

# Identification of a 220 kDa membrane-associated plant cell protein immunologically related to human $\beta$ -spectrin

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Electrophoretic analysis of low ionic strength extracts of tomato plant leaves revealed the presence of two proteins of apparent molecular weights of 240 kDa and 220 kDa which co-migrated with purified human erythrocyte  $\alpha$ - and  $\beta$ -spectrin subunits. Immunochemical analyses employing an affinity-purified polyclonal antibody to human erythrocyte  $\beta$ -spectrin reacted specifically with the 220 kDa plant cell protein. Immunofluorescence microscopy indicated that the  $\beta$ -spectrin antibody recognized an antigen which was primarily restricted to the peripheral areas of the cells. Collectively, these results suggest that the cells of higher plants contain polypeptides related to the spectrin family of proteins. It is proposed that the plant cell possesses a membrane skeleton which is structurally and perhaps functionally analogous to that of the animal cell.

Membrane skeleton;  $\beta$ -Spectrin; Actin-binding protein; Higher plant

## 1. INTRODUCTION

The plant cytoskeletal system is generally considered to consist of two distinct components, microtubules (MT) and microfilaments (MF). Evidence is accumulating which suggests that intermediate filaments (IF) constitute a third and distinct cytoskeletal filament structure within this cell [1–3]. In animal cells, the spectrin-based membrane skeleton (MS) is a topographically distinct cytoskeletal structure which, depending on the cell type, can be physically related to various other intercellular cytoskeletal systems [4,5]. The MS is known to interact with various cytoplasmic and membrane proteins [6,7]. For example, spectrin, the major component of the MS binds to actin, a process which may be mediated by a minor MS protein now referred to as adducin [5].

In plant cells, the microfilament system has been well characterized, although little is known of how or if this particular filament structure is anchored to the plant cell membrane [8,9]. Both MF and MT are known to be associated with the plasma membrane which suggest that the plant cell may contain a spectrin-based MS. Our objective in the present investigation was to carry out a series of immunochemical experiments designed to test the hypothesis that the plant cell contains spectrin or proteins related to spectrin.

## 2. MATERIALS AND METHODS

### 2.1. Protein extraction and gel electrophoresis

Fresh leaves from 30-day tomato plants were ground to a fine powder in liquid nitrogen. The tissue was incubated at 37°C for 15 min in spectrin extraction buffer (0.3 mM phosphate, pH 8.0, 0.1 mM EDTA, 1 mM PMSF, 5 mg/ml each of leupeptin, pepstatin and anti-pain) [10]. The extract was centrifuged at 20 000  $\times g$  and saturated to 50% with ammonium sulfate. The precipitate was collected, resuspended and dialysed for 24 h at 4°C against three changes of 50 mM Tris-HCl, pH 7.6, 1 mM EDTA containing the same protease inhibitor cocktail. SDS-PAGE was performed according to [11] using a 7–15% gradient and the gels were stained with Coomassie blue.

### 2.2. Affinity purification of $\beta$ -spectrin antibody and immunoblotting

Membrane skeleton proteins were prepared from fresh human blood [10]. Antibodies to human  $\beta$ -spectrin were affinity-purified [12]. Immunoblotting [13] was carried out using nitrocellulose membranes and an alkaline phosphatase goat anti-rabbit secondary antibody diluted 1:40 000 in TBST (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.05% Tween-20). Chromogenic development using 5-bromo-4-chloro-3-indolylphosphate and Nitro blue tetrazolium was employed to visualize antibody-antigen complexes.

### 2.3. Immunofluorescence microscopy

Immunocytochemical localization was carried out on sections of leaves embedded in polyethylene glycol (PEG). Samples were fixed for 3 h at room temperature in a mixture of 4% paraformaldehyde + 0.1% glutaraldehyde buffered with 100 mM sodium cacodylate (pH 7.2), then treated with 50 mM sodium borohydride for 30 min to block free aldehyde groups. After dehydration in ethanol, samples were transferred for 24 h in each of the following: PEG of molecular weight 1000 (BDH, ST-Laurent, Canada) at 48°C, then in PEG 1500 at 52°C and finally in PEG 1500:4000 (1:7.3) at 58°C. Sections were rehydrated with 0.5% bovine serum albumin (BSA-PBS) in 100 mM phosphate-buffered saline and then incubated with a blocking agent (5% non-fat dry milk in PBS). The sections were incubated overnight with the affinity-purified anti- $\beta$ -spectrin antibody diluted 1:10 in PBS, then transferred on a biotin-conjugated goat anti-rabbit antibody (Bio/Can Scientific, Mississauga, Canada) for 60 min. The antigen was visual-

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ized using Cy3-conjugated streptavidin (Bio/Can Scientific) according to the manufacturer's recommendations. Finally, sections were mounted on slides and observed with a Zeiss microscope equipped for epifluorescence. Specificity of the immunolabeling was evaluated following adsorption of the antibody with immobilized  $\beta$ -spectrin, incubation of the sections omitting the first antibody and incubation with the secondary antibody alone. These controls revealed a complete absence of non-specific fluorescence.

### 3. RESULTS

#### 3.1. Electrophoretic analysis

Proteins were extracted from tomato leaves using a modification of the basic protocol designed to prepare spectrin from human red blood cell. Caution was required during the sample preparation since spectrin and MS associated proteins are particularly susceptible to proteolysis and plant tissues have a high level of proteolytic activity [14]. Electrophoresis of the plant cell extracts revealed the presence of two polypeptides demonstrating apparent molecular weights of 240 kDa and 220 kDa (Fig. 1). These two polypeptides migrated in SDS gels to positions near or identical to those of purified human red blood cell  $\alpha$ - and  $\beta$ -spectrins. The stained protein pattern shown in Fig. 1 also indicates that the two polypeptides in question constitute relatively minor protein components in this particular preparation.

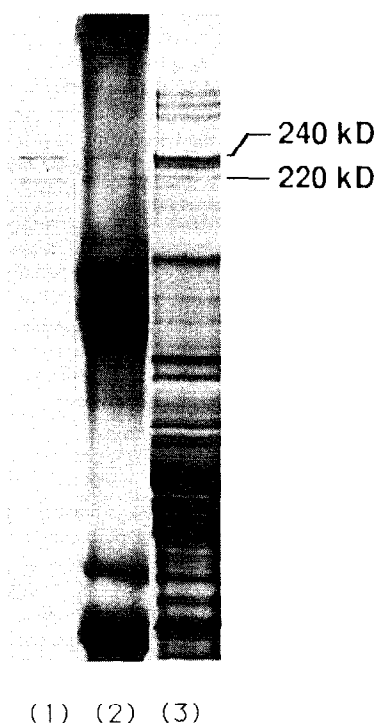


Fig. 1. Electrophoretic analysis of tomato plant leaf extracts. Purified  $\alpha$ - and  $\beta$ -spectrin subunits from human red blood cell are shown in lane 1. The plant cell extracts (lane 2) contain two proteins showing apparent molecular weights of 240 and 220 kDa. The proteins found in rat brain homogenate (lane 3) is shown for comparison ( $\alpha$ -spectrin: 240 kDa;  $\beta$ -spectrin: 235 kDa).

#### 3.2. Immunoblotting

Immunochemical analysis of the erythrocyte protein extracts using affinity-purified  $\beta$ -spectrin antibody revealed a highly specific reaction with the 220 kDa spectrin subunit (Fig. 2). This antibody also clearly recognized the 220 kDa protein in the plant leaf extract. As illustrated in Fig. 2, the affinity-purified antibody demonstrated a clear and unique specificity for this protein. Parallel experiments with root cell extracts revealed that these cell types also contain a 220 kDa protein immunologically related to human red blood cell  $\beta$ -spectrin (not shown).

#### 3.3. Immunocytochemistry

Immunofluorescence microscopy revealed fluorescent labeling restricted to the periphery of the cells (Fig. 3). The leaf epidermis as well as trichomes showed identical labeling profiles. In the mesophyll cells, chloroplasts emitted a yellowish glow recognized as autofluorescence as the appropriate control experiments revealed identical staining. In some cells, the region of the nuclear membrane appeared faintly fluorescent, this

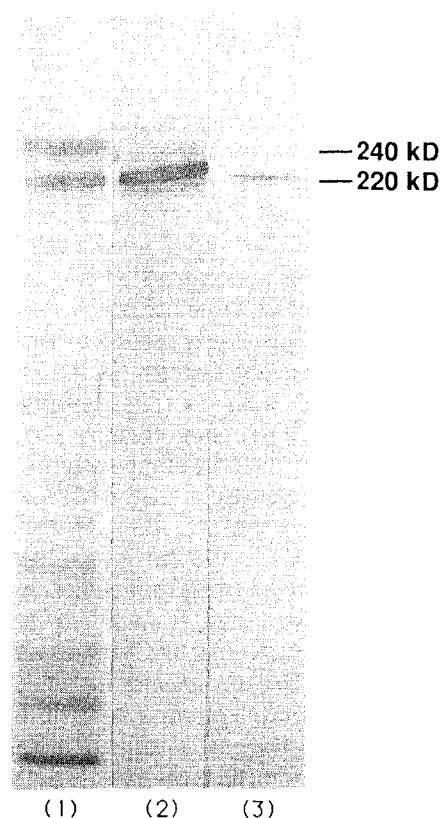


Fig. 2. Identification of a 220 kDa plant cell protein immunologically related to human red blood cell  $\beta$ -spectrin. A standard preparation of red blood cell spectrin [12] was electrophoresed, transferred to nitrocellulose and stained with Indian ink (lane 1) to reveal the  $\alpha$ - and  $\beta$ -spectrin subunits. A parallel gel lane was used to evaluate the specificity of the  $\beta$ -spectrin antibody (lane 2). The plant leaf extract (refer to Fig. 1) was processed for immunoblotting (lane 3) and revealed that the 220 kDa plant protein reacts specifically with the  $\beta$ -spectrin antibody.

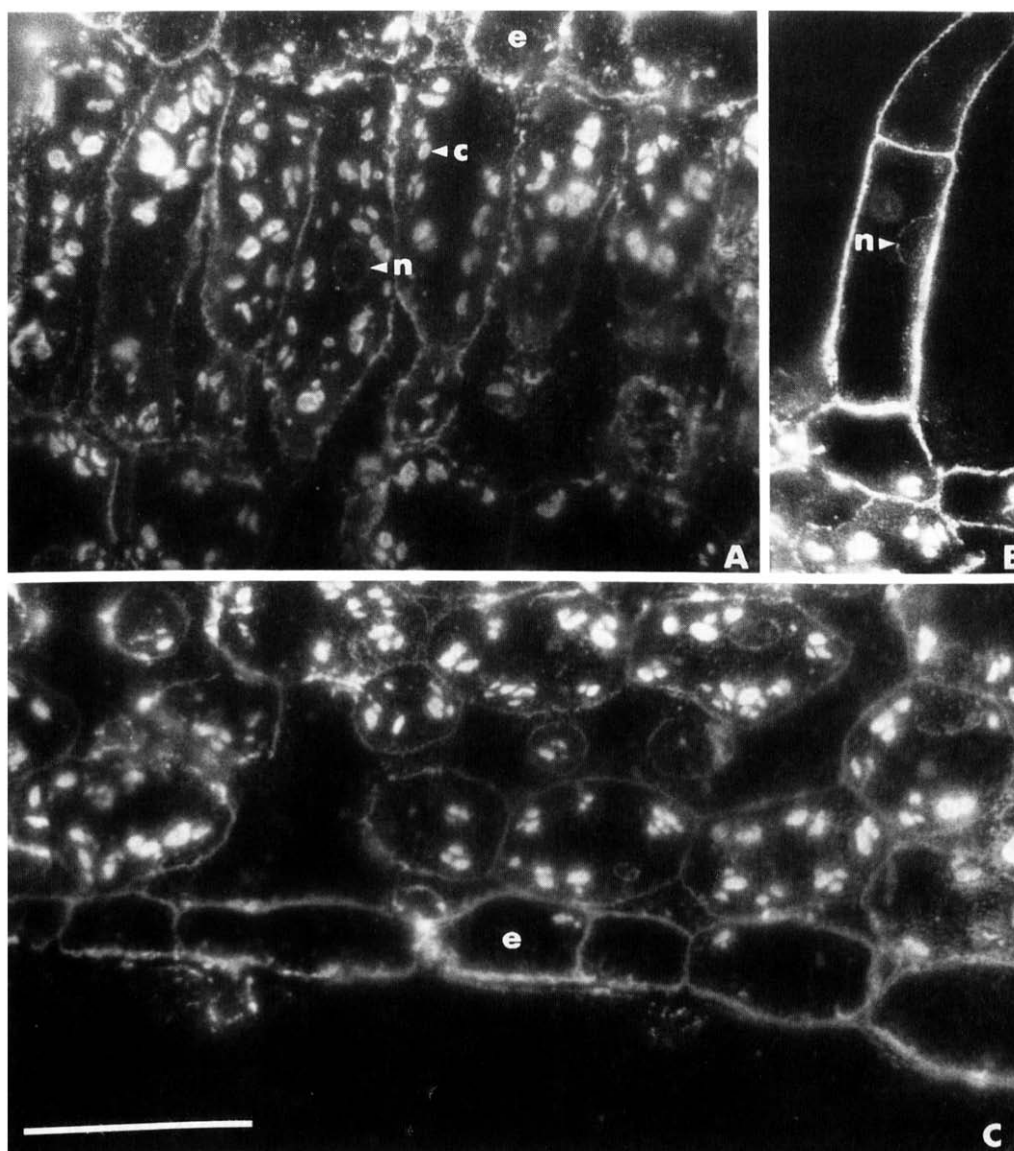


Fig. 3. Immunocytochemical localization of the 220 kDa spectrin-like protein in tomato leaf cells. Panel A illustrates a trichome on the leaf abaxial epidermis; the fluorescence is localized at the whole periphery of the trichome and the epidermal cells. Panel B shows an adaxial portion of the mesophyll with palisade parenchyma cells that display fluorescence in the perimeter of each cell; chloroplasts reveal an autofluorescence which is also observed in control preparations. Panel C illustrates the abaxial region of the mesophyll with the organization of spongy parenchyma cells and lacunae. Note the identical fluorescent labeling profiles as in the other cell types. Abbreviations: C, chloroplast; E, epidermis; N, nucleus. Scale bar = 40  $\mu$ m.

was observed in the cells of either palisade or spongy parenchyma of the mesophyll as well as in the trichomes (Fig. 3).

#### 4. DISCUSSION

The present investigation provides new evidence that tomato plant cells contain a 220 kDa polypeptide which appears to be a member of the spectrin family of proteins. Application of the procedure developed to prepare spectrin from erythrocytes [10] to plant leaf cells resulted in poor yields and degradation of the 220 kDa plant protein. However, it was found that the 37°C

low ionic strength extraction performed directly on plant leaf homogenates yielded a 220 kDa protein which was visible following SDS-PAGE and showed no evidence of significant degradation. Experiments are now underway in order to develop a purification protocol specific for the 220 kDa plant protein.

The results of the immunochemical experiments constitute strong evidence in support of the contention that the plant cell protein is the counterpart to animal cell  $\beta$ -spectrin. The affinity purified polyclonal antibody to human red blood cells reacted monospecifically with the plant 220 kDa protein. It was predicted that if the plant cell protein in question was indeed a bona fide compo-

nent of a putative MS then it would be expected to be localized to the same restricted domain characteristic of animal spectrin. This prediction was borne out as the results of the present immunocytochemical experiments indicate. The data obtained from the immunofluorescence experiments are in agreement with the typical localization of spectrin in the various types of animal cells. We anticipate that the identification of a putative MS in the plant cell may carry a significant impact with respect to research on the cytoskeletal structures particularly on studies directed at elucidating the role of the microfilaments.

In summary, we have demonstrated that the cells of a higher land plant possess a 220 kDa protein, immunologically related to human  $\beta$ -spectrin, which demonstrates an electrophoretic mobility indistinguishable from  $\beta$ -spectrin and is localized to the cytoplasmic face of the plasma membrane. It is concluded that this protein is the plant cell form of  $\beta$ -spectrin. It is therefore proposed that the plant cell possesses a MS which, in some respects, may be analogous to the MS of animal cells. The results of molecular cloning experiments presently underway in this laboratory have identified cDNAs which bear significant homology to the spectrin family of proteins (unpublished results). Further efforts at the biochemical and molecular level are now required to fully characterize the proposed MS of the plant cell and to begin to understand its physiological significance in this cell type.

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