A Protein Cross-Reacting with Anti-Spectrin Antibodies is Present in Higher Plant Cells

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Proteins that react with anti-human spectrin antibodies raised in rabbit were found in pea seedlings and leaves. The immunoreactive proteins seem to be associated with the membranes and can be extracted with low ionic strength solutions.

Introduction

Spectrin is the main constituent of the erythrocyte membrane skeleton. It forms heterodimers composed of two nonidentical subunits of M_r 280,000 (α) and 247,000 (β) which are 200 nm long, flexible rods [1]. Heterodimers associating head to head form heterotetramers which appear to be the physiologically relevant unit of the erythrocyte membrane skeleton [2]. In the early 1980s the existence of spectrin-like proteins in cells of almost all animal tissues was demonstrated (for a review see *e.g.* ref. [3]). Spectrin-like proteins were also found in *Acanthamoeba* [4] and *Dictyostelium* [5] cells. In this communication we report the presence of a protein in higher plant cells which reacts with antibodies raised against human erythrocyte spectrin.

Materials and Methods

Plant material

Pea seeds (*Pisum sativum* L.) were surface sterilized in 1% H_2O_2 solution, soaked in water for 2–3 h, sown in a moist germinating-bed in a plastic cuvette (30 × 40 × 5 cm), and allowed to germinate for 7 days in the dark at 22–24 °C. Roots of seedlings were excised up and frozen in liquid nitrogen. For some experiments we used also leaves from 12-day-old light grown pea plants.

Plant cell extracts

The pea seedlings (roots) or leaves were homogenized (300 mg) in 1 ml of either (1) buffer containing 50 mM Tris-Glycine (pH 8.5), 10 mM EGTA, 0.1% SDS, PMSF (1 mg/ml), leupeptin (100 μg/ml), pepstatin A (100 μg/ml), or in 1 ml of (2) low ionic strength buffer containing 0.1 mM EDTA (pH 7.6), PMSF (1 mg/ml), leupeptin (100 μg/ml), pepstatin A (100 μg/ml), or in 1 ml of (3) "SDS buffer" containing 50 mM Tris-HCl (pH 8.0), 12.5% SDS, 2.5% β-mercaptoethanol, 5 mM EDTA, 20% glycerol. Homogenates were centrifuged at 5850 × *g* for 5 min. Supernatants were used for analysis.

Isolation of subcellular fractions

The crude nuclear fraction was obtained according to Luthe and Quatrano [6]. The 5800 × *g* supernatant was centrifuged at 16,000 × *g* and the resulting supernatant was finally centrifuged at 105,000 × *g* to obtain membrane and cytosolic fractions.

Electrophoresis and immunoblotting

SDS-polyacrylamide gel electrophoresis (4–20% concentration gradient gel) was carried out in the Laemmli system [7] and electrophoretic transfer onto nitrocellulose filters according to Towbin *et al.* [8]. The filters were stained for protein with Ponceau S.

Abbreviations: EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid; PBS, 0.15 M NaCl in 5 mM sodium phosphate, pH 7.4; PMSF, phenylmethylsulfonyl fluoride; SDS, sodium lauryl sulfate; TPBS, 0.05% Tween-20 in PBS.

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For immunospecific staining the nitrocellulose sheets were incubated first with newborn calf serum diluted (1:100) with 5% nonfat dry milk in 0.05% Tween 20 in PBS for 120 min, then washed 3 × 10 min with TPBS. Then they were incubated for 120 min with diluted (1:250–1:600 in TPBS) serum of a rabbit immunized with human erythrocyte spectrin. Antibodies against human erythrocyte spectrin were obtained as described by others [9]. After washing with TPBS the filters were treated for 1 h with goat IgG directed against rabbit IgG conjugated to horseradish peroxidase (Bio-Rad) diluted 1:2000 in PBS. The filters were then washed in PBS three times. The immune complexes were detected by incubation of filters with a solution prepared by mixing 4 ml of 4-chloro-1-naphthol solution in methanol (3 mg/ml) with 20 ml PBS. 30% H_2O_2 (14 µl) was added to develop color reaction.

Preparation of protoplasts and immunofluorescence

Preparation of protoplasts was carried out as described by others [10]. For immunofluorescence, isolated protoplasts were pipetted onto a cover slip treated with 0.1% Alcian blue [11] and incubated at room temperature for 30 min. Then the preparations were washed in PBS, fixed with 2% formaldehyde in PBS and permeabilized in 10% methanol in acetone at −20 °C for 5 min, washed again with PBS and incubated with 1% fetal calf serum in PBS (15 min at room temperature). Then the preparations were incubated with anti-spectrin antibody diluted in PBS (1:100) for 30 min, washed several times with PBS and incubated 30 min with fluorescein isothiocyanate conjugated goat anti-rabbit IgG diluted 1:250, washed with PBS and stored in a humid chamber until microscopic examination in an Axiophot-Opton microscope.

Results and Discussion

Two antibodies were used: (1) obtained after immunization with the β-subunit of the spectrin band cut out of nitrocellulose after transfer of human erythrocyte ghost proteins separated in a SDS-polyacrylamide gel. This antibody was generally specific for the β-subunit, however with a substantial cross-reactivity towards the α-subunit. This antibody, called anti-spectrin antibody, was used in the experiments described unless stated otherwise; (2) obtained as above but by immunization of a rabbit with the α-spectrin subunit. This antibody was α-subunit specific (see Fig. 5).

Extracts of pea seedlings obtained by extraction either with the buffer containing SDS or with low ionic strength were subjected to SDS-polyacrylamide gel electrophoresis, and the proteins were transferred onto nitrocellulose and treated with anti β-spectrin antibodies (Fig. 1 D). The electrophoretic mobility of a polypeptide reacting with the anti-spectrin antibodies corresponded to M_r 100,000. The same results were obtained, when the frozen pea seedlings were extracted in boiling SDS buffer; this may suggest that this protein is not the product of proteolysis (not shown). The presence of a protein sharing antigenic determinants with spectrin could also be demonstrated in plant cells by the observation of permeabilized plant protoplasts, treated with anti-spectrin antibodies, then with FITC-conjugated goat IgG directed against rabbit IgG and observed in the fluorescence microscope (Fig. 2A). Control protoplasts treated with preimmune rabbit serum showed no visible fluorescence (not shown).

In order to test the specificity of the reaction of the plant protein with the anti-spectrin antibodies, prior to the reaction with the blotted proteins, the anti-spectrin antibodies were incubated with isolated human red blood cell spectrin. After centrifugation and dilution of the antibodies, they were allowed to react with the blots containing the same

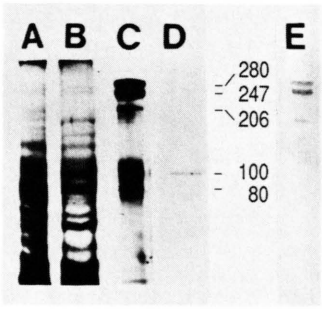

Fig. 1. Electrophoretic analysis of plant cell extracts. A. extract of 30 mg pea seedlings in buffer (1); B. D. low ionic strength extract of 30 mg pea seedlings; C. E. bovine erythrocyte ghosts; (A–C). Coomassie blue staining; (D–E). proteins transferred onto nitrocellulose probed with anti-spectrin antibodies (1:250). Details see in Materials and Methods.

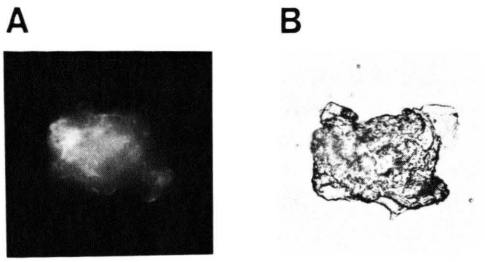

Fig. 2. Indirect fluorescent antibody staining of protoplasts from pea seedlings (roots) with antibodies to human spectrin. The fluorescent picture (A) is paired by a light micrograph (B) of the same object. Magnification 400×.

amounts of electrophoretically separated pea membrane fraction proteins. As can be seen in Fig. 3, the intensity of the immune reaction decreased with the increasing amount of spectrin used for the adsorption. This suggests that the reaction with the membrane polypeptide is specific.

The polypeptide reacting with anti-spectrin antibodies was co-purified with the membrane fraction. Our results indicate that the membrane fraction contained a relatively high proportion of polypeptide reacting with anti-spectrin antibodies. Nuclear and cytosolic fractions did not contain substantial amounts of this protein (Fig. 4). Also, in the 16,000 × *g* pellet the reaction with anti-spectrin antibodies was similar to that in the

Fig. 3. Spectrin inhibits the reaction of anti-spectrin antibodies with the plant protein of M_r 100,000. Serum of a rabbit immunized with human spectrin was incubated with 100 (A) and 200 (B) µg of human erythrocyte spectrin. The samples were centrifuged and supernatants were diluted to obtain a final dilution of 1 : 250, and used for the reaction with the electrophoretically separated membrane material from pea seedlings (roots) (8 µg protein).

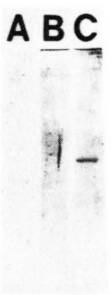

Fig. 4. A polypeptide reacting with anti-spectrin antibodies is probably a membrane protein. Immunoblot analysis of electrophoretically separated proteins from pea seedlings (roots) A. crude nuclear fraction, 46 µg protein; B. cytosol, 35 µg protein; C. membrane fraction, 24 µg protein.

105,000 × *g* pellet (data not shown) which was probably due to the presence of membrane fragments and membranous structures in this pellet in addition to mitochondria [12]. Our results indicate, that in cells of higher plants, a protein reacting with anti-human erythrocyte spectrin antibodies occurs. It seems to co-purify with the membrane fraction and can be extracted with low ionic strength solution. In addition to the reaction with anti-spectrin antibodies, its co-purification with the membrane fraction, as well as the extraction with a low ionic strength solution also substantiate its similarity to spectrin. The use of extraction buffer without Triton X-100 did not affect the amount of the M_r 100,000 protein in the membrane fraction but the amount of membrane material was decreased. The resistance to extraction with non-ionic detergents is also one of the features of spectrin, the element of the membrane skeleton. The plant protein displayed however, a much lower M_r (about 100,000) than erythroid and non-erythroid spectrin (M_r 280,000, 247,000). Experiments with anti-α-spectrin antibodies were also performed (Fig. 5). Extracts of pea seedlings and leaves were compared by immunoblotting. Our results showed that in the low ionic strength extract of both pea seedlings and pea leaves a protein is present, which cross-reacts with antibodies raised against human erythrocyte α-spectrin. The M_r of this protein is about 200,000. Moreover, in the extract of pea leaves obtained by extraction with boiling SDS buffer, a polypeptide of a high molecular weight, which corresponded to the molecular weight of the

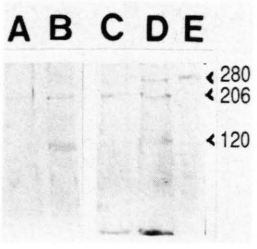

Fig. 5. A polypeptide cross-reacting with antibodies against the α-subunit of spectrin is present in pea seedlings and leaves. Immunblot analysis of plant extracts. A. B. pea seedlings; C. D. leaves; A. C. low ionic strength extracts; B. D. SDS buffer extracts; E. bovine erythrocyte ghosts. Proteins transferred onto nitrocellulose filter were probed with anti-α-spectrin antibodies. Matching Coomassie blue staining patterns were similar to those presented in Fig. 1 A and B.

α-subunit of human erythrocyte spectrin (M_r 280,000) was found in addition to the 200,000 band.

In the present analysis two factors should be considered: 1. the specificity of antibodies used and 2. the possibility of the occurrence of a protein containing a homologous, 106 amino acid residue segment which is found in both human and avian spectrin and also in other proteins like α-actinin and dystrophin [13, 14]. The latter possibility was excluded by immunoblot analysis of a myofibril preparation containing α-actinin. Even at large excess of the material a reaction with anti-spectrin antibodies was not observed (not shown).

The specificity of the reaction with the antibodies was tested in several ways. First of all, it was found that the obtained serum reacted only with spectrin. On the blots of erythrocyte membranes the antibodies were found to react only with spectrin bands. Nitrocellulose filters obtained by electrophoretic transfer of plant proteins separated on SDS-polyacrylamide gel electrophoresis, and incubated with preimmune rabbit serum (1:250), did not show unspecific reactions with rabbit IgG. The fact that protein cross-reacting with anti-spectrin antibodies is present in plant tissue seems to be a rather unexpected phenomenon. Particularly, the presence of the α-reactive protein seems to be novel observation in the literature, since the β-subunit reactive protein has recently been reported by others [15]. Further studies on the oligomeric structure and mutual interactions of spectrin-like polypeptides are currently conducted.

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