The EMBO Journal vol.3 no.1 pp.227 – 233, 1984

Identity of p36K phosphorylated upon Rous sarcoma virus transformation with a protein purified from brush borders; calcium-dependent binding to non-erythroid spectrin and F-actin

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Communicated by K. Weber

Membrane vesicles derived from the apical side of porcine intestinal epithelial cells retain, after demembranation in the presence of calcium, two major proteins (I, II) which are released by the addition of calcium chelators. We have purified and characterized these two calcium-binding proteins. Protein I has a mol. wt. of 85 000 and contains two copies of a 36-K subunit and an additional 10-K subunit. It binds in a calcium-dependent manner to F-actin as well as to non-erythroid spectrin. Immunofluorescence microscopy reveals protein I-related antigens in the terminal web of the intestinal cell and in a submembraneous cortical layer in various tissue culture cells. Biochemical and immunological results document that the 36-K subunit of protein I is identical with the cellular p36K recognized as a major substrate for tyrosine phosphorylation by the *sarc* gene kinase in Rous sarcoma virus-transformed cells. The biochemical properties of protein I agree with its location seen in immunofluorescence microscopy and cell fractionation and suggest that the actin-spectrin network in the cortical layer may be affected by virus transformation.

Key words: phosphotyrosine/Rous sarcoma virus/calcium/spectrin/actin/brush border

Introduction

The microfilament organization of the chicken intestinal epithelial cell has been characterized in detail (reviewed by Weber and Glenney, 1982). Ultrastructurally intact as well as demembranated brush broders and microvilli have been analyzed for their major actin-binding proteins. Fimbrin and villin act as bundling proteins in the microvillus core filament bundle, which is connected by fine cross-bridges to the inner side of the plasma membrane (Bretscher and Weber, 1980; Mooseker *et al.*, 1980; Glenney *et al.*, 1981a; Bretscher, 1981). These connections seem to arise from a 110-K protein (Matsudaira and Burgess, 1979), which binds calmodulin (Glenney and Weber, 1980) and is thought to be anchored at a 140-K transmembrane protein (Coudrier *et al.*, 1983). Within the terminal web, the core filament bundles are connected via the long rod-like molecules of TW260/240, a non-erythroid spectrin (Glenney *et al.*, 1982a, 1983; Hirokawa *et al.*, 1983). These structural studies have relied heavily on the use of EGTA-containing buffers to avoid destruction of F-actin by the Ca^{2+}-activation of villin (Bretscher and Weber, 1980; Mooseker *et al.*, 1980). In an attempt to search for further microfilament-binding proteins, which may depend on Ca^{2+}, we have explored the protein composition of brush border vesicles known to result from fractionation experiments in the absence of chelating agents (Kessler *et al.*, 1976; Schmitz *et al.*, 1973; Coudrier *et al.*, 1983). Using this approach for the isolation of mammalian villin and fimbrin from porcine intestinal cells (Gerke and Weber, 1983) we recognized two major proteins (I and II) whose presence in demembranated vesicles depended on the presence of Ca^{2+}. We have now purified and characterized these calcium-binding proteins. We show that protein I contains a polypeptide of 36 K which seems identical with the major cellular protein phosphorylated by the *sarc* gene kinase in Rous sarcoma virus (RSV)-transformed cells (reviewed by Hunter and Sefton, 1982).

Results

Purification and physical-chemical properties of protein I

Proteins I and II present in porcine brush border-derived membrane vesicles were purified and characterized (Figure 1). They are retained upon Triton X-100 demembranation in the presence of Ca^{2+} but are specifically released by subsequent addition of EGTA. This property provided a high enrichment of the two proteins in the resulting supernatant. Separation of I and II was possible by ion-exchange chromatography on DEAE-cellulose since protein I is not retained in low salt buffer. Final purification was obtained by gel filtration and, if necessary, by chromatography on CM-cellulose. About 5 – 10 mg of both proteins can be obtained starting with 20 small intestines, and preliminary experiments show that a similar purification can be performed with chicken intestine.

The Stokes radii of I and II were found to be 41 ± 1 Å and 21 ± 1 Å, respectively, and were not influenced by the presence or absence of Ca^{2+} in the elution buffer. Polyacrylamide gel electrophoresis in the presence of SDS revealed a single band of mol. wt. 32 K for II and two polypeptides of 36 K and 10 K for I (Figure 1). Standard proteins used in this determination included muscle glyceraldehyde phosphate dehydrogenase assumed to have a mol. wt. value of 37 K and the villin headpiece (Glenney *et al.*, 1981b) known by amino acid sequence analysis to be 8.6 K. Scans of Coomassie blue-stained gels indicated that the two polypeptides were present in a molar ratio of ~2 copies of 36 K for one copy of 10 K. Direct mol. wt. determination of protein I performed by analytical ultracentrifugation yielded a value of 85 000 ± 3000. The combined results point to a rather globular protein, which does not change its oligomeric character upon exposure to Ca^{2+}. Thus protein I has a dimeric organization as far as the 36-K subunit is concerned. Given the gel filtration data, protein II is most likely a monomer. Isoelectric focussing gels performed in 9.5 M urea showed that the 36-K subunit has a pI of ~7.4 (Figure 1). Overloaded gels reveal a second isoelectric variant (~15%) at the same apparent mol. wt. but with a slightly more acidic pI of ~7.2. Protein II has a pI of ~6.5. All three polypeptides – p36K, p10K and p32K – were characterized by their amino acid composition (Table I).

Because of their release by EGTA from demembranated vesicles, both proteins were subjected to Ca^{2+} binding experiments using a gel filtration procedure (Hummel and Dreyer, 1962). In the presence of 50 mM NaCl, 0.1 mM

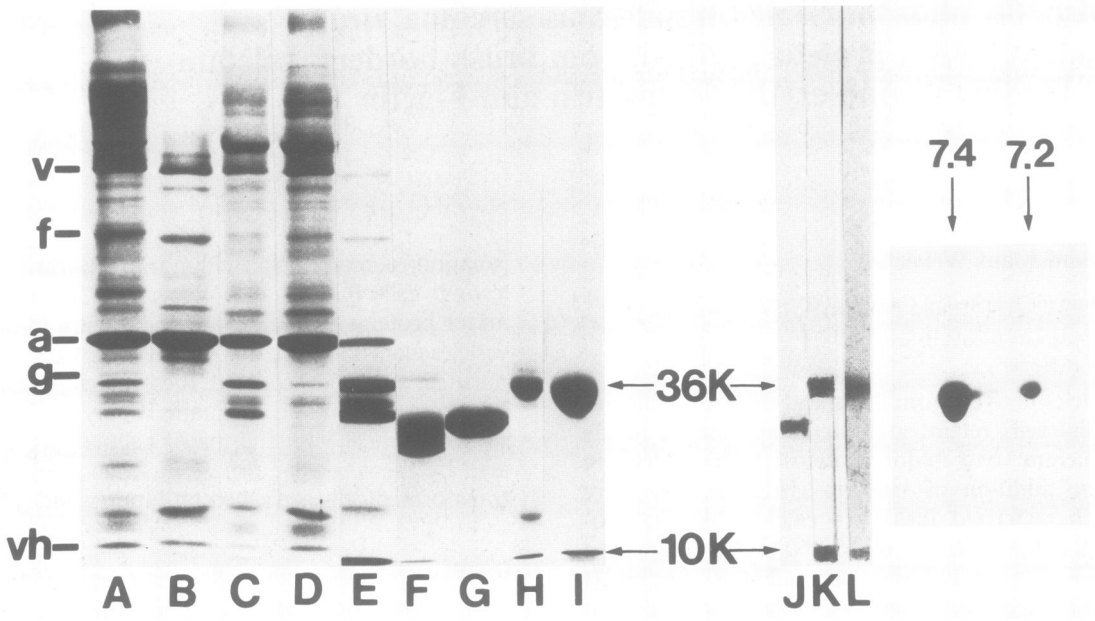

Fig. 1. Purification and immunological characterization of proteins I and II. Demembranated brush border fragments (**lane A**) were washed with buffer A containing Ca^{2+} (see Materials and methods). The supernatant (**lane B**) containing villin, fimbrin and actin was discarded and the pellet (**lane C**) extracted with EGTA in buffer B. After high speed centrifugation proteins I and II were almost absent from the pellet (**lane D**) and enriched in the supernatant (EGTA extract, **lane E**), which was applied to a DE-52 column in buffer C. The column was developed with a salt gradient to provide protein II (**lane F**), which was gel filtered through Sephadex G-100 for final purification (**lane G**). The flow-through fraction of the DE-52 column (**lane H**) was subjected to gel filtration on G-100 and chromatography on CM-52 cellulose (**lane I**) to provide protein I comprised of p36K and p10K. All samples were run on 10% polyacrylamide slab gels. The positions of some brush border proteins and mol. wt. standards are indicated. v: villin (95 000), f: fimbrin (68 000), a: actin (42 000), g: glyceraldehyde phosphate dehydrogenase (37 000), vh: villin headpiece (8600). The EGTA extract (see **lane E**) was electrophoretically transferred to nitrocellulose paper and stained with antibodies against protein II (**lane J**) and protein I (**lane K**). **Lane L** shows the result with antibodies raised against the p36 *sarc* kinase substrate from chick fibroblasts. Note the same reaction pattern in **lanes K** and **L**. The right side of the figure shows the relevant part of a two-dimensional gel analysis on protein I. A major and a minor component of pI ~ 7.4 and 7.2 are seen for the 36-K subunit.

Table I. Amino acid composition (mol%) of protein I, its subunits p36K and p10K, and protein II

Amino acid	Protein I	p36K	p10K	Protein II
Asp	10.9	11.4	10.6	11.9
Thr	5.3	5.1	6.0	4.8
Ser	6.7	10.0	4.0	8.5
Glu	11.8	12.1	11.8	12.3
Pro	3.2	3.1	3.2	1.0
Gly	8.8	8.4	12.2	8.1
Ala	6.5	6.6	5.9	9.0
Val	4.5	4.5	4.6	4.7
Met	3.6	2.3	6.7	3.5
Ile	4.1	4.4	2.8	4.6
Leu	8.8	8.8	7.3	10.4
Tyr	4.1	4.3	1.6	3.7
Phe	4.4	3.5	6.1	3.3
Lys	10.8	9.5	11.9	6.7
His	1.9	1.2	3.1	0.9
Arg	4.6	4.8	2.2	6.6

Tryptophan and cysteine not determined. p36K and p10K were separated under denaturing conditions by chromatography on DE-52 cellulose in the presence of 8 M urea.

dithiothreitol (DTT), 50 mM Tris-HCl, pH 8.5, protein II showed Ca^{2+} binding indicating a dissociation constant of $\sim 2 \times 10^{-5}$ M. Protein I also revealed Ca^{2+} binding in the same buffer but because of a weaker affinity a direct evaluation of the binding constant was postponed, since ligand binding studies with fodrin and F-actin (see below) allowed an approximate assessment.

Protein I is present in many cell types

Rabbit antibodies were raised to both proteins and purified by antigen affinity chromatography. Using Western blots, no immunological cross-reactivity was detected between the two proteins. Antibodies to protein I detected both the p36K and p10K polypeptide (Figure 1). Immunofluorescence microscopy performed on porcine intestinal epithelial cells showed strong labelling of the terminal web by antibodies against protein I (Figure 2). When various cell lines including rat mammary RMCD, rat NRK, hamster BHK21, and mouse 3T3 cells were examined, a submembraneous display of protein I-related antigens was obtained (Figure 2). The patterns were very similar to those found with various antibodies to non-erythroid spectrins (Levine and Willard, 1981; Glenney *et al.*, 1982a; Burridge *et al.*, 1982; Rapasky *et al.*, 1982). Antibodies to protein II gave a general but not structured fluorescence picture.

Ca^{2+}-dependent interaction of protein I with non-erythroid spectrin and F-actin

Given the location of protein I in the cortical layer we searched for its possible binding partners. So far we have concentrated on non-erythroid spectrin and F-actin. A mixture of protein I and fodrin, the major brain spectrin-like molecule, subjected to gel filtration on Sepharose 4B at pH 7.4 in the presence of 2 mM Mg^{2+}, 1 mM EGTA, 10 mM imidazole-HCl, 100 or 150 mM KCl, gave clean separation of the two

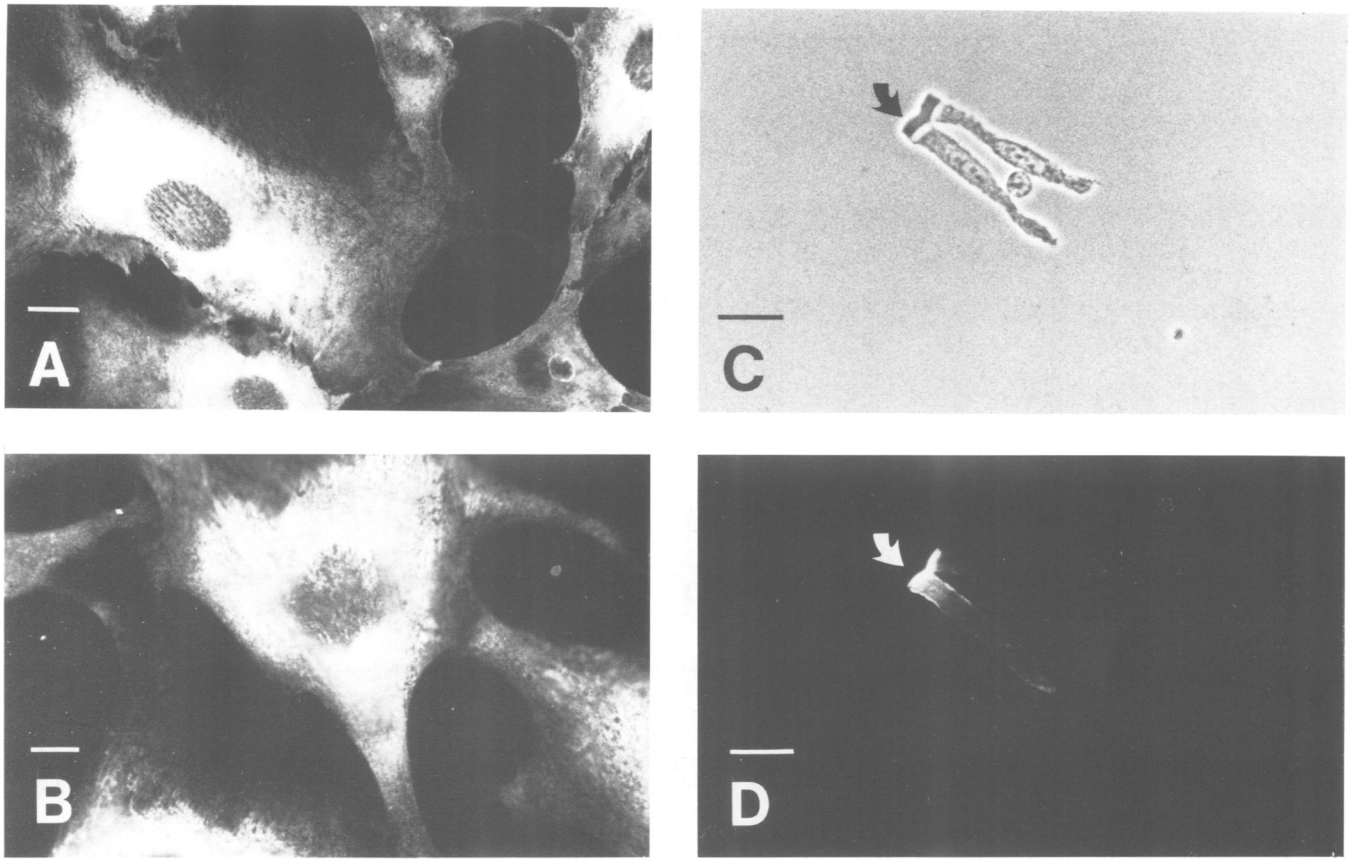

Fig. 2. Immunofluorescence microscopy on cells with anti-protein I and anti-fodrin antibodies. Affinity-purified antibodies to protein I (**A**) and fodrin (**B**) were used to localize the antigens in rat mammary cells. Immunofluorescence staining of isolated pig intestinal cells with anti-protein I is demonstrated in **D**. **C** shows the corresponding phase image. Arrowheads indicate the same relative position at the tips of the microvilli. Note the strong labelling of the terminal web in **D** and the similar submembraneous localization of protein I and fodrin antigens in **A** and **B**. Bars = 15μm.

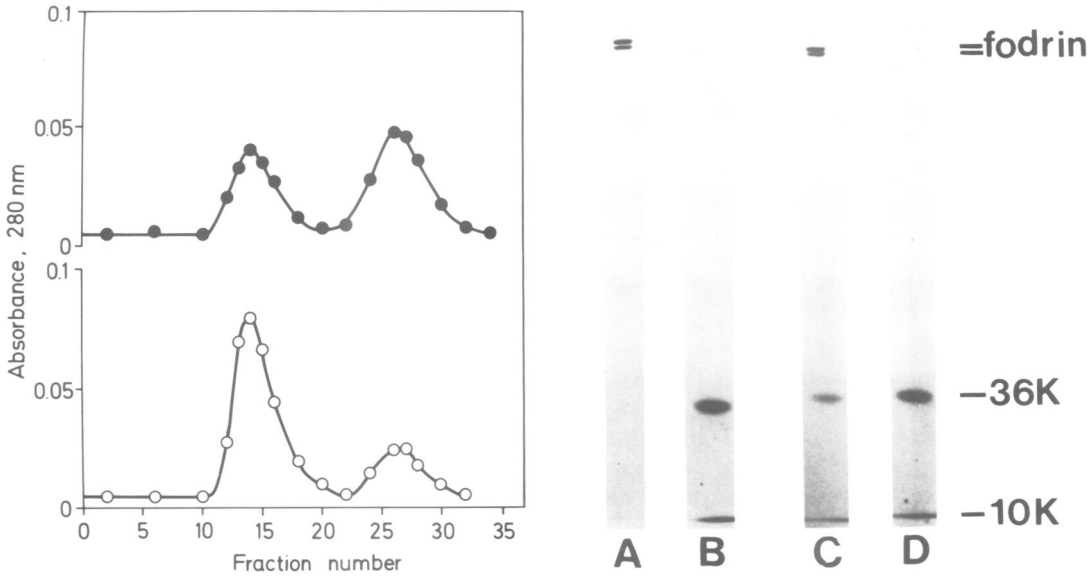

Fig. 3. Interaction of protein I with fodrin. Protein I (1 mg/ml) was mixed with pig brain fodrin (1 mg/ml) and applied at room temperature to gel filtration on Sepharose 4B-Cl in 100 mM KCl, 10 mM imidazole-HCl (pH 7.4), 0.5 mM DTT, 1 mM NaN_3. Fractions were collected, monitored for absorbance at 280 nm and analyzed by SDS-PAGE (**lanes A, B, C, D**). The elution profile in 1 mM EGTA is shown in the upper part (—●—). The polypeptide composition of the first peak (fraction 14) and second peak (fraction 27) are given in **lane A** and **B**, respectively. Gel filtration in 1 mM $CaCl_2$ is given in the second profile (—○—). The corresponding polypeptide compositions of the two peaks are shown in **lane C** (fraction 14) and **lane D** (fraction 27). Note the Ca^{2+}-dependent complex formation documented by SDS-PAGE (compare **lanes A** and **C**) and the increase in absorbance for the first peak. Protein I without fodrin elutes at the position of the second peak in the presence of EGTA or Ca^{2+}.

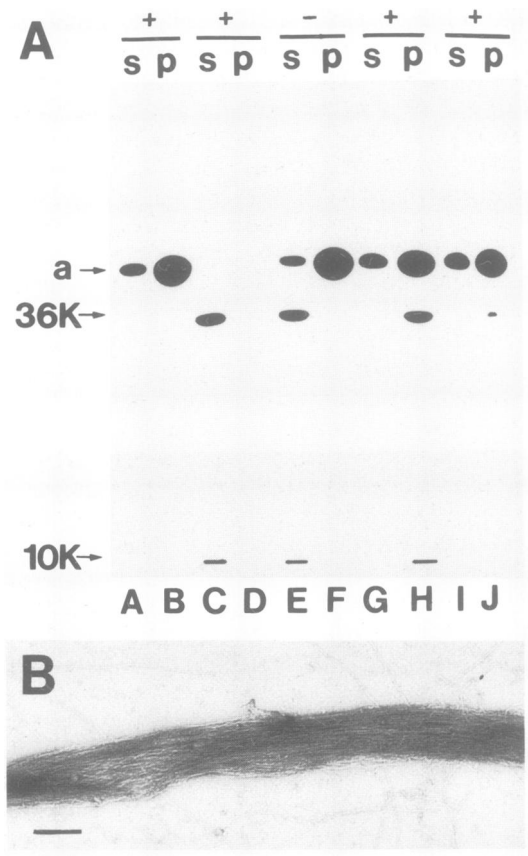

Fig. 4. Ca^{2+}-dependent binding of protein I to actin (**A**) and electron micrograph of the F-actin-protein I complex (**B**). Actin (0.5 mg/ml), protein I (0.1 mg/ml) or actin-protein I mixtures were incubated in 10 mM imidazole-HCl, pH 7.4, 100 mM KCl, 2 mM $MgCl_2$, 1 mM NaN_3 and 0.2 mM DTT containing either 1 mM $CaCl_2$ (+) or 1 mM EGTA (−). After centrifugation for 20 min at 150 000 *g*, equivalent amounts of supernatants (s) and pellets (p) were analyzed by SDS-PAGE. The results with actin and protein I alone are shown in **lanes A, B** and **C, D**, respectively. Mixtures were comprised of actin and protein I at a molar ratio of 5 to 1 (**lanes E, F, G, H**) or 50 to 1 (**lanes I, J**). Note the binding of protein I to F-actin in Ca^{2+}-containing buffers (**lanes G, H** and **I, J**) but the absence of protein I from the F-actin pellet in the absence of calcium (**lanes E, F**). Also note that the 10-K polypeptide moving near the dye front always appears in the same fractions as the 36-K subunit of protein I. The electron micrograph shows negatively stained bundles of F-actin induced by protein I in the presence of Ca^{2+}. Prior to examination in the electron microscope protein I and actin were mixed at a molar ratio of 1 to 5 and incubated at room temperature for 1 h in the presence of 1 mM $CaCl_2$ using the buffer given above. Magnification 40 000 x; bar = 0.2 μm.

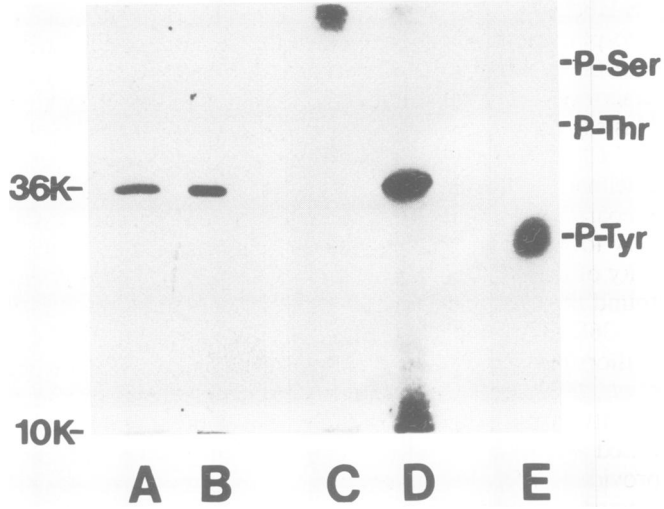

Fig. 5. Immunoprecipitation and phosphoamino acid analysis of protein I from normal and RSV-transformed cells. Normal (**lanes A, C**) and RSV-transformed NRK cells (**lanes B, D**) were labelled overnight with [^{35}S]-methionine (**A, B**) or [^{32}P]phosphate (**C, D**), lysed with Triton X-100 and the EGTA extract immunoprecipitated with protein I antibodies as described in Materials and methods. The immunoprecipitates were subjected to SDS-PAGE (10%) and the labelled polypeptides analyzed by autoradiography. The ^{32}P-labelled and immunoprecipitated 36-K band from the extract of RSV-transformed cells (**lane D**) was hydrolysed and the phosphoamino acids were separated by thin layer electrophoresis and visualized by autoradiography (**lane E**). Indicated marker phosphoamino acids were mixed with the sample and visualized by ninhydrin staining.

proteins without any indication of interaction (Figure 3). When, however, EGTA was substituted by Ca^{2+} (1 mM) protein I became complexed with fodrin. The resulting change of elution position was dependent on the presence of fodrin, since protein I alone eluted as expected at a position corresponding to an 85-K protein (see above). Complex formation between protein I and fodrin was studied under several different buffer conditions. It always required Ca^{2+} but was weakened at lower pH such as 6.5 and suppressed at higher ionic strength such as 500 mM KCl (pH 7.4).

Interaction of protein I with F-actin was analyzed by low and high speed centrifugation assays and monitored by electron microscopy using negative staining (Figure 4). Binding of protein I was Ca^{2+}-dependent and became detectable in the high speed centrifugation assay at a molar ratio of 1 molecule protein I for 50 actin molecules. Increase of the relative concentration of protein I to a value of 1−5 actins resulted in structures which could be harvested by Eppendorf centrifugation. Electron microscopy of such solutions inspected prior to centrifugation revealed closely packed filaments rather similar to the actin bundles induced by the bundling factors villin and fimbrin (Bretscher and Weber, 1980; Bretscher, 1981; Glenney *et al.*, 1981a).

Preliminary experiments designed to assess the free Ca^{2+} concentration necessary for the interaction of protein I with fodrin or F-actin showed that complex formation became observable at a Ca^{2+} concentration of $0.5-1 \times 10^{-4}$ M in the presence of 2 mM Mg^{2+} and was optimally displayed at 5×10^{-4} M or above. Control experiments also showed that complexes were reversibly formed since a further addition of EGTA leading to a free Ca^{2+} concentration of 10^{-6} M or below abolished the binding of protein I to both fodrin and F-actin. So far we have not detected any interaction between protein II and actin or calmodulin.

Immunological cross-reactivity between protein I and the p36K polypeptide phosphorylated upon RSV transformation

Various properties of 36 K present in protein I purified from porcine intestinal cells pointed to a possible identity with the major cellular p36 protein specifically phosphorylated in RSV-transformed cells (see Discussion). In order to decide whether protein I and p36 are indeed the same protein we performed two experiments. First, we used protein I antibodies on extracts of RSV-transformed NRK cells radiolabelled with [^{35}S]methionine and $^{32}PO_4$ (see Materials and methods). Cell fractionation was essentially done as for protein I (see Materials and methods). Immune precipitates collected from the final EGTA supernatant revealed a 36-K phosphoprotein only in RSV-transformed cells, whereas both normal and transformed NRK cells showed immune precipitation of a

^{35}S-labelled 36-K polypeptide (Figure 5). Analysis for phosphoamino acids documented the presence of ^{32}P-labelled phosphotyrosine in p36K from transformed cells (Figure 5) as described in several studies of RSV-transformed chick fibroblasts (Erikson and Erikson, 1980; Radke *et al.*, 1980; Cheng and Chen, 1981; Cooper and Hunter, 1982; Greenberg and Edelman, 1983a). Contrary to these reports the 36 K prepared from RSV-transformed NRK cells showed only a very low level of phosphoserine. This seems to be a peculiarity of the NRK system, since Courtneidge *et al.* (1983) also found that phosphotyrosine is the major phosphoamino acid of p36K in this transformed cell line. In agreement with these authors but in contrast with an earlier suggestion (Ruebsamen *et al.*, 1982) we were unable to detect malate dehydrogenase activity in purified protein I. Second, a rabbit IgG fraction raised against p36K isolated from chicken fibroblasts, kindly provided by Dr. R.Ralston, was used on purified protein I. It reacted strongly with the native protein in the dotting test and, on Western blots, clearly recognized not only the 36-K polypeptide but also the 10-K subunit (Figure 1).

Discussion

We have analyzed the membrane vesicles resulting from cell fractionation in the presence of calcium (Kessler *et al.*, 1976; Schmitz *et al.*, 1973) and thus extended previous studies on the microfilament organization of the intestinal epithelial cell (Weber and Glenney, 1982) to potential actin-binding proteins which depend on Ca^{2+}. Two major cellular proteins which are retained after demembranation of the vesicles in the presence of Ca^{2+} but are released upon subsequent exposure to EGTA were purified. One of these Ca^{2+} binding proteins – protein I – is now characterized by various biochemical properties including its Ca^{2+}-dependent interaction with F-actin and non-erythroid spectrin. Although our results on protein II are still incomplete, the detailed characterization of protein I is of general interest, particularly for those interested in RSV-induced transformation.

During the characterization of protein I we noticed a number of biochemical and cell biological properties very similar to those established recently for a major 36-K protein phosphorylated in RSV-transformed cells. These included a similar polypeptide mol. wt., an isoelectric point of ~7.4 for the larger subunit (Radke and Martin, 1979; Erikson *et al.* 1979), a dimeric character on gel filtration (Erikson and Erikson, 1980) and the inability to bind to DEAE-cellulose in low salt buffer (Erikson and Erikson, 1980; Courtneidge *et al.*, 1983). Additional evidence concerns the cellular location of p36K and protein I (see Results). Earlier indications of an anchorage of p36K to the cytoskeletal matrix or to the inner side of plasma membranes (Cheng and Chen, 1981; Cooper and Hunter, 1982; Amini and Kaji, 1983) were recently extended by immunofluorescence microscopical studies, which pointed to a submembraneous location very similar to that of non-erythroid spectrin (Courtneidge *et al.*, 1983; Nigg *et al.*, 1983; Lehto *et al.*, 1983; Greenberg and Edelman, 1983a, 1983b). Improved cell fractionation studies emphasized again a membrane anchorage (Greenberg and Edelman, 1983b) and the solubilization of p36K by high salt or EDTA seen in this and earlier studies are in agreement with the release of non-erythroid spectrin (fodrin) and the Ca^{2+}-dependent binding of protein I to fodrin and F-actin (see Results). Proof of the expected relationship between p36K, defined as the cellular target of the pp60 *sarc* tyrosine kinase and the 36-K subunit of protein I from the intestinal epithelium, has been obtained by two experiments. First, we found that rabbit antibodies obtained against p36K purified from RSV-transformed chicken fibroblasts reacted with homogeneous protein I and its 36-K subunit. Second, rabbit antibodies against protein I precipitate from cell extracts of RSV-transformed NRK cells a phosphoprotein of mol. wt. 36 K, which in untransformed NRK cells lacks phosphorylation. Analysis of phosphoamino acids revealed, as expected, phosphotyrosine (Radke *et al.*, 1980; Erikson and Erikson, 1980; Cheng and Chen, 1981).

Protein I contains, in addition to the major 36-K subunit, a 10-K polypeptide. This finding could raise the possibility of a proteolytic nicking of a larger polypeptide, particularly since epithelial cells have a wealth of proteases. However, all previous studies on fibroblast p36K have not detected such a putative precursor. Although the presence of a 10-K subunit has not been reported in previous p36K preparations we note that certain gel patterns on immunoprecipitates indicate such a band at or close to the dye front (see Figure 1 in Greenberg and Edelman, 1983a, 1983b). In addition, the rabbit antibody against p36K prepared from chick fibroblasts recognizes in Western blots not only the 36-K but also the 10-K subunit of protein I (Figure 1). The 10-K subunit co-purifies with protein I through gel filtration and cation-exchange chromatography and is retained in the complexes formed between protein I and actin or fodrin. Separation of the two subunits under denaturing conditions followed by renaturation experiments may allow a decision as to which subunits are involved in the different binding activities so far observed with protein I.

The Ca^{2+} binding of protein I and the Ca^{2+} requirement for its interaction with F-actin and non-erythroid spectrin seem in line with the submembraneous location of the protein in a number of cultured cells and its presence in the terminal web of the intestinal epithelial cell. These properties seem also to explain the release of protein I and p36K from crude membrane preparations by EDTA and/or high salt seen in various studies. However, several questions as to the binding activities of protein I remain unresolved. First, certain microfilament-associated proteins and particularly regulatory proteins of F-actin assembly show Ca^{2+} dissociation constants around micromolar levels (Yin and Stossel, 1980; Glenney *et al.*, 1981a, 1981b; Burridge and Feramisco, 1981). This is clearly not the case for protein I, where our preliminary estimates point to a value of the order of 50 – 100 μM in the presence of 2 mM Mg^{2+}. We note, however, that a Ca^{2+}-activated submembraneously located protease thought to be physiologically important in the destruction of the receptor for epidermal growth factor is triggered by an even higher Ca^{2+} requirement (Yeaton *et al.*, 1983). Interestingly this receptor is an intrinsic plasma membrane protein with an associated tyrosine kinase activity (Ushiro and Cohen, 1980), which can rapidly phosphorylate p36K in A-431 cells exposed to the growth factor (Erikson *et al.*, 1981; Hunter and Cooper, 1981). Thus it remains to be seen if certain submembraneous compartments could be regulated by higher local Ca^{2+} concentrations than the micromolar levels assumed to act on other cytoskeletal interactions. Alternatively we cannot yet exclude the possibility that protein I also binds to actin and fodrin in the absence of Ca^{2+} albeit with such a weak affinity that we have been unable to detect this interaction, since the assays used select for high affinity binding. Second, the Ca^{2+}-dependent complexes formed between protein I and actin as well as fodrin need further characterization. We feel that the actin bundles

induced *in vitro* at a high relative molar ratio of protein I are not necessarily a physiological reflection of the F-actin state below the membrane but rather indicate an F-actin binding ability in a minimal component system devoid of other binding proteins. Thus for instance vinculin, thought to connect *in vivo* microfilaments with the plasma membrane, seems to act *in vitro* as a Ca^{2+}-independent bundling protein (see for instance Isenberg *et al.*, 1982). Future experiments have particularly to assess the stoichiometry of the fodrin-protein I complexes, since binding of the dimeric protein I to the tetrameric or dimeric fodrin molecule raises the possibility that protein I cross-links molecules of non-erythroid spectrin. Preliminary evaluation of the observed gel filtration profiles support such a possibility. At least under the experimental conditions used there seems to be more than one molecule of protein I bound per fodrin molecule.

In conclusion, we have shown that a major cellular target of RSV-tyrosine kinase, studied for several years as a possible modulator of tumor virus transformation, can be easily purified in large amounts from a readily available and inexpensive source. Since p36K can be phosphorylated *in vitro* (Erikson and Erikson, 1980) it should now be possible to see if any of the currently known biochemical properties of protein I are modulated by tyrosine phosphorylation.

Materials and methods

Purification of proteins I and II

Pig small intestines were washed with ice-cold 0.14 M NaCl, 10 mM imidazole-HCl, pH 7.3, and then slit lengthwise. The mucosa was scraped off with a glass slide and placed into cold 50 mM mannitol, 2 mM Tris-HCl, pH 7.1. All subsequent steps were carried out at 4°C with 0.25 mM phenylmethylsulfonylfuoride (PMSF) present in every buffer following the Ca^{2+} precipitation (Schmitz *et al.*, 1973). Mucosal scrapings were disrupted in a Waring Blendor model 91-358 operated at full speed twice for 60 s. Solid $CaCl_2$ was added under stirring to a final concentration of 10 mM. After standing for 20 min at 4°C the homogenate was centrifuged first for 15 min at 2900 *g* and then for 40 min at 27 000 *g*. The pellet of brush border membrane fragments was resuspended in buffer A (100 mM KCl, 0.5 mM DTT, 1 mM $CaCl_2$, 1 mM NaN_3, 1% Triton X-100, 10 mM imidazole-HCl, pH 7.4), stirred for 20 min and centrifuged (25 000 *g*, 30 min). The pellet was washed twice in buffer A without Triton and resuspended in buffer B (100 mM KCl, 0.5 mM DTT, 5 mM EGTA, 1 mM NaN_3, 10 mM imidazole-HCl, pH 7.4) by Dounce homogenization. The supernatant of a 45 min spin at 100 000 *g* (EGTA extract) was extensively dialyzed against buffer C (0.5 mM DDT, 1 mM NaN_3, 10 mM imidazole-HCl, pH 7.4), clarified by high speed centrifugation and applied to a DE-52 cellulose column (Whatman) equilibrated in buffer C. Protein I was purified from the unadsorbed fraction of the column (see below). The column was developed with a gradient of 0–300 mM NaCl in buffer C. Protein II eluted at 20–35 mM NaCl. It was concentrated by ammonium sulfate precipitation (0–70%) and dialyzed against buffer D (100 mM NaCl, 0.5 mM DTT, 1 mM NaN_3, 1 mM EGTA, 10 mM imidazole-HCl, pH 7.4). Gel filtration on a Sephadex G-100 column (Pharmacia) in buffer D yielded electrophoretically pure protein II. Protein I, present in the flow through fractions of the DE-52 column, was concentrated by ammonium sulfate precipitation (0–70%), dialyzed and gel filtered through a G-100 column in buffer D. Peak fractions containing protein I were subjected to chromatography on CM-52 cellulose (Whatman) in buffer E (0.5 mM DTT, 1 mM NaN_3, 20 mM Na-acetate/acetic aicd, pH 5.6). Protein I eluted at 0.1–0.15 M NaCl when the column was developed with a gradient of 0–0.5 M NaCl in buffer E.

F-actin and fodrin binding experiments

Actin purified from a bovine skeletal muscle acetone powder was gel filtered through Sephadex G-150. High speed and low speed centrifugation sedimentation assays were performed as described (Glenney *et al.*, 1981a). Fodrin was isolated from pig brain (Glenney *et al.*, 1982b). Binding of protein I to fodrin was monitored by gel filtration. Briefly, protein I and fodrin (both at 1 mg/ml) were mixed in 100 mM KCl, 0.5 mM DTT, 1 mM NaN_3, 10 mM imidazole-HCl at varying pH and free Ca^{2+} concentrations. After incubation at 20°C for 30 min the mixture was applied to a Sepharose 4B-Cl column (0.6 x 26 cm) which was developed at a constant flow rate in the specified buffers. The elution profile was read at 280 nm and fractions were characterized by polyacrylamide gel electrophoresis in the presence of SDS.

Isotopic labelling of NRK cells, immunoprecipitation and phosphoamino acid analysis

Normal rat kidney cells (NRK) and a line of NRK cells transformed by the Schmidt-Ruppin strain of RSV (SR-NRK) were grown 10–16 h in a medium containing either 10 μCi [^{35}S]methionine (1020 Ci/mmol, Amersham) or 50 μCi ^{32}P (Carrier-free, Amersham) in 1 ml. After labelling, cells were washed with 130 mM NaCl, 0.4 mM Na_2HPO_4, 20 mM Tris, pH 7.4, and lysed for 20 min on ice using 100 mM KCl, 0.1 mM DTT, 2 mM $CaCl_2$, 1 mM EDTA, 1% Triton X-100, 1% Aprotinin (Sigma), 10 mM imidazole-HCl, pH 7.4. The remaining insoluble material was extracted with the same buffer but substituting 5 mM EGTA for the 2 mM $CaCl_2$. After centrifugation for 40 min at 100 000 *g* the supernatant was processed for immunoprecipitation using antigen affinity-purified rabbit IgGs directed against protein I. The antigen-antibody complexes were adsorbed to *Staphylococcus aureus* protein A-Sepharose 4B-Cl (Pharmacia). The protein A-Sepharose was washed several times and then boiled in SDS buffer (70 mM Tris-HCl, pH 6.8, 3% SDS plus 5% 2-mercaptoethanol) to release the immunocomplexes, which were analyzed by SDS-PAGE and subsequent autoradiography of the dried gels. ^{32}P-Labelled immunoprecipitates were also lyophilized and subjected to hydrolysis in 6 N HCl for 2 h at 110°C (Karess and Hanafusa, 1981). The dried hydrolysates were dissolved in 5–10 μl of pH 3.5 buffer (pyridine/acetic acid/water, 1:10:189), mixed with standards of phosphoserine, phosphothreonine and phosphotyrosine (Sigma), spotted onto cellulose-coated α-plates (Merck) and run in the pH 3.5 buffer at 900 V for 90 min. The ^{32}P label seen by autoradiography was compared with the ninhydrin staining of the standards.

Miscellaneous techniques

Negative stain analysis of actin bundles was carried out as before (Glenney *et al.*, 1981a, 1981b). Antibodies to purified protein I and II were elicited in rabbits. Monospecific IgGs were selected from the total IgGs by affinity chromatography on the corresponding antigen coupled to Sepharose 4B (Weber *et al.*, 1976). 'Western blotting' of proteins separated in SDS-polyacrylamide gel electrophoresis and electrophoretically transferred to nitrocellulose paper was performed according to Burnette (1981). Instead of radio-iodinated protein A, peroxidase-coupled swine anti-rabbit IgGs (DAKO) were used and visualized by 4-chlor-1-naphthol and perhydrol. Analytical gel filtration experiments were performed at 4°C in a column (0.8 x 70 cm) packed with Sephadex G-100 or Sephacryl S-200 (Pharmacia), equilibrated in 100 mM KCl, 0.5 mM DTT, 1 mM NaN_3, 10 mM imidazole-HCl, pH 7.4, 1 mM $CaCl_2$ or 1 mM EGTA and calibrated with different proteins of known Stokes radii (R_s). Analytical ultracentrifugation studies were carried out as described before (Gerke and Weber, 1983). Protein concentrations were determined by the method of Lowry *et al.* (1951). The antigen affinity-purified rabbit antibodies to fodrin have been described and so has the indirect immunofluorescence microscopy (Glenney *et al.*, 1982a, 1982b).

Acknowledgements

We thank Dr. M.Osborn for helpful discussions and immunofluorescence microscopical results and Dr. J.Wehland for providing the NRK cells. Dr. Robert Ralston, Department of Microbiology and Immunology, University of California, San Francisco, kindly provided a sample of anti-p36K rabbit IgGs. U.Plessmann is thanked for the amino acid analyses. V.Gerke was supported by a Max Planck predoctoral fellowship.

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Received on 26 October 1983