

Some Properties of Spectrin-Like Proteins from *Pisum sativum*

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Z. Naturforsch. **52c**, 180–186 (1997); received August 11/November 22, 1996

Plant Spectrin, Spectrin (Fodrin), Membrane Skeleton, Ankyrin-, Ca^{2+} -Calmodulin-,
Actin-Binding

Proteins cross-reacting with antibodies directed against α - and β -spectrin were recently detected in plant cells. In this report we have studied the ability of these proteins to interact with other components of membrane skeleton such as ankyrin, f-actin and calmodulin. It was found that the polypeptide of high molecular weight reacting with anti- α -spectrin antibody binds calmodulin in Ca^{2+} -dependent manner. Protein complexes containing polypeptides cross-reacting with anti-spectrin antibodies interact with muscle f-actin (in co-sedimentation assay) and with erythrocyte ankyrin (ELISA-type assay). These data further substantiate a possibility of occurrence of spectrin-based membrane skeleton in higher plant cells.

Introduction

Spectrin, the main peripheral protein of the red blood cell membrane, present also in other cells of many animal species, both vertebrates and invertebrates (Goodman *et al.*, 1981; review: Hartwig, 1995) was also found in several protozoan cells (Alcina *et al.*, 1988; Choi and Yeon, 1989; Kwiatkowska and Sobota, 1992; Pollard, 1984; Schneider *et al.*, 1988). Structural and functional similarities occurring between spectrin and several other proteins became a basis of a family of homologous proteins: spectrin- α -actinin-dystrophin (for a review see Hartwig 1995). Major features of these proteins are: a presence of 106–120 amino acid residue repeated sequences of triple α -helical structure (Yan *et al.*, 1993). Spectrin α subunit contains 20 homologous 106 amino acid residue repeats (segments $\alpha 10$ and C-terminal 22 do not fit to this structural motif) while β -subunit contains 17 such segments (Wasenius *et al.*, 1989; Winkelman *et al.*, 1990a; Winkelman *et al.*, 1990b; Sahr *et al.*, 1990; Ma *et al.*, 1993; Matsudaira, 1991). Alpha-actinin contains 4 repeat segments consisting of 122 amino acid residues and dystrophin contains 24–25 segments homologous to 106 residue repeat segments of spectrin (Davison and Critchley, 1988; Byers *et al.*, 1989). The second fea-

ture of these proteins is the presence of EF-hand domains (i.e. domains homologous to the calcium-binding domain of calmodulin). The third characteristic property of these proteins is the presence of highly conserved N-terminal domain responsible for binding of actin filaments (Karinch *et al.*, 1990). Moreover, this domain is present also in other actin-binding proteins as fimbrin that contains two actin-binding domains in tandem without inserted sequence, and in so called ABP-280 and ABP-120, high molecular weight actin-binding proteins with repeated segments, that are unrelated to the 106-residue repeats of spectrin (for detailed reviews see Matsudaira, 1991 and Hartwig, 1995).

In addition to the above mentioned f-actin binding domains animal spectrins also contain in the β -subunits conservative ankyrin-binding sequence (Kennedy *et al.*, 1991). Most of animal α -subunits contain a calmodulin binding site (Leto *et al.*, 1989) (only red blood cell spectrin contains a calmodulin binding site in β -subunit (Sears *et al.*, 1986)). Segment $\alpha 10$ has a short stretch of amino acids which is homologous to the regulatory, SH3 domain of the src family of proteins (Sahr *et al.*, 1990).

The physiological role of erythrocyte spectrin seems to be more obvious in comparison to spectrins from non-erythroid cells. The red blood cell spectrin acts as a support for the plasma membrane, which is responsible for its elasticity and stability. Non-erythroid spectrins differ from ery-

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throid ones in their distribution inside the cell. Apart from plasma membrane the non-erythroid spectrins are present in the cytosol, suggesting also other than membrane stabilising function(s) of these proteins (Schatten *et al.*, 1986; Bonder *et al.*, 1989; Fowler and Ermone, 1992; Pescareta *et al.*, 1989; Black *et al.*, 1988).

In the nineties, several reports have appeared indicating the presence of spectrin, or rather polypeptides sharing antigenic determinants with spectrin in higher and lower plants (Michaud *et al.*, 1991; DeRuijter and Emons, 1993; Faraday and Spanswick, 1993; Sikorski *et al.*, 1993; Lorenz *et al.*, 1995) as well as in fungal (Kaminskyj and Heath, 1995) and bacterial (Bisikirska *et al.*, 1996) cells. However, the evidence supporting the existence of spectrin-like membrane skeletal proteins in nonanimal organisms is based so far mainly on the immunoreactivity of these polypeptides. In the present study we report the data on interactions of the spectrin immunoanalogues from higher plant cells with other proteins such as a calmodulin, actin and ankyrin.

Materials and Methods

Plant material

For experiments we used two types of plant material: 1. dry pea (*Pisum sativum*) seeds and/or 2. roots and leaves from 12-day old pea plants grown as previously described (Sikorski *et al.*, 1993).

Plant cell extracts

The plant tissue was homogenised in low ionic strength buffer containing 0.1 mM EDTA (pH 7.6), 1 mM PMSF*, leupeptin (100 µg/ml), pepstatin A (100 µg/ml), 1 mM iodoacetamide (1:5 w/v) (Sigma Chem. Co.) and extracted at 0 °C for 1 hour. Homogenates were centrifuged at 20 000 x g for 30 min. Supernatants were used for experiments.

* Abbreviations: PMSF, phenylmethylsulfonyl fluoride; SDS, sodium lauryl sulfate; SDS-PAGE, SDS-polyacrylamide gel electrophoresis; TPBS, Tween 20 containing phosphate buffered saline.

Antibodies

Polyclonal antibodies were raised in rabbits against red blood cell spectrin subunits were obtained as was previously described. The characteristics of the specificity of these antibodies was also presented previously (Lorenz *et al.*, 1995). For experiments unfractionated sera were used.

Calmodulin-binding assay

Biotinylated calmodulin (Gibco BRL) was used for the detection of calcium/calmodulin-binding proteins immobilised on nitrocellulose filter in analysis according to the procedure described in manufacturer's manual.

Actin co-sedimentation assay

Rabbit skeletal muscle actin was prepared from acetone-extracted skeletal muscles according to Spudich and Watt (1971). Actin-binding assay was performed by cosedimentation with preformed f-actin (repolymerised at 3 mg/ml by incubation in 10 mM Tris-HCl, pH 7.4, 130 mM KCl, 20 mM NaCl, 2 mM MgCl₂, 1 mM β-mercaptoethanol) according to methods described by others (Karinich *et al.*, 1990; Mische *et al.*, 1987). Actin (0.5 ml) and the extract of pea seeds (0.5 ml, 4.5 mg/ml) were mixed and layered onto 2.5 ml 10% sucrose cushion in centrifuge tubes and incubated at room temperature for 1 hour. After incubation, the samples were centrifuged at 100 000 x g for 30 min at 4 °C. Pellets and supernatants were analysed by SDS-PAGE and immunoblotting.

Ankyrin-binding assay by ELISA-type test

Erythrocyte ankyrin (3 µg) in 50 mM sodium carbonate buffer, pH 9.6 was dispensed into each well of a 96-well microtiter ELISA plate (Corning) and incubated at 30 °C for 2 h. After incubation, the wells were washed three times with phosphate-buffered saline containing 0.05% Tween 20 (TPBS) and then incubated for 1 h at room temperature with 5% non-fat dry milk dissolved in TPBS. The wells were washed three times with TPBS. To each well various concentrations of pea seeds extract proteins were added and incubated at room temp. for 2 h. After washing (3x with TPBS) to each well 100 µl of anti-α-spectrin antibodies (1:1000) were added and incubated for 1 h.

The wells were washed again (3 x with TPBS) and to each well 100 µl of goat anti-rabbit IgG conjugated to horseradish peroxidase (1:10 000) was added. After incubation (1 h, room temp.) the wells were washed (3 x with TPBS). The colour reaction was developed by incubation with a solution of 0.04 mg/ml of freshly prepared o-phenylenediamine dissolved in 0.1 M sodium citrate buffer, pH 5.0 containing 0.01% H₂O₂ for 30 min in the dark. The reaction was stopped with 100 µl of 1 N sulphuric acid and absorbance was read at 493 nm. Control samples were performed in the same way except that plant extract was omitted. Erythrocyte ankyrin prepared as described previously (Białkowska *et al.*, 1994), was a gift from Dr. K. Białkowska, Institute of Biochemistry, University of Wrocław.

Electrophoresis and immunoblotting

SDS-polyacrylamide gel electrophoresis and subsequent electrophoretic transfer onto nitrocellulose filters (Gelman Sci., Ann Arbor USA) in solution containing 200 mM glycine-NaOH, 20% methanol and 0.01% SDS, pH 9.2 were carried out according to the methods mentioned previously (Sikorski *et al.*, 1993; Lorenz *et al.*, 1995). Immune reaction was carried out using rabbit antibodies raised against to erythrocyte α - and β -spectrin subunits followed by peroxidase-conjugated goat anti-rabbit antibodies (Sigma Chem. Co.).

Results and Discussion

As previously described (Sikorski *et al.*, 1993), the cells of pea (*Pisum sativum*) contain protein immuno analogues of erythrocyte spectrin. Immunoblotting analysis showed the presence of analogues both α - and β -subunits of spectrin molecule. Moreover, it was shown that this protein(s) could be extracted with low ionic strength solution and are associated with the membranes (Sikorski *et al.*, 1993; Lorenz *et al.*, 1995). When low ionic strength solution extracts from seeds, young roots and leaves were analysed in SDS-polyacrylamide gel electrophoresis and immunoblotting each extract contained many polypeptide fractions (up to 300 kD) which were stained with Coomassie blue (compare Fig. 1A. and B. for seed and leaf extracts), while only few were "stained" with anti-spectrin antibodies. Namely, seed extract con-

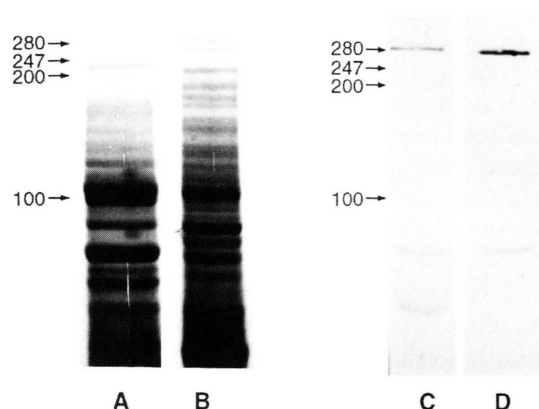


Fig. 1. Binding of biotinylated-calmodulin to spectrin-like proteins from *Pisum sativum* seeds and leaf cells extracts. Electrophoresis in 7% SDS-PAGE. Seed extract proteins – A. C.: Leaf extract proteins – B. D.: Coomassie blue staining – A. B.: Proteins transferred onto nitrocellulose probed with biotinylated calmodulin in the presence of Ca²⁺ – C. D.: Red blood cell membrane proteins (α -spectrin, β -spectrin, ankyrin and band 3 protein) served as molecular weight standards. Their position in the gel is denoted with arrows at the left column. Colour reaction in lanes C.-D. was developed by peroxidase reaction using 4-chloro-1-naphthol/H₂O₂.

tained α -immunoreactive polypeptide chains of mobility corresponding to molecular weight 280, 170 (major component), 110 (minor component) and 70 kD. In young leaf extract polypeptide chains of mol. wt 280, 200 (major component), 100 and 70 (minor components) and in young root extract polypeptides of mol. wt 200 (major component), 100 and 70 kD (minor components) reacting with antibodies directed with anti- α -spectrin were detected (not shown). Beta-reactive polypeptides in seeds, young roots and leaves were of molecular weight 120 and 100 kD (not shown). Our data indicate that spectrin immunoreactive polypeptides extracted from pea leaves with low ionic strength solution form in solution a high molecular weight complex that eluted from Sephacryl S-500 column at a volume corresponding to the molecular weight between 500 and 1000 kD. This peak contained polypeptides reacting with both kinds of antibodies (anti- α and anti- β spectrin). Electrophoretic analysis of the proteins of the low ionic strength extract in nondenaturing conditions also revealed a major component of molecular weight ~800 kD. If one takes into the consideration the fact that a β -spectrin immunoreactive polypeptide in

higher plants is (at least in our hands) of 100–120 kD, the plant spectrin tetramer could be of molecular weight ~800 kD, i.e. $2 \times 280 + 2 \times 120$ kD (Bisikirska, 1995).

As was mentioned above, a reactivity of spectrin, particularly of α -chain with calmodulin in a Ca^{2+} -dependent manner is one of characteristic features of (nonerythroid) spectrins. Low ionic strength extracts of seeds and leaves were subjected to SDS-polyacrylamide gel electrophoresis (Fig. 1A. and B.), then electrophoretically transferred onto nitrocellulose filter and treated with biotinylated calmodulin in the presence of calcium ions. As can be seen in Fig. 1C and D, mainly α -immunoreactive polypeptides of molecular weight about 280 kD show an ability to bind calmodulin. In control samples, when the calcium ions were substituted with 10 mM EGTA, no positive reaction was observed (not shown). This experiment indicates, that calmodulin-binding activity seems to be linked exclusively to polypeptides homologous to α -subunit of spectrin. Interestingly, of high molecular weight polypeptides (>100 kD) reacting positively with anti- α -spectrin only "intact", 280 kD polypeptide reacts with calmodulin, what may suggest that protease-sensitive site may be related to calmodulin-binding domain. In vertebrate α -

spectrin, a calmodulin-binding site is located in 11th, while in insect α -spectrin in 14th repeat unit, therefore more or less close to the centre of the molecule. If this domain was located similarly in plant homologue, breaking of polypeptide chain at the site close to one of the ends of the molecule would be responsible for the loss of calmodulin-binding activity, possibly by inducing a conformational change. On the other hand, a presence of fragments of molecular weight of ~70 kD reacting with Ca^{2+} /calmodulin (Fig. 1C. and D.) may suggest its location in this fragment and explains the lack of reactivity in the case of polypeptides of molecular weight of 200, 170 and 100 kD.

All known spectrins (erythroid and nonerythroid, vertebrate and invertebrate) interact with f-actin. This ability was tested for spectrin-like protein(s) extracted from pea seeds with low ionic strength solution, by f-actin co-sedimentation assays as described in Materials and Methods. After incubation of low ionic strength extract with f-actin and ultracentrifugation, supernatants and pellets were electrophoretically analysed (Fig. 2). Coomassie blue staining as well as immunoreaction indicated that both α - and β -immunoreactive polypeptides (although proteolysed) were present in the high-speed sedimentation pellets (Fig. 2A,

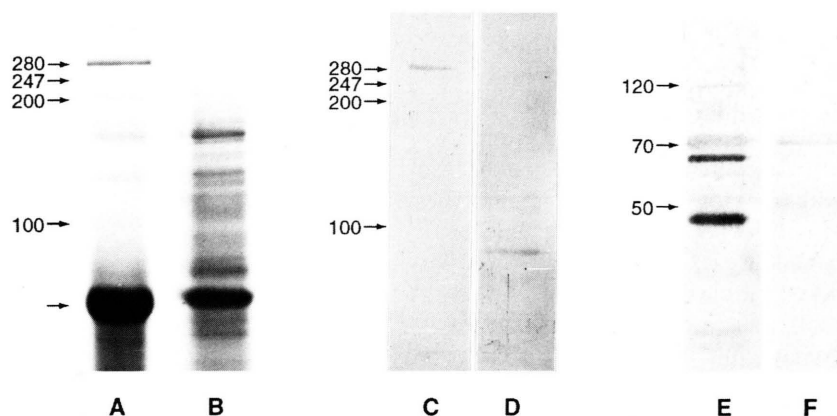


Fig. 2. Interaction of plant extract spectrin-like proteins with rabbit muscle f-actin. F-actin (0.5 ml, repolymerised at 3 mg/ml) by incubation in 10 mM Tris-HCl, pH 7.4, 130 mM KCl, 20 mM NaCl, 2 mM MgCl_2 , 1 mM 2-mercaptoethanol) was mixed with 0.5 ml of the seed extract (0.5 ml, 4.5 mg protein/ml) and layered onto 2.5 ml 10% sucrose cushion in centrifuge tube and incubated 1 hour at room temperature, then centrifuged at $100000 \times g$ for 30 min at 4°C . Pellets and supernatants were subjected to SDS-PAGE and immunoblotting. Pellets – A. C. E., supernatants – B. D. F., Coomassie blue staining – A. B., proteins transferred electrophoretically onto nitrocellulose filter probed with anti- α -spectrin antibodies (1:250) – C. D. and with anti- β -spectrin antibodies (1:250) – E. F. On each lane ~40 μg protein was applied. Left columns: (A-D) positions of the erythrocyte membrane proteins (as in Fig. 1), arrow = muscle actin, E,F – positions of molecular weight standards (β -galactosidase – 120, bovine serum albumin – 70 and ovalbumin – 50 kD) are also indicated by arrows.

C, E.) together with f-actin, what may suggest that they exist as a protein complex which binds filamentous actin. These polypeptides did not sediment in the absence of f-actin in the sample (not shown).

Different isoforms of β -subunit of spectrin molecule have a conservative ankyrin-binding domain (Kennedy *et al.*, 1991). To analyse the interactions of spectrin-like proteins from pea seeds with erythrocyte ankyrin the ELISA-type test was developed. Immobilised on microtiter ELISA plate ankyrin was incubated with low ionic strength extract proteins from seeds. For the detection of immunoreactive proteins bound to the ankyrin, the antibodies raised against α -subunit of erythrocyte spectrin were used. As can be seen in Fig. 3 an increasing amount of added extract induced a proportional increase of bound secondary antibody. When plant protein extract was absent the change of absorbance was small (treated as a control) since the system was blocked with casein-containing solution (Materials and Methods). Also, when ankyrin was absent and plant extract was incubated with "blocked" microtiter plate surface, reasonable binding of antibodies could not be observed. Similar results were obtained, when fraction from gel filtration column (Sephacryl S-500) containing macromolecular weight complex of polypeptides reacting with anti-spectrin antibodies were subjected to the test of ankyrin-binding (data not shown).

Presented above results may indicate that spectrin-like protein(s) from plant cells interact with ankyrin, Ca^{2+} -calmodulin as well as with f-actin. These properties together with reactivity with anti-spectrin antibodies, extractability and localisation in the cell are the major features of spectrins. Functional similarities of spectrin-like proteins to spectrin may indicate a presence of membrane skeletal structure similar to this present in animal cells. However, unanswered question is the function of such a structure in plant cell that is furnished with a cell wall.

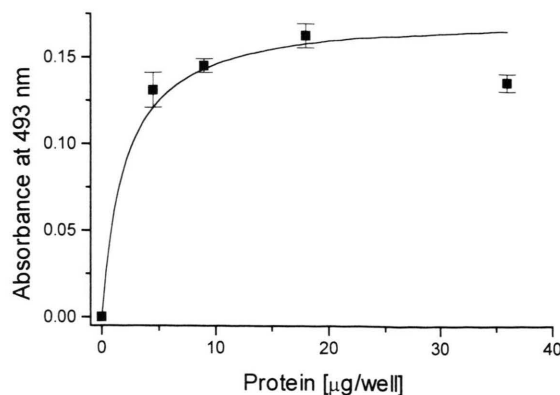


Fig. 3. **Binding of plant extract spectrin-like proteins by immobilised ankyrin.** Purified erythrocyte ankyrin, attached to the microtiter plate was blocked and incubated with various concentrations of low ionic strength extract proteins from pea seeds. After washing off the unbound material, incubations with primary anti- α -spectrin antibodies, followed by secondary goat anti-rabbit antibodies were carried out. Then, after developing peroxidase reaction absorbance at 493 nm was read. Error bar = standard deviation ($n=4$). For the details see Materials and Methods.

It should be noted, however, that these results are of qualitative nature, since high proteolysis, in spite of using many protease inhibitors, prevents isolation of native protein. This does not allow the appropriate, quantitative analyses of these interactions leading to the determination of parameters such as K_D and B_{max} . Therefore further experiments on purification of spectrin from plant cells are carried out in this laboratory. In order to obtain an evidence on the genomic level screening for clones hybridising with spectrin probes in plant cDNA libraries is under way.

Acknowledgement

This work was supported by KBN grants to University of Wrocław and to A. F. S. Authors acknowledge also a material grant from Gelman Science, Ann Arbor MI, U. S. A.

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