A novel isoform of cytoplasmic actin that binds poly-L-proline

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An actin-like protein was purified to apparent homogeneity from chick-embryo homogenates and chick-embryo fibroblasts by the use of poly-L-proline–agarose affinity chromatography; we therefore refer to this protein as PBP (poly-L-proline-binding protein). PBP binds to deoxyribonuclease–agarose, co-migrates with known actin standards on SDS/polyacrylamide-gel electrophoresis, and has an amino acid composition similar to that of actin. Linear peptide maps after digestion with *Staphylococcus aureus* proteinase reveal its apparent homology with γ -actin; however, isoelectric-focusing experiments show that PBP is clearly more acidic than any of the three major isoforms of actin. PBP polymerizes in the presence of ATP to form fibrillar structures resembling actin paracrystalline aggregates. In chick-embryo fibroblasts, immunofluorescence with antibodies to PBP shows that its distribution is cytoplasmic: perinuclear staining of the cytoplasm, generalized cytoplasmic staining and peripheral fibrillar structures are evident. In contrast, antibodies specific for the (α , γ)-actins reveal the typical stress fibre structures characteristic of fibroblastic cells. PBP appears to constitute a novel isoform of cellular actin, distinct from the known actin isoforms in terms of its lower isoelectric point, its ability to bind poly-L-proline and its distinct subcellular localization.

INTRODUCTION

Actin is a ubiquitous eukaryotic protein, which in higher organisms exists in several distinct highly conserved classes or isoforms. The chemistry of the muscle actins has been studied in considerable detail (for review see Clarke & Spudich, 1977), but, however, the functional significance of multiple non-muscle forms of actin is still obscure. Three major electrophoretic classes of actin have been defined (Garrels & Gibson, 1976; Whalen *et al.*, 1976), one of which predominates in muscle (α) whereas the other two classes, β and γ , predominate in various non-muscle cell lines and tissues (Whalen *et al.*, 1976; Rubenstein & Spudich, 1977). Although the different isoforms of actin show considerable homology, some differences have been demonstrated (Gruenstein & Rich, 1975; Gruenstein *et al.*, 1975; Gordon *et al.*, 1977; Vandekerckhove & Weber, 1978; Cavadore *et al.*, 1985), and at present at least six distinct actin polypeptides have been identified in mammals (Elizaga *et al.*, 1976; Garrels & Gibson, 1976; Vandekerckhove & Weber, 1978). The isoforms are highly conserved across species barriers.

Certain clonal cell lines contain multiple forms of actin (Garrels & Gibson, 1976; Sakiyama *et al.*, 1981), suggesting that the cytoplasmic isoactins may have specialized functions within the cell, analogous to the tissue-specific functions of the muscle isoforms of actin. Immunological studies with isotype-specific antibodies have demonstrated different subcellular distributions of the cytoplasmic actin species (Lubit & Schwartz, 1980; Morgan *et al.*, 1980; Pardo *et al.*, 1983), whereas other data suggest that the isoforms differ in subcellular organization (Rubenstein *et al.*, 1982) or in developmental

distribution (Storti *et al.*, 1976). Furthermore, novel isoforms of non-muscle actin have been reported (Bremer *et al.*, 1981; Kumar *et al.*, 1984) that appear to be nuclear rather than cytoplasmic. Selective distribution of the isoactins appears likely; however, in order for the isoactins to control distinct aspects of intracellular movement, they must also be subject to separate control mechanisms.

Traditional methods of actin purification rely on cycles of polymerization and depolymerization, which result in overall low yields and which could potentially select against the recovery of minor species. The purification of actin by virtue of its ability to bind DNAase I (Lindberg & Eriksson, 1971; Lazarides & Lindberg, 1974) has helped to surmount this problem, but it is not yet clear whether all existing isoforms of actin have been recognized. The studies described in the present paper indicate that a previously uncharacterized isoform of actin can be purified by its affinity for PLP, an affinity that other isoforms of actin do not possess. In addition to its unique binding properties, this novel actin exhibits a lower isoelectric point than the major actin isoforms, and furthermore shows a subcellular distribution distinct from that of (α , γ)-actin.

MATERIALS AND METHODS

Purification of PBP from chick embryos

Chick embryos (13-day) were homogenized in an equal volume of buffer A (0.01 M-Tris/0.2 M-NaCl/0.1 M-glycine/10 μ M-dithiothreitol, pH 7.8 at 4 °C), fractionated with (NH₄)₂SO₄ and applied to a PLP–agarose affinity column as described previously (Kedersha & Berg,

Abbreviations used: PLP, poly-L-proline; PBP, poly-L-proline-binding protein; DNAase, deoxyribonuclease.

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Fig. 1. Tandem DEAE-cellulose/concanavalin A-Sepharose chromatography of PLP-agarose affinity-column eluate

Chick-embryo homogenate was prepared, affinity-purified and subjected to tandem ion-exchange-concanavalin A-Sepharose chromatography as described in the text. The A_{230} of the material eluted from the DEAE-cellulose column that was not trapped by the in-line concanavalin A-Sepharose column is shown as $\circ$ — $\circ$. ---- indicates the NaCl gradient used to elute the columns. Roman numerals refer to peaks, and Arabic numerals indicate fractions that were sampled and subjected to SDS/polyacrylamide-gel electrophoresis as shown in Fig. 2.

1981). The eluate was dialysed against buffer B (0.05 M-Tris/0.05 M-NaCl/0.05 M-glycine/10 μ M-dithiothreitol/1.0 mM- CaCl_2 , pH 7.4 at 4 °C, then applied to a DEAE-cellulose (Whatman) column (1.5 cm $\times$ 10.0 cm). The ion-exchange column was washed, then connected to a second column containing concanavalin A-Sepharose (bed volume 3 ml, previously equilibrated with buffer B). A linear gradient of 0.05–0.35 M-NaCl in buffer B was applied to the linked columns. As described previously (Kedersha & Berg, 1981), this gradient was sufficient to elute both prolyl hydroxylase and PBP from the DEAE-cellulose; however, the glycoprotein prolyl hydroxylase was retained by the concanavalin A-Sepharose, whereas the PBP was not. The PBP thus obtained appeared quite pure by SDS/polyacrylamide-gel electrophoresis (Fig. 2).

Binding studies

Cells were isolated from 17-day chick-embryo tendons as described previously (Kao *et al.*, 1977) and frozen. Thawed cells were homogenized, by ten strokes of a Teflon/glass tissue grinder, in either buffer A (see above) or buffer G (0.01 M-Tris/0.2 mM- CaCl_2 /0.2 mM-ATP/10 μ M-dithiothreitol, pH 7.4 at 4 °C), to yield a final concentration of 10^8 cells/ml. The homogenates were clarified by centrifugation (20000 *g* for 30 min), and 0.5 ml portions of the resulting supernatant were incubated with either PLP-agarose or DNAase-agarose. All incubations were performed in Microfuge tubes at 4 °C for 4–16 h, after which the tubes were centrifuged (12000 *g*) for 5 min and the supernatants removed. The affinity-gel pellets were washed three times with buffer C (0.01 M-Tris/0.15 M-NaCl/0.5% Nonidet P40/0.5% sodium deoxycholate/0.01% SDS, pH 7.4 at 4 °C), and the final pellets were boiled for 5 min in slab-gel sample buffer containing 5.0% 2-mercaptoethanol. The extracted material was then applied to a 10% polyacrylamide slab gel (Laemmli, 1970).

Fig. 2. SDS/polyacrylamide-gel electrophoresis of the material eluted from the columns indicated in Fig. 1

Lane 1, affinity-purified starting material; lane 2, material that did not bind to the DEAE-cellulose column and appeared in the void volume; lane 3, fraction 34; lane 4, fraction 38; lane 5, fraction 42; lane 6, fraction 49. PH- α and PH- β indicate the migration positions of the α -subunit and β -subunit of prolyl hydroxylase respectively.

Peptide mapping

Chicken gizzard actin (kindly supplied by Dr. Susan Craig) and purified chick PBP were iodinated by use of an immobilized enzyme system (Enzymobeads; Bio-Rad Laboratories) and subjected to one-dimensional peptide

Fig. 4. Binding studies using serial PLP-agarose and DNAase-agarose treatment of the same chick-embryo-fibroblast extracts

In these experiments chick-embryo-fibroblast extracts were prepared and precipitated first with one affinity gel, then the other, and subjected to SDS/polyacrylamide-gel electrophoresis. Lane 1, chick-embryo-fibroblast extract in buffer G from which all PLP-binding material was removed, treated with DNAase-agarose; lane 2, post-PLP-agarose extract in buffer A, treated with DNAase-agarose; lane 3, post-PLP-agarose extract supplemented with 0.2 mM-ATP and 0.2 mM- CaCl_2 , treated with DNAase-agarose; lane 4, post-DNAase-agarose extract in buffer G, treated with PLP-agarose; lane 5, post-DNAase-agarose extract in buffer G supplemented with 5% formamide, treated with PLP-agarose; lane 6, chick-embryo-fibroblast extract in buffer A, treated with DNAase-agarose. Lanes 7–11 contain material purified from constant amounts of chick-embryo-fibroblast extract in buffer A, treated with increasing amounts of PLP-agarose; lane 7, 50 μl of PLP-agarose suspension; lane 8, 100 μl of PLP-agarose suspension; lane 9, 150 μl of PLP-agarose suspension; lane 10, 200 μl of PLP-agarose suspension; lane 11, 250 μl of PLP-agarose suspension. Lane 12, post-PLP-agarose material treated with DNAase-agarose; lane 13, chick-embryo-fibroblast extract in buffer G, treated with PLP-agarose; lane 14, purified PBP in buffer A, treated with PLP-agarose; lane 15, purified PBP in buffer G, treated with DNAase-agarose; lane 16, purified PBP standard; lane 17, chick-embryo-fibroblast extract supplemented with 3 mg of PLP/ml, treated with DNAase-agarose. PH- α and PH- β indicate the migration positions of the α -subunit and β -subunit of prolyl hydroxylase respectively.

were focused for 16 h at 400 V and for 2 h at 800 V, removed from the tubes, and both fixed and washed with several changes of methanol/acetic acid/water (1:1:8, by vol.) to remove the Ampholines, then stained with Coomassie Brilliant Blue R.

Amino acid analysis of PBP

Purified PBP was hydrolysed in 6 M-HCl containing 0.2% (v/v) 2-mercaptoethanol for 16 h at 116 °C. The sample was evaporated to dryness and resuspended in 0.067 M-sodium citrate buffer, pH 2.5, and analysed on a JEOL model 6H amino acid analyser containing Durrum ion-exchange resin DC6A and with the three-buffer Pico Buffer II system commercially available (Pierce Chemical Co.). Loss of serine and threonine during the hydrolysis was corrected for in tabulating the results; however, no adjustment was made for partial loss of methionine.

Antibody preparation

Antibodies to PBP were prepared in three rabbits as follows. Approx. 200 μg of PBP was emulsified in Freund's complete adjuvant (GIBCO) and injected into the leg muscle of a male New Zealand White rabbit. Subsequent boosters were made with Freund's incomplete

adjuvant and were administered every 4 weeks. Rabbits were bled from an ear vein 7–10 days after each booster, the sera collected and antibodies purified by passage of the sera over a PBP-agarose column, made by coupling 1 mg of PBP to 25 ml of CNBr-activated agarose (Porath *et al.*, 1967). The column was washed with phosphate-buffered saline (0.50 M-sodium phosphate/0.15 M-NaCl, pH 7.4) and the antibodies eluted with 3 M-NaCNS in phosphate-buffered saline, dialysed extensively against phosphate-buffered saline and stored frozen until use.

Electron microscopy

Purified PBP was dialysed against buffer containing 2 mM-Tris/HCl, 0.2 mM-ATP, 0.5 mM-2-mercaptoethanol and 0.2 mM- CaCl_2 , pH 8.0, at 25 °C as described by Spudich & Watt (1971). The dialysed material was centrifuged at 100 000 g for 1 h, and concentrated solutions of KCl and MgCl_2 were added to achieve final concentrations of 50 mM and 2 mM respectively. The solution was incubated at room temperature for 30 min, then applied to carbon-coated Parlodian grids and negatively stained with 1% (w/v) phosphotungstic acid, pH 7.4. The specimens were examined on a JEOL JEM-100B electron microscope.

Fig. 5. Linear SDS/polyacrylamide-gel-electrophoresis peptide maps of PBP and known actins

[^{125}I]Iodinated PBP and chicken gizzard actin were digested with *S. aureus* V8 proteinase during electrophoresis as described in the text. The lanes were scanned with a densitometer and the tracings aligned for comparison. Inset: autoradiogram of the linear peptide map.

Immunofluorescence

Secondary cultures of chick-embryo tendon fibroblasts were sparsely plated on coverslips and grown for 1–3 days in Dulbecco's medium (GIBCO) containing 4% (v/v) foetal-calf serum. Cells were fixed in phosphate-buffered saline containing 3.5% (v/v) formaldehyde at room temperature for 20 min, washed twice (5 min each) in phosphate-buffered saline, permeabilized by a 5 min immersion in methanol at -20°C and air-dried. The coverslips were incubated with antibodies (10–20 μg) at 37°C for 90 min, washed three times (5 min each) with phosphate-buffered saline and incubated for 60 min at 37°C in 30 μl of a 1:10 dilution of goat anti-(rabbit immunoglobulin) antibody conjugated to fluorescein (Behring Diagnostics, American Hoechst Corporation). Coverslips were washed as before, air-dried, mounted in

glycerol and viewed on a Zeiss photomicroscope equipped with epifluorescence illumination.

RESULTS

Purification of PBP

By use of a combination of affinity chromatography, ion-exchange chromatography and lectin affinity chromatography (see Fig. 1), PBP was purified to apparent homogeneity (Fig. 2, lanes 3, 4 and 5). As previously shown, the initial PLP-agarose column eluate contained very few species by SDS/polyacrylamide-gel electrophoresis (Fig. 2, lane 1), two of which were identified as the non-identical subunits of prolyl hydroxylase (Berg & Prockop, 1973). The combined DEAE-cellulose/concanavalin A-Sepharose columns removed all of the contaminating species and resulted in a single species on SDS/polyacrylamide-gel electrophoresis (Fig. 2, lanes 3, 4 and 5).

Fig. 6. Isoelectric focusing of PBP and actin standards

Samples were subjected to isoelectric focusing as described in the text. Lane 1, rabbit skeletal-muscle actin; lanes 2 and 3, chicken gizzard actin; lane 4, chick-embryo PBP. The α -, β - and γ -isoforms of the actin standards are indicated. Anode is at the bottom.

Characterization of PBP

The apparent M_r of PBP as determined by SDS/polyacrylamide-gel electrophoresis (Fig. 2) was identical with that of both rabbit skeletal-muscle actin and chicken gizzard actin (results not shown). When compared with protein standards, the M_r was estimated to be 45000. PBP was also found to resemble the rabbit and chicken skeletal-muscle actins in terms of amino acid composition (Table 1). The major significant difference in amino acid content was that PBP contained more histidine and arginine than did either of the skeletal-muscle actins. Although PBP appeared to contain less methionine than did the actins, it is very probable that losses occurred during hydrolysis, as no precautions were taken to prevent the oxidation of methionine.

The binding properties of PBP and of the non-muscle actins with respect to PLP and to DNAase were investigated by using batch adsorption on agarose affinity gels, to which either PLP or DNAase had been conjugated by CNBr activation. Both crude chick tendon-cell homogenates and purified PBP were used. In all cases the quantity of PLP-agarose used was in excess of that required to bind all the available PBP, as demonstrated by treating equal amounts of cell extracts with increasing amounts of PLP-agarose (Fig. 4, lanes 7–11). As indicated in Fig. 3, approximately equal amounts of PBP were detected in cell homogenates regardless of the buffer conditions used for extraction (Fig. 3, lanes 1 and 7). The addition of 0.2 mM-ATP and 0.2 mM- CaCl_2 to the media did not alter the amount of PBP detected (lane 3), nor did the addition of 5 mM-EDTA. However, the addition of 0.2 mM- CuNO_3 to the cell homogenate completely blocked PBP-PLP

binding; it also prevented the binding of prolyl hydroxylase (lane 4). The addition of 5% (v/v) formamide to the cell homogenate seemed to diminish slightly the amount of PBP bound to PLP-agarose (Fig. 3, lane 6). When equal amounts of cell extracts were treated with DNAase-agarose, considerably more actin-like material was recovered (Fig. 3, lanes 8–10) than in similar extracts treated with PLP-agarose. In order to ensure that quantitative amounts of actin were recovered by this technique, increasing amounts of formamide were used in order to ensure the complete depolymerization of actin (Zechel, 1980) into the monomer (DNAase-binding) form. No increase in the amount of bound actin was seen when increasing amounts of formamide were used (Fig. 3, lanes 8–10).

Further binding studies (Fig. 4) indicated that increasing amounts of PLP-agarose precipitated only a finite amount of PBP (lanes 7–11). The post-PLP-agarose supernatants were shown to contain large amounts of DNAase-binding actin-like material (lanes 1 and 12), indicating that most of the cellular actin does not bind PLP. However, purified PBP was shown to bind DNAase (lane 15); this binding was not affected by the addition of 3 mg of PLP/ml (lane 17), which is sufficient to prevent the binding of PBP to PLP-agarose. It therefore appears that PBP contains two independent binding sites: one for DNAase and another for PLP. The known actins bind only DNAase; they do not bind to PLP-agarose. This suggests that PBP constitutes a minor species of cellular actin that, unlike the bulk of cellular actin, binds PLP.

PBP and actin were examined for detectable structural differences by linear peptide mapping according to the method of Cleveland *et al.* (1977). PBP and chicken

Fig. 7. Electron microscopy of polymerized PBP

Purified soluble PBP was polymerized as described in the text, and the sample was negatively stained with phosphotungstic acid. Bar indicates 100 nm.

gizzard actin yielded identical peptide maps (Fig. 5). Slight differences between PBP and rabbit skeletal-muscle actin were noted (results not shown).

Isoelectric focusing revealed that PBP appears to consist of at least two major electrophoretically distinct species, both of which possess lower isoelectric points than any of the known actins (Fig. 6). This result was consistently obtained with several separate preparations of PBP; the same result was obtained with PBP and actin that had been affinity-purified from the same cell homogenate (results not shown). Typical pI values observed for PBP were 5.7 to 5.9; rabbit skeletal-muscle actin showed a pI of 6.2, and chicken gizzard actin gave a pI value of 6.4. PBP purified from cells labelled for 4 h with [32 P]P_i contained no detectable radioactivity (results not shown), so it does not seem likely that phosphorylation accounts for this electrophoretic difference.

Polymerization of PBP to form fibrils

The ability of PBP to polymerize to form microfibrillar structures similar to those formed by the known actins was investigated. Polymerized structures were obtained (Fig. 7); however, they formed aggregated fibrils rather than the discrete fibrils considered representative of polymerized muscle actins. The diameter of the individual filaments within the aggregates (6 nm) was in the range characteristic of actin filaments. The polymerized PBP therefore resembled actin 'paracrystalline aggregates' (Abramowitz *et al.*, 1975; Bray & Thomas, 1976). These results show that PBP clearly polymerizes into actin-like structures; however, further studies are necessary to characterize its polymerization characteristics and the polymerized form(s) of PBP in detail.

Immunological studies

Antibodies were prepared against PBP affinity-purified as described above. Characterization of the affinity-purified antibodies revealed the following: by using Western-blot analysis, no cross-reactivity was observed with denatured rabbit skeletal-muscle actin, whereas the antibody strongly reacted with denatured chicken gizzard actin (results not shown). The anti-PBP antibodies and antibodies specific for (α , γ)-actin (Pardo *et al.*, 1983) were used to stain cultured chick tendon fibroblasts (Figs. 8 and 9). The immunofluorescent patterns obtained with the different antibodies were markedly different (Figs. 8 and 9). The anti-(α , γ)-actin antibodies revealed the expected pattern of stress fibres classically obtained during staining of fibroblasts for actin (Fig. 8). In contrast, anti-PBP antibodies did not show well-defined stress fibres, but instead stained the cytoplasm and brightly outlined the nucleus and cytoplasmic vesicles (see Fig. 9). In some cells a peripheral fibrous pattern was observed. This staining pattern suggests that PBP is present largely as free monomers or in very small filaments beyond the resolving power of light microscopy. It is also possible that PBP is associated with cellular organelles, causing the perinuclear ring and the brightly highlighted perinuclear vesicles. In either case it appears clear that PBP and γ -actin are segregated into morphologically distinct structures within the cell.

DISCUSSION

PBP may be classified as an actin according to several criteria: M_r , amino acid composition, peptide maps,

DNAase binding and its ability to polymerize into actin-like filaments. Its isoelectric point is at least 0.4 pH unit lower than that of the most acidic isoform (α) of the known actins, suggesting that it constitutes a novel isoform(s) of actin physically distinguishable from the known actins. Binding studies indicate that the affinity of PBP for PLP is a function unique to PBP, and not a general characteristic of the actins as is DNAase binding, the functional significance of which is unknown. It therefore appears that PBP is both physically and functionally distinct from the known actins, physically characterized by its lower isoelectric point and functionally characterized by its ability to bind PLP.

From the ratio of PBP to non-PBP actins found in the same sample (Fig. 4, lanes 11 and 12), it appears that PBP is a minor species of actin comprising less than 10% of the total amount of actin found in fibroblasts; such a minor species of non-muscle actin might have previously escaped detection (Ponte *et al.*, 1983). In addition, the subcellular organization of PBP may preclude its purification by routine actin purification schemes, as it appears to be partially associated with the nuclear envelope and with perinuclear vesicles (Fig. 9). Other forms of actin have been shown to possess an affinity for unfractionated cell membranes (Gruenstein *et al.*, 1975) and for lysosomal membranes (Mehrabian *et al.*, 1984). If PBP represents an isoform of actin that preferentially binds to such membranous structures, as suggested by the immunofluorescence data, it might be preferentially lost from conventional cytoplasmic actin preparations by the high-speed centrifugation steps that precede actin purification by DNAase-agarose chromatography.

PLP does not inhibit the binding of PBP to DNAase (Fig. 4, lane 17), suggesting that the PBP binding sites for PLP and DNAase are independent. PLP has an unusual conformation in solution, possessing an extended rod-like structure (Carver & Blout, 1967). This conformation is sufficiently similar to that of collagen to enable PLP to serve as an affinity ligand for prolyl hydroxylase, an enzyme involved in collagen biosynthesis (Tuderman *et al.*, 1975). PBP does not bind *Ascaris* cuticle collagen, which contains numerous Xaa-Pro-Gly sequences (Berg & Prockop, 1973) typical of all collagens, although it strongly binds to PLP-agarose under identical buffer conditions. This suggests that PBP interacts specifically with polyproline sequences rather than collagen-like sequences, and that a PBP-binding protein might possess a proline-rich domain.

The existence of the distinct actin isoforms is thought to reflect functional specificities that have thus far not been elucidated. This hypothesis requires not only that the different isoforms are subject to some sort of specific regulation, but also that they control separate aspects of cell movement, suggesting that the different isoforms are associated with specific organelles or structures within the cell. Several studies have shown that different isoforms may indeed be located in preferential intracellular regions (Lubit & Schwartz, 1980; Bremer *et al.*, 1981; Rubenstein *et al.*, 1982; Pardo *et al.*, 1983; Kumar *et al.*, 1984). PBP is of special interest as it constitutes the first actin species that exhibits a specific binding ability in addition to a distinct subcellular localization. Its resolution into multiple species by isoelectric focusing suggests that it may in fact comprise more than a single species.

Cells stained with anti-(α,γ)-actin antibody

Fig. 8. Immunofluorescence of chick-embryo fibroblasts stained with anti-(α,γ)-actin antibody

Cells were grown on glass slides, and half of each slide was stained with affinity-purified PBP IgG and the other half was stained with affinity-purified anti-(α,γ)-actin antibodies. Fluorescein-conjugated goat anti-(rabbit immunoglobulin) antibodies were used to locate the sites of primary antibody binding. Non-immune controls showed essentially no fluorescence.

Cells stained with anti-PBP antibody

Fig. 9. Immunofluorescence of chick-embryo fibroblasts stained with affinity-purified anti-PBP antibody

Cells were prepared and stained as described in the legend to Fig. 8, differing only in the antibodies used.

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