of DTT from the medium.

Amino acid analysis

Hydrolysis of the samples was carried out at 108°C for 24 h in 6 M HCl, 0.1% mercaptoethanol (6 μg protein in 20 μl). Amino acid analysis of acid digests was performed by HPLC on a Pico-Tag column (0.3 × 25 cm; Waters) after the derivatization of amino acids with phenylisothiocyanate as described previously (Bidlingmeyer *et al.*, 1984).

Calcium-binding measurements

Ca^{2+} binding to scinderin was determined by equilibrium dialysis. The protein was dialysed overnight against 0.1 M KCl, 0.1 mM EGTA and 10 mM imidazole-HCl (pH 7.1) to remove Ca^{2+} bound to the protein. For equilibrium dialysis the microdialysis procedure of Overall (1987) was used. One ml sample of the protein (200 μg/ml) in a microfuge tube was dialysed with shaking at 4°C against 300 ml of above solution containing 16 μCi of $^{45}CaCl_2$ (New England Nuclear) for 24 h. In order to achieve a desired free Ca^{2+} concentration, $^{40}CaCl_2$ was added to the dialysis buffer in the amount required according to the method of Caldwell (1970). The pH of the buffers was maintained at 7.1. Following equilibration, 100 μl samples taken from the solutions inside and outside the dialysis bags were added to 10 ml vials containing Aquasol scintillation fluid (New England Nuclear) and the radioactivity was measured in a scintillation spectrometer (Beckman). Ca^{2+}-binding data was first subjected to Marguard non-linear least-squares procedure SSQMIN (Potter *et al.*, 1983) and then analysed with a curve-fitting computer program EBDA to determine K_d and B_{max} values (McPherson, 1983) by the method of Scatchard (1949).

Protein determination

Protein assays in the range of 1–20 μg were performed according to Bradford (1976) using a commercially available product (Bio-Rad protein assay, Bio-Rad). Bovine serum albumin (Sigma Chemical Co.) was used as a standard for the establishment of calibration curves.

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