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Cellular distribution of three mammalian Ca^{2+}-binding proteins related to *Torpedo* calelectrin

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Addition of Ca^{2+} to post-microsomal fractions of bovine adrenal or liver produced a sedimentable complex of membrane vesicles and cytoplasmic proteins. Proteins with apparent mol. wts. 70 000, 36 000 and 32 500 were solubilized from this complex by Ca^{2+} chelation. The 36 000 mol. wt. protein (p36) was immunoprecipitated by an antiserum specific for pp36, a major substrate for Rous sarcoma virus *src*-gene tyrosine kinase. This protein was present in many mesenchymal cells and associated with membrane cytoskeleton of bovine fibroblasts in a Ca^{2+}-dependent manner. The 70 000 and 32 500 mol. wt. proteins were widely distributed in established cell lines, but were not clearly associated with cell organelles in tissue sections, nor retained in cytoskeleton preparations. On immunoblots p36 reacted strongly with antibodies produced against the electric fish protein *Torpedo* calelectrin and the similar Ca^{2+}-binding properties and subunit mol. wts. of these proteins suggests that they might be functionally related. Since *Torpedo* calelectrin, p70, p36 and p32.5 were bound by lipid vesicles or microsomal membranes at micromolar free Ca^{2+} concentrations, regulated association with intrinsic membrane components may be involved in the functions of these widespread proteins.

Key words: adrenal medulla/membrane-binding protein/calcium/calelectrin/*src*-gene kinase

Introduction

Ca^{2+} is a universal second messenger in eukaryotic cells and its effects are transduced through specific Ca^{2+}-binding proteins. We have identified new intracellular Ca^{2+}-binding proteins in adrenal and liver (Geisow and Burgoyne, 1982; Walker *et al.*, 1983; Südhof *et al.*, 1983), two of which (p70 and p32.5) were recently purified to homogeneity (Südhof *et al.*, 1984). Both proteins have similar properties to *Torpedo* calelectrin, a 34 000 mol. wt. Ca^{2+}-binding protein from *T. marmorata* (Walker, 1982; Walker *et al.*, 1982; Südhof *et al.*, 1982) and combine with *Torpedo* calelectrin antiserum (Sudhof *et al.*, 1984). In the presence of Ca^{2+}, *Torpedo* calelectrin binds liposomes made from adrenal chromaffin granule membrane lipids (Südhof *et al.*, 1982) and the mammalian proteins bind chromaffin granule membranes (Geisow and Burgoyne, 1982; Südhof *et al.*, 1984).

The functions of *Torpedo* calelectrin and the two mammalian proteins are unknown. Because a number of Ca^{2+}-binding proteins which interact with cell membranes or with cytoskeleton have been recognised (Geiger, 1983), it was initially important to rule out identity with known cellular proteins. Secondly, it was necessary to determine the cellular distribution of the new proteins to assist in determining their functions. We also report a rapid method of isolation of *Torpedo* calelectrin-related Ca^{2+}-binding proteins and a third, previously undescribed protein.

Results

Isolation of adrenal p70, p36 and p32.5

Post-microsomal supernatants from bovine adrenal medulla prepared in the presence of EGTA were made 100 μM in free Ca^{2+} using a Ca^{2+}-sensitive electrode. The sedimentable complex which formed contained membrane vesicles and protein aggregates, shown by electron microscopic observation using negative stain. No fibrillar structures were seen. After washes, 1 mM EGTA extracted proteins from this pellet which gave a reproducible pattern on 2-dimensional gel electrophoresis (Figure 1a). The major proteins were resolved by anion-exchange chromatography of the crude EGTA eluate at pH 7 into three fractions (Figure 1a). Unretarded by the column: mol. wt. 36 000 pI′ 7–7.4 (p36); at 0.1 M NaCl: mol. wt. 32 500 pI′ 5.8 (p32.5) and at 0.22 M NaCl: mol. wts. 70 000 pI′ 6.4; 35 000 pI′ 5.3; 32 500 pI′ 5.4 (p70, p35, p32.5). pI′ refers to the pH at the position of the polypeptide in the first dimension gel. p36 migrated as a closer pair of bands on SDS gels and as two components of slightly different pI′ on 2-D gels. p70 was completely resolved from p35 and p32.5 by chromatography on Ultrogel AcA 44.

Adrenal p70 and p32.5 pI′ 5.8 were identical with Ca^{2+}-binding proteins isolated by hydrophobic interaction chromatography on phenyl-Sepharose (Südhof *et al.*, 1984) on the basis of 2-D gel electrophoresis and peptide mapping. The presence of microsomal membrane vesicles in the initial supernatant was necessary for the isolation of these proteins, and is probably the basis for the initial purification method using phenyl-Sepharose. These vesicles adsorbed essentially irreversibly to the hydrophobic matrix. In the absence of membrane the purified proteins neither sedimented at 100 000 g_{av}, nor bound significantly to hydrophobic matrices, even at 1 mM Ca^{2+}.

Affinity purification and specificity of anti-p70, anti-p32.5 and anti-Torpedo calelectrin

Rabbit antisera were raised against bovine liver p70, p32.5 and *Torpedo* calelectrin as described (Südhof *et al.*, 1984). Affinity-purified immunoglobulin was obtained by adsorption onto Sepharose CL-4B columns conjugated with 0.5–1.0 mg of the homogeneous antigens. After a high salt wash, 400 μg anti-*Torpedo* calelectrin, 320 μg anti-p32.5 were obtained from 1 ml and 120 μg of anti-p70 from 0.5 ml of the respective antisera by elution with pH 2.4 buffer. Affinity-purified anti-p70 and anti-p32.5 immunoprecipitated [^{35}S]methionine-labelled 70 000 and 32 500 mol. wt. polypeptides from embryonic bovine tracheal (EBTr) fibroblasts

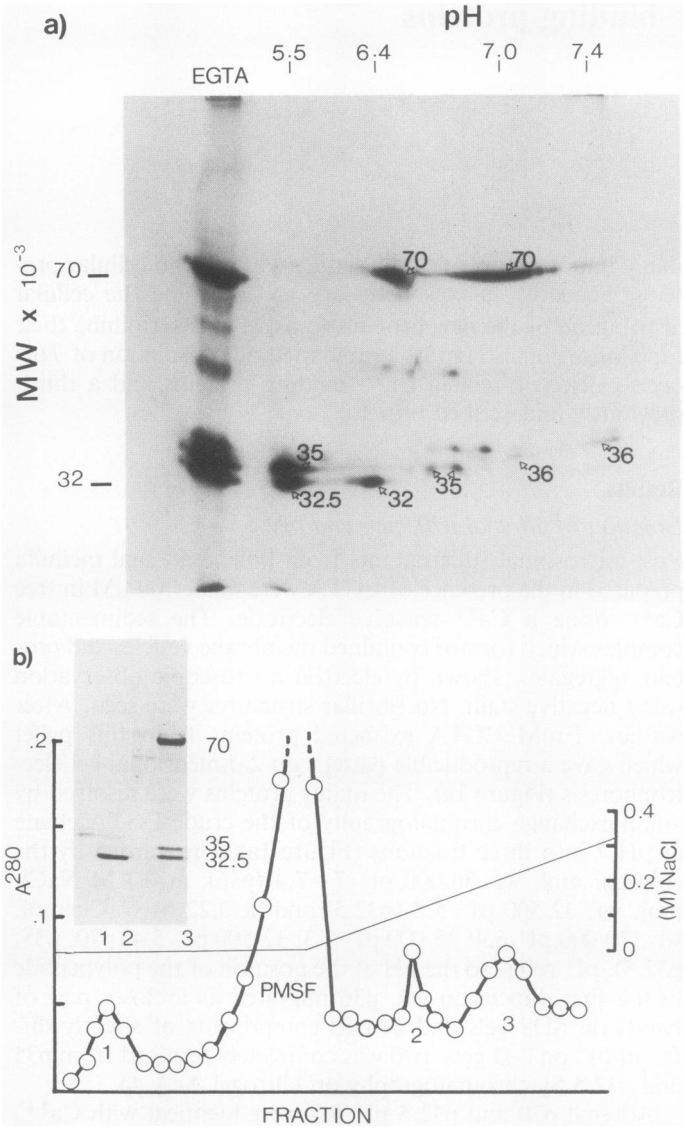

Fig. 1. Ca^{2+}-dependent adrenal medulla membrane-binding proteins. **(a)** The ^{125}I-labelled EGTA-extract (EGTA) analysed by narrow pH range 2-D gel. The polypeptide p32 is referred to in the text as p32.5 pI′ 5.8. **(b)** Separation of p36 (Peak 1), p32.5 pI′ 5.8 (Peak 2) and p70, p35 and p32.5 pI′ 5.4 (Peak 3) on DEAE-Sephacel pH 7.0. The inset shows (L to R) Peak 1, Peak 2, PMSF and Peak 3. PMSF refers to elution position of protease inhibitors PMSF and leupeptin.

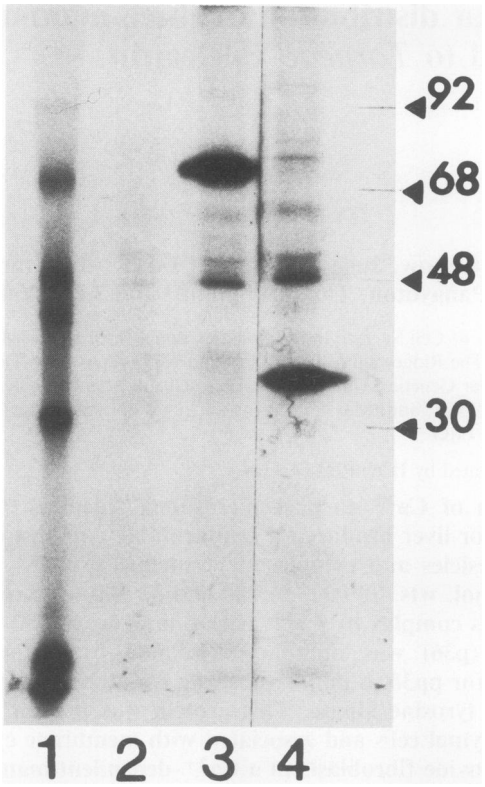

Fig. 2. Immunoprecipitation of [^{35}S]methionine-labelled proteins from EBTr fibroblasts. **Lane 1**, mol. wt. markers: phosphorylase b, albumin, ovalbumin, carbonic anhydrase and lysozyme. **Lane 2**, proteins bound by protein A-Sepharose alone. **Lane 3**, anti-p70 immunoprecipitate. **Lane 4**, anti-p32.5 immunoprecipitate. Minor bands in **lanes 3** and **4** represent non-specific adsorption (i.e., also present in **lane 2**).

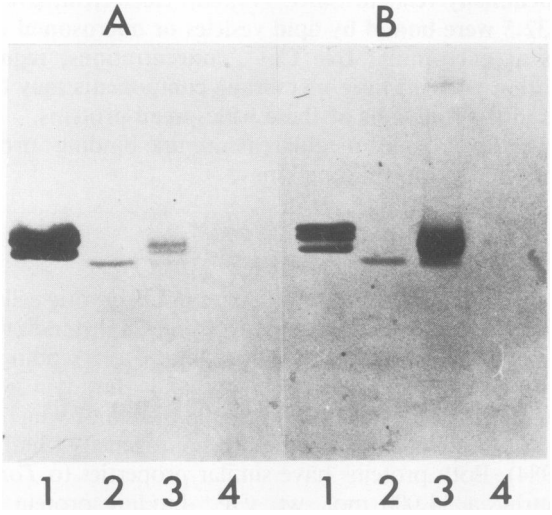

Fig. 3. Immunoblots of p36, p32.5 and *Torpedo* calelectrin. 0.5 μg of **1**, p36; **2**, p32.5; **3**, *Torpedo* calelectrin; **4**; chromaffin granule contents transferred to nitrocellulose were immunostained either with **A**: rat pp36 antiserum (1:400) or **B**: anti-*Torpedo* calelectrin (1:100).

(Figure 2). Anti-*Torpedo* calelectrin did not immunoprecipitate any labelled components. When whole antisera were used in place of the immunoglobulin, there was no significant difference in the pattern of immunoprecipitated polypeptides, confirming that the original antisera were monospecific.

Recognition of p36 by anti-Torpedo calelectrin

Polypeptides of mol. wts. 34 000 – 36 000 in the crude adrenal EGTA extract were strongly labelled after transferring SDS-polyacrylamide gels to nitrocellulose and staining with anti-*Torpedo* calelectrin. After separating the components by DEAE-chromatography, most of this immunoreactivity was found to be associated with p36 (Figure 3b, lane 1). A minor faster migrating protein was also stained and probably represents a p36 proteolysis product as it appears after storage of p36. *Torpedo* calelectrin (lane 3) and p36 were comparably immunoreactive, p32.5 (lane 2) was relatively poorly stained and adrenal chromaffin granule content proteins (lane 4), used as a control for unspecific labelling, were not labelled at all. Bands corresponding to p36 and *Torpedo* calelectrin were excised from an SDS-polyacrylamide gel and one-dimensional peptide maps pro-

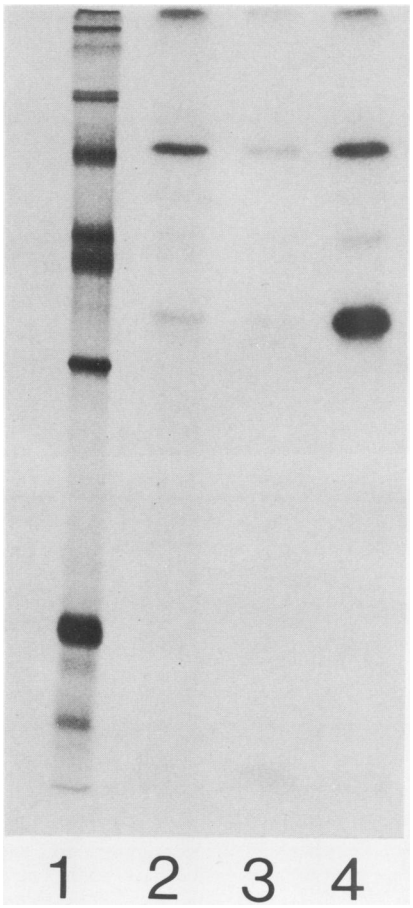

Fig. 4. Immunoprecipitation of ^{125}I-labelled adrenal medulla proteins from the radio-iodinated initial EGTA extract (Figure 1). **Lane 1**, mol. wt. markers. **Lane 2**, non-immune serum. **Lane 3**, *Torpedo* calelectrin antiserum. **Lane 4**, pp36 antiserum (70 000 mol. wt. polypeptide represents non-specific binding because it is also present in **lane 2**).

duced using *Staphylococcus aureus* V8 protease (not shown). A single 14-K peptide was immunoreactive with anti-*Torpedo* calelectrin in the p36 peptide map, while most *Torpedo* calelectrin peptides were labelled. *Torpedo* calelectrin itself was not labelled on blots by any of the antisera raised against mammalian proteins.

Homology of bovine adrenal p36 with Rous sarcoma virus src-gene kinase substrate pp36

Purified p36 eluted in positions corresponding to globular proteins of mol. wts. 80 000–90 000 and 37 000 after gel filtration on AcA 44. The native form of the protein may thus consist of at least two 37 000 mol. wt. polypeptides. After blotting (Figure 3a, lane 1) the bands associated with p36 were strongly immunoreactive with a well-characterised antiserum for rat pp36 (Courtneidge *et al.*, 1983). p32.5 and *Torpedo* calelectrin were only labelled at background levels (lanes 2 and 3). A 36 000 mol. wt. polypeptide was immunoprecipitated from the initial ^{125}I-labelled adrenal EGTA extract (Figure 4, lane 4) by pp36 antiserum, but not by preimmune serum or *Torpedo* calelectrin antiserum (Figure 4, lanes 2 and 3).

Immunocytochemistry of p70, p36 and p32.5

The distribution of these antigens was assessed by indirect immunofluorescence in cryostat sections of bovine adrenal, rat liver and gut. The p36 (pp36) was present in connective tissue and vascular endothelial cells of all the tissues, but was

Table I. Expression of p70, p36 and p32.5

Cell type		p70	p36	p32.5
Fibroblast	EBTr	+ +	+	+
Epidermoid	A431	+ +	+	+
Neuronal	PC12	+	+	+
Erythroid	Friend	–	–	–
	Erythrocyte	–	–	–

+ + and + refer to size of immunoprecipitated band and – means no detectable immunoprecipitate.

undetectable in parenchymal cells, neurones (adrenal) and smooth muscle. The protein was most strongly expressed in gut epithelium and was highly localized at brush borders of isolated rat jejunum epithelial cells. This site was also weakly labelled by anti-*Torpedo* calelectrin, but all other regions of the tissues examined were negligibly stained by the immunoglobulin. Rat and bovine tissues were very weakly and rather generally stained by anti-p70 and anti-p32.5.

A number of primary cells and cell lines were positive for p70, p36 and p32.5 by immunoprecipitation, immunoblotting or both methods (Table I). Established cell lines appeared to express each protein, although erythroid cells were negative for p70, p36 and p32.5 by immunoprecipitation. EBTr fibroblasts were selected to determine the subcellular distribution of the antigens, because single polypeptides were obtained by immunoprecipitation with anti-p70, anti-p32.5 and pp36 antiserum. Positive labelling relative to non-immune controls was absolutely dependent upon permeabilization, confirming that all the antigens were intracellular. Anti-p70 produced diffuse staining (Figure 5). Antiserum to pp36 also produced diffuse labelling, while anti-p32.5 labelled discrete, irregular structures around the nucleus. A similar fragmented pattern was produced by a rabbit antiserum to stripped dog pancreas endoplasmic reticulum (not shown).

To determine whether any of the antigens were associated with cytoskeleton, Triton shells were prepared from EBTr cells by standard methods. Ca^{2+}-dependence was assessed by the inclusion of either 1 mM Ca^{2+} or 1 mM EGTA in the extraction buffer. Antiserum to pp36 stained an extensive reticular network after Triton extraction at high Ca^{2+} (Figure 5d). Labelling was prevented when the initial extraction buffer contained EGTA or after the antiserum was pre-incubated with adrenal p36. There was essentially no anti-p70 or anti-p32.5 immunofluorescence after detergent extraction, irrespective of the Ca^{2+} concentration of the buffer. In double-labelling experiments neither p70, p36 nor p32.5 immunofluorescence corresponded with the distribution of actin, vimentin or microtubule labelling.

Relationship of p70 and p32.5 to calcium-binding proteins of known function

Purified p70 and p32.5 were compared with Ca^{2+}-binding proteins of similar mol. wts. by immunoblotting using antisera obtained as gifts and, where appropriate, by functional assay carried out essentially as described in the references given. Identity of p70 and p32.5 with the following proteins was ruled out: protein kinase C (Kikkawa *et al.*, 1982), calpains, heat-shock proteins, fimbrin, intermediate filament subunits, clathrin light chains, tubulin-binding protein (Weingarten *et al.*, 1976) and actin-capping protein (Kiliman and Isenberg, 1982). No correspondence was found with any pro-

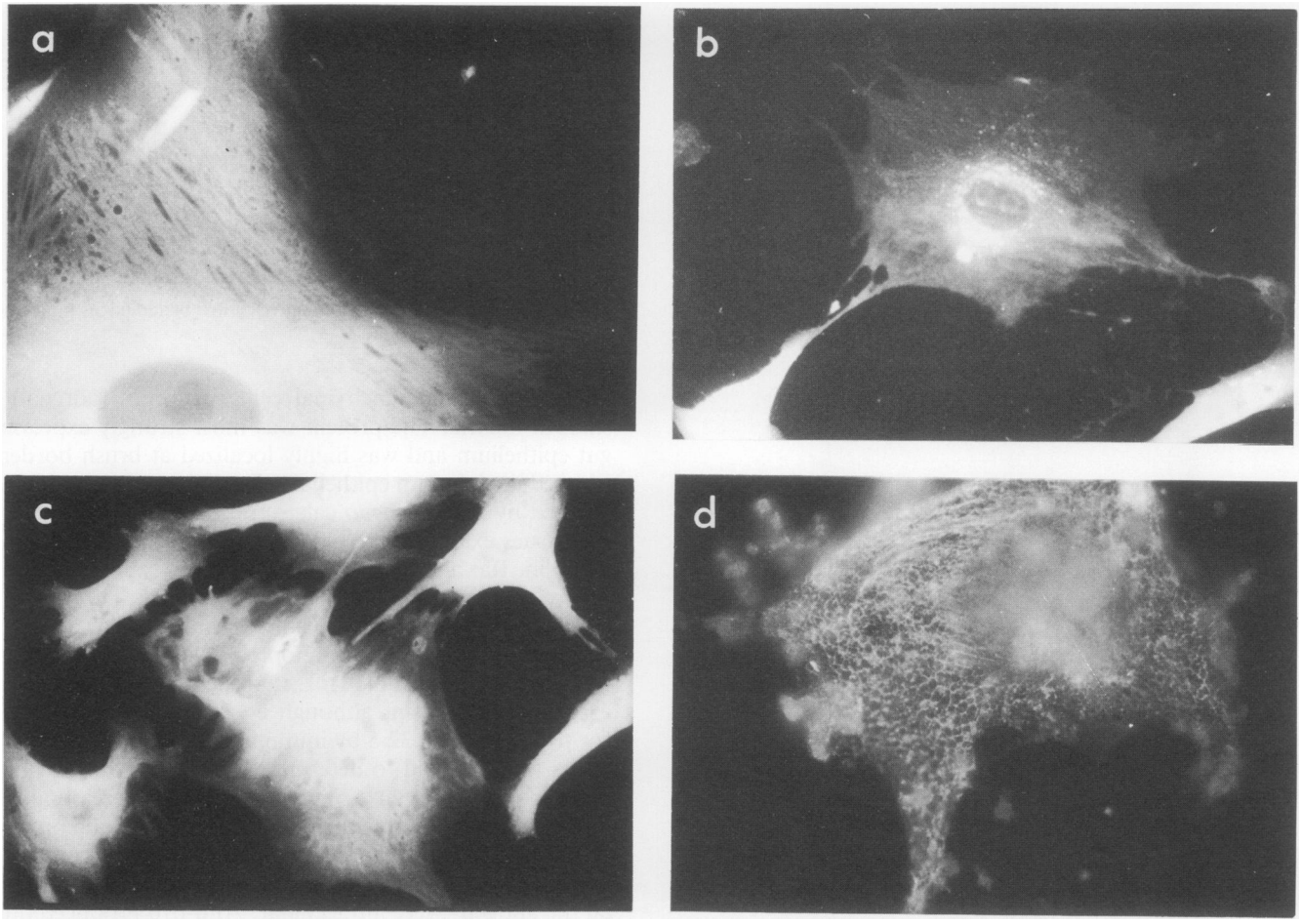

Fig. 5. Indirect immunofluorescence of p70, pp36 and p32.5 in EBTr fibroblasts. Cells fixed with 3% paraformaldehyde and permeabilized by NP-40 were labelled with anti-p70 (**a**), anti-p32.5 (**b**) and anti-pp36 (**c**). In (**d**) the cells were extracted with NP-40 in the presence of 1 mM Ca^{2+} prior to fixation. Magnifications **a** x 650; **b,c,d** x 300.

tein of known function. However, a 68 000 mol. wt. protein extracted by EGTA from human B lymphoblastoid cells (BRI 8) plasma membrane cytoskeletons (Owens and Crumpton, 1983) was labelled by immunoblotting with *Torpedo* calelectrin antiserum. A 32-K protein extracted from pig intestinal epithelial cell brush border membranes by EGTA (Gerke and Weber, 1984) was also labelled on immunoblots by *Torpedo* calelectrin antiserum (data not shown).

Discussion

We have described the rapid isolation, antigenic properties and cellular distribution of three Ca^{2+}-dependent membrane-binding proteins from bovine adrenal. Adrenal p36, which has not previously been described, was homologous with pp36, a cellular substrate for the oncogenic protein tyrosine kinase of Rous sarcoma virus (Radke and Martin, 1979), based upon several different criteria. Adrenal p36 had a native mol. wt. of 80 000–90 000, while pp36 has a native mol. wt. of 70 000–90 000 (Erikson *et al.*, 1984; Gerke and Weber, 1984). p36 focussed at neutral pH on 2-D gel electrophoresis and was not bound by DEAE-Sephacel at low salt. The same physical properties have been reported for pp36 (Erickson *et al.*, 1984; Hunter and Cooper, 1981). Adrenal p36 was immunoprecipitated by anti-pp36 serum.

The strong labelling of p36 on immunoblots by anti-*Torpedo* calelectrin and anti-pp36 suggests that *Torpedo* calelectrin and pp36 are related. This antigenic relationship is not extensive, since only one p36 peptide was immunoreactive with anti-*Torpedo* calelectrin. Also *Torpedo* calelectrin antiserum was unable to immunoprecipitate [^{35}S]methionine-labelled polypeptides from cells positive for pp36, suggesting that the relevant epitopes are masked unless p36 is denatured. Absence of any significant cross-reaction between *Torpedo* calelectrin and pp36 antiserum indicates that non-overlapping determinants are recognised by anti-pp36 and anti-*Torpedo* calelectrin.

Antiserum to pp36 produced both diffuse and reticular cell surface immunofluorescence in fibroblasts as expected from previous studies (Courtneidge *et al.*, 1983; Greenberg and Edelman, 1983). The reticular staining pattern obtained after extraction with non-ionic detergent prior to fixation, was Ca^{2+}-dependent and was prevented by pre-incubation of the antiserum with purified adrenal p36. This is indirect evidence that p36 has a similar subcellular localization to pp36 and strongly suggests that p36 also interacts with detergent-insoluble cytoskeleton in a Ca^{2+}-dependent manner.

In contrast to p36, neither p70 nor p32.5 were retained after detergent extraction of EBTr cells at high Ca^{2+} concentrations. This indicates a fundamental difference in the subcellular interactions of these Ca^{2+}-binding proteins. The weak immunofluorescence of p70 and p32.5 in bovine and rodent issues is incompatible with expected levels of these proteins (10 mg/kg liver for p70), unless the proteins are largely present in a soluble, extractable form. *In vitro*, p70

and p32.5 were quantitatively released from microsomal membrane vesicles or chromaffin granule membranes (Südhof *et al.*, 1984) at normal intracellular Ca^{2+} concentrations (100–200 nM). If the membrane binding sites are lipid in character as implied by previous studies (Südhof *et al.* 1982, 1984) the observed extraction of the proteins by detergent at high Ca^{2+} would be expected. Immunocytochemistry is evidently an unreliable means of assessing the levels of cellular expression of p70 and p32.5, but the weak labelling of cell organelles described might represent sites of recruitment during elevated intracellular Ca^{2+} levels.

Ca^{2+}-binding proteins with similar mol. wts. have been reported elsewhere. The proteins called 'chromobindins' from post-microsomal supernatant of adrenal medulla, appear to contain p70, p35 and p32.5 from inspection of the published 2-D gel analysis (Creutz *et al.*, 1983). The 'Calcemedins' obtained from chicken gizzard extracts by Ca^{2+}-dependent hydrophobic interaction chromatography (Moore and Dedman, 1982) correspond with p70, p35 and p32.5 on the basis of the purification protocol. The Ca^{2+}-binding protein of mol. wt. 68 000 obtained by Owens and Crumpton (1983) in EGTA extracts of lymphocyte plasma membrane cytoskeleton is homologous with p70 on the basis of labelling on immunoblots by *Torpedo* calelectrin antiserum. This lymphocyte protein also appears to exist partly in association with cell surface and partly in soluble form (Owens *et al.*, 1984).

Proteins I and II from intestinal epithelial brush border membranes described by Gerke and Weber (1984) are homologous with p36 and p32.5 respectively, since protein I was shown to be identical with pp36 and protein II labelled on immunoblots with *Torpedo* calelectrin antiserum (Südhof, personal communcation). Protein II and p32.5 pI′ 5.8 have very similar amino acid compositions. Lack of identity of bovine p70, p36 and p32.5 with Ca^{2+}-binding proteins of similar mol. wts. and known functions suggests that these proteins represent members of a new group. However, the immunological properties, Ca^{2+} binding and similar monomeric mol. wts. indicate that mammalian p36 (pp36) and the electric fish protein calelectrin are related. The observation that pp36 is a substrate for growth-related protein tyrosine kinases prompts experiments to determine whether *Torpedo* calelectrin is a phosphoprotein. Conversely, the established lipid-binding properties of *Torpedo* calelectrin (Südhof *et al.*, 1982) and the association of p36 with microsomal membranes raise an important question whether pp36 has affinity for lipids in addition to its interaction with membrane cytoskeleton. Further biochemical studies of the relationships between these proteins should be profitable in understanding their function, the significance of their Ca^{2+}-binding and phosphorylation (pp36) by oncogene-specified tyrosine kinases.

Materials and methods

Materials and cells

Beef adrenal glands were supplied by British Beef, Watford. Primary chromaffin cell cultures were obtained as described by Wilson and Viveros (1981). A431 cells were a gift of the Imperial Cancer Research Fund, London. Primary chromaffin cells (80% pure) were established in 50% DMEM and 50% Ham's F12 with 10% foetal calf serum (FCS). EBTr cells (GIBCO) were grown in Ham's F12 with 10% FCS and A431 cells were grown in DMEM with 5% new born calf serum. Friend cells were cultured in MEM with 10% FCS. Primary cells from rat liver and gut were obtained by collagenase perfusion and shake-off in EDTA/hyaluronidase respectively. Isotopically-labelled reagents were supplied by Amersham International.

Rabbit antisera against *Torpedo* calelectrin, p70 and p32.5 were obtained as previously described (Südhof *et al.*, 1984). Homogeneous *Torpedo* calelectrin (1.3 mg), p70 (0.24 mg) and p32.5 (0.7 mg) were coupled to CNBr-activated Sepharose CL-4B (Sigma) in 0.1 M $NaHCO_3$ buffer pH 8.3. After washing, the gels were incubated with 1 M ethanolamine pH 8, cycled with high and low pH buffers and finally equilibrated with $NaCl/P_i$. The antisera were run on the affinity columns which were washed with 0.5 M $NaCl/P_i$, before eluting immunoglobulin with 0.2 M glycine HCl pH 2.4 The eluate was neutralized with solid Tris base and assayed by dot blotting with pure antigen.

Ca^{2+}-dependent membrane-binding protein isolation

A post-microsomal supernatant was obtained from adrenal medulla (Geisow and Burgoyne, 1982) by centrifugation (100 000 g_{av}; 1 h). Excess lipid was removed by filtration and the solution made 1 mM with respect to Ca^{2+} in the presence of 0.2 mM phenylmethylsulphonyl fluoride (PMSF) and 10 μg/ml leupeptin. After 15 min at 20°C the solution was centrifuged (100 000 g_{av}; 1 h). The pellet was resuspended and washed 2 x in 0.1 mM Ca^{2+}, 10 mM Tris-HCl pH 7.0 containing the protease inhibitors. Finally, the pellet was extracted with 2 ml 2 mM EGTA in the same buffer. After centrifuging insoluble material at 100 000 g_{av}; 60 min, the supernatant was fractionated on 4 ml DEAE-Sephacel in 10 mM Tris-HCl pH 7.0 using a 50 ml 0–0.4 M NaCl gradient. Proteins were also isolated from the post-microsomal supernatant by Ca^{2+}-dependent hydrophobic interaction chromatography (Südhof *et al.*, 1984). Stokes radius determinations were carried out as described by Südhof *et al.* (1984).

Electrophoresis and peptide mapping

Single-dimension peptide mapping performed according to Cleveland *et al.* (1977) used *S. aureus* V-8 protease. Two dimensional gel electrophoresis used the methods of O'Farrell (1975) with measurement of the pH gradient by protein standards (Biorad Ltd.) and electrode measurement of eluted 1st dimension gel slices. Unstained bands in SDS-PAGE were elecrophoretically transferred to nitrocellulose. Antigenic polypeptides were detected after rinsing the blot in $NaCl/P_i$ and incubating for 5 h in 5% hemoglobin in $NaCl/P_i$. First antibody was applied at dilutions of 1:200 to 1:400 in $NaCl/P_i$-hemoglobin. After 5 x 10 min washes, either protein A (1 μCi, 1 μg protein) or horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin was added for 1 h in $NaCl/P_i$-hemoglobin. After five washes, the blot was dried and auto-radiographed or visualized with 1 mg/ml diaminobenzidine and 0.01% H_2O_2 in $NaCl/P_i$. Protein was detected on blots by staining with Ponceau Red S.

Radiolabelling and immunoprecipitation

Cultures were equilibrium-labelled with [^{35}S]methionine (1430 Ci/mmol), 50 μCi/ml in 1/10 methionine medium for 16 h. After washing in $NaCl/P_i$ cells were lysed with 0.2–0.5 ml 50 mM Tris-HCl pH 7.4 containing 1 mM PMSF, 10 μg/ml leupeptin, 5 mM EDTA and 1% Nonidet P-40 (NP-40).

Lysates were pre-adsorbed with protein A-Sepharose and mixed with 10 μl protein A-Sepharose-antibody conjugates (1 μg immunoglobulin or 1 μl antiserum) for 1 h by gentle agitation. The immunoadsorbant was washed 5 x with lysis buffer containing 0.4 M NaCl, 1 x with $NaCl/P_i$ and boiled 5 min in SDS-PAGE sample buffer prior to electrophoresis. Gels containing ^{35}S were pre-soaked in 'Amplify' (Amersham International) before drying and autoradiography.

Adrenal Ca^{2+}-dependent membrane-binding proteins were iodinated using IODOGEN (Pierce and Warriner Ltd.) coated tubes as described (Burgoyne and Geisow, 1981).

Indirect immunofluorescence

Single and double-label indirect immunofluorescence used 1:100 affinity purified FITC or TRITC-labelled anti-species IgG (Miles Ltd.). Fibroblasts were fixed at 37°C with 3.7% w/v *p*-formaldehyde in $NaCl/P_i$ and permeabilized with 0.5% NP-40 in $NaCl/P_i$ or fixed and permeabilized with methanol for 10 min at −20°C. Triton shells were prepared by extraction at room temperature with 1% v/v Triton X-100 or 1% v/v NP-40 in 10 mM Hepes buffer pH 7.2, 100 mM KCl, 2 mM $MgCl_2$, 1 M glycerol, 1 mM PMSF, 1 mM iodoacetic acid and either 1 mM $CaCl_2$ or 1 mM EGTA. After 5 min cells were washed with the same buffer, followed by 4% w/v *p*-formaldehyde in $NaCl/P_i$. Cells were mounted in 50% glycerol and photographed using a Leitz microscope equipped with epifluorescence illumination. Small tissue blocks from rat liver and gut and bovine adrenal medulla were snap frozen in embedding medium and 4 μm cryostat sections cut, freeze-dried and fixed with acetone for 10 min. After re-hydration, sections were processed for indirect immunofluorescence as described above.

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References

Burgoyne,R.B. and Geisow,M.J. (1981) *FEBS Lett.*, **131**, 127-131.
Cleveland,D., Fischer,S., Kirschner,J. and Laemmli,U. (1977) *J. Biol. Chem.*, **252**, 1102-1106.
Courtneidge,S., Ralston,R., Alitalo,K. and Bishop,J.M. (1983) *Mol. Cell Biol.*, **3**, 340-350.
Creutz,C.E., Dowling,L.G., Sando,J.J., Villar-Palasi,C., Whipple,J.H. and Zaks,W.J. (1983) *J. Biol. Chem.*, **258**, 14664-14674.
Erikson,E., Tomasiewicz,H.G. and Erikson,R.L. (1984) *Mol. Cell Biol.*, **4**, 77-85.
Geiger,B. (1983) *Biochem. Biophys. Acta*, **737**, 305-341.
Geisow,M.J. and Burgoyne,R.D. (1982) *J. Neurochem.*, **38**, 1735-1741.
Gerke,V. and Weber,K. (1984) *EMBO J.*, **3**, 227-233.
Greenberg,M.E. and Edelman,G.M. (1983) *J. Biol. Chem.*, **258**, 8497-8502.
Hunter,T. and Cooper,J.A. (1981) *Cell*, **24**, 741-752.
Kikkawa,U., Takai,Y., Minakuchi,R., Inohara,S. and Nishizuka,Y. (1982) *J. Biol. Chem.*, **257**, 13341-13348.
Kilimann,M.W. and Isenberg,G. (1982) *EMBO J.*, **1**, 889-894.
Moore,P.B. and Dedman,J.R. (1982) *J. Biol. Chem.*, **257**, 9563-9667.
Owens,R.J. and Crumpton,M.J. (1983) *Biochem. Soc. Trans.*, **11**, 156-157.
Owens,R.J., Gallagher,C.J. and Crumpton,M.J. (1984) *EMBO J.*, **3**, 945-952.
O'Farrell,P.H. (1975) *J. Biol. Chem.*, **250**, 4007-4021.
Radke,K. and Martin,G.S. (1979) *Proc. Natl. Acad. Sci. USA*, **76**, 5212-5216.
Sudhof,T.C., Walker,J.H. and Obrocki,J. (1982) *EMBO J.*, **1**, 1167-1170.
Sudhof,T.C., Zimmerman,C.W. and Walker,J.H. (1983) *Eur. J. Cell Biol.*, **30**, 214-218.
Sudhof,T.C., Ebbecke,M., Walker,J.H., Fritsche,U. and Boustead,C. (1984) *Biochemistry (Wash.)*, **23**, 1103-1109.
Walker,J.H. (1982) *J. Neurochem.*, **39**, 815-823.
Walker,J.H., Obrocki,J. and Sudhof,T.C. (1983) *J. Neurochem.*, **41**, 139-145.
Weingarten,M.D., Lockwood,A.H., Hwo,S.Y. and Kirschner,M.W. (1976) *Proc. Natl. Acad. Sci. USA*, **72**, 1858-1862.
Wilson,S.P. and Viveros,O.H. (1981) *Exp. Cell. Res.*, **133**, 159-169.

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