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Evidence for a membrane skeleton in higher plants

A spectrin-like polypeptide co-isolates with rice root plasma membranes

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A fraction enriched in plasma membranes was isolated from rice roots by differential centrifugation and aqueous polymer two-phase partitioning. Analysis of the fraction by SDS-PAGE showed the presence of several low mobility polypeptides (M_r > 100 kDa). One of these polypeptides (M_r ~ 230 kDa) was specifically recognized by polyclonal antibodies to human erythrocyte spectrin. This finding suggests that a higher plant spectrin-based membrane skeleton may be preserved and studied using high-purity plasma membrane fractions obtained by aqueous polymer two-phase partitioning.

Aqueous polymer two-phase partitioning; Fodrin; Peripheral protein; Cytoskeleton–plasma membrane interaction

1. INTRODUCTION

A spectrin-based membrane skeleton has been characterized in a diverse variety of cell types and organisms [1–4]. Proteins of the spectrin family serve as major structural components of the membrane skeleton by their association with membrane proteins and cytoskeletal filaments, especially actin [1–4]. A possible role has been suggested for spectrin in the mediation of microtubule and microfilament associations with the plant plasma membrane [5,6]. Only recently, however, has a spectrin-like protein been identified in plants [7]. Tomato leaf extracts were found to contain polypeptides that co-migrated in electrophoretic analysis with the 220 kDa and 240 kDa α- and β-subunits of human erythrocyte spectrin. Antibodies to the β-subunit of human erythrocyte spectrin recognized the tomato M_r 220 kDa polypeptide and, in immunofluorescence microscopy studies, recognized antigens in the peripheral region of tomato leaf cells [7].

The aqueous polymer two-phase partitioning method of plant plasma membrane purification yields a fraction that is highly enriched in right-side-out plasma membrane vesicles [8]. The right-side-out orientation may protect and preserve electrostatic interactions important to the integrity of protein complexes and peripheral protein associations with the membrane [9]. In the present study, aqueous polymer two-phase partitioning was used to obtain a purified plasma membrane fraction from rice roots. A spectrin-like polypeptide was identified in this fraction by relative electrophoretic mobility and antigenicity.

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Abbreviations: BSA, bovine serum albumin; BTP, 1,3-*bis*[*tris*(hydroxymethyl)-methylamino]propane; DTT, dithiothreitol; MES, 2-[*N*-morpholino]ethanesulfonic acid; PMSF, phenylmethylsulphonyl fluoride; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.

2. MATERIALS AND METHODS

2.1. *Membrane purification and characterization*

Rice plants (*Oryza sativa* L. cv. IR36) were grown in hydroponic culture [9]. Roots (80 g) were harvested 11 days after imbibition and chopped in homogenization buffer (250 ml) by a food processor. The homogenization buffer consisted of 50 mM BTP/MES, pH 8.0, 250 mM sucrose, 10% (v/v) glycerol, 2 mM EGTA, 0.5% (w/v) BSA, and 1 mM PMSF. The homogenate was centrifuged at 9 950 × *g* for 10 min. The supernatants were filtered through nylon mesh screens (149 μm openings) and a microsomal fraction was obtained by centrifugation at 85 000 × *g* for 35 min. A U_4U_4' plasma membrane fraction was obtained from the microsomal fraction by aqueous polymer two-phase partitioning [8] as presented in detail by [9]. The U_4U_4' fraction was diluted four-fold with dilution/resuspension buffer (10 mM potassium phosphate, pH 8.0, 300 mM sorbitol, 10% (v/v) glycerol, 0.1 mM Na_2EDTA, 0.8 mM PMSF) and centrifuged at 150 000 × *g* for 40 min. The plasma membrane pellets were resuspended in dilution/resuspension buffer and stored in liquid nitrogen. Protein was assayed [10] with BSA as a standard. Membrane preparations were diluted into phosphate-free 300 mM sorbitol and 10% (v/v) glycerol before aliquots were taken for ATPase assays, which were conducted as in [9].

2.2. *SDS-PAGE and immunodetection*

Membrane protein was solubilized and diluted to 1 μg/μl in sample preparation buffer (0.125 M Tris-HCl, pH 6.8; 8% (w/v) SDS; 20% (v/v) glycerol; 20 mM DTT; 0.2 mM PMSF, 0.001% (w/v) Bromphenol blue). The time and temperature of the solubilization step were critical to membrane sample preparation. Optimal conditions are presented

with the figure explanation. Purified human erythrocyte spectrin (Sigma Chemical Co., St. Louis, MO, USA) was prepared for electrophoresis by incubation in the above buffer at 100°C for 5 min. Discontinuous SDS-PAGE [11] was conducted on 7 × 8 cm gels of 0.75 mm thickness using an SE 250 unit (Hoefer Scientific Instruments, San Francisco, CA, USA) with the central chamber cooled to 10°C by a circulating water bath. Resolving gels were of 5% acrylamide as detailed on page 34 of [12]. Samples were run at 20 mA per gel.

Electrotransfer to nitrocellulose [13,14] was conducted at 0.5 A for 2 h at 10°C in the presence of 0.01% (w/v) SDS. Nitrocellulose blots were washed with tris-buffered saline (TBS: 10 mM Tris-HCl, pH 8.0, 150 mM NaCl) three times for 10 min and blocked for 15 h at 5°C in TBST + BSA (TBS that contained 0.05% (v/v) tween 20 and 1% (w/v) BSA). Polyclonal anti-human erythrocyte spectrin (Sigma product number S-1515, lot no. 079F4826) was diluted to 1:400 in TBST+BSA and incubated with the blots for 3 h at 24°C. After reaction with primary antibody, blots were washed with TBST three times for 5 min each. Antibody binding was detected using anti-rabbit IgG conjugated to alkaline phosphatase (Promega Corp., Madison, WI, USA) at 1:7,500 dilution in TBST+BSA. After incubation for 1.5 h, blots were washed with TBST+BSA and antibody binding was visualized by color development with an alkaline phosphatase substrate kit (Bio-Rad Laboratories, Richmond, CA, USA). Nonimmune rabbit serum, omission of the primary antibody, and the color development reaction alone served as controls. Immunoblots of BSA alone were processed as a control for contaminating animal spectrin.

3. RESULTS

3.1. *Membrane characterization*

ATPase activities and the effects of ATPase inhibitors were used to characterize the microsomal and phase-partitioned U_4U_4' membrane fractions obtained from rice roots. A summary of specific activities is presented in Table I.

An increase from the microsomal to the U_4U_4' fraction in pH 6.5 ATPase specific activity and its inhibition by vanadate served as an indicator of plasma membrane enrichment. The vanadate-sensitive activity of the U_4U_4' fraction was 96% of the control and was enriched 1.4-fold over that of the microsomal fraction.

The depletion of tonoplast and mitochondrial membranes by phase partitioning was indicated by decreases in nitrate-sensitive, azide-sensitive, and control ATPase specific activities at pH 8.0. Nitrate-sensitive activity, which indicates contamination by tonoplast and mitochondrial ATPases, was reduced 4.6-fold by phase partitioning. Azide-sensitive activity, which indicates contamination by mitochondrial ATPases, was reduced 9.9-fold by phase partitioning. Molybdate-sensitive activity at pH 8.0 served as an indicator of nonspecific alkaline phosphatases and was reduced by 1.5-fold. Control activity at pH 8.0 was diminished by 2.6-fold. Relative sensitivities to the inhibitors served as markers of purity and contamination. Nitrate-sensitive activity at pH 8.0 as a proportion of vanadate-sensitive activity at pH 6.5 decreased from 49.7% in the microsomal fraction to 7.8% in the U_4U_4' fraction. Azide-sensitive activity as a proportion of vanadate-sensitive activity decreased from 56.8% in the microsomal fraction to 4.1% in the U_4U_4' fraction.

3.2. *SDS-PAGE and immunodetection*

The high molecular mass complement of polypeptides associated with purified rice root plasma membranes was examined by SDS-PAGE in resolving gels of 5% acrylamide. Several discrete bands of $M_r > 100$ kDa were consistently observed (Fig. 1, lane 1). The low mobility polypeptides were a small proportion of the total protein, hence the overstained appearance of the lower half of the lane.

Antibodies to human erythrocyte spectrin recognized the 220 kDa α- and and 240 kDa β-subunits of spectrin (Fig. 1, lane 5) as well as a single band of rice plasma membrane protein at $M_r \sim 230$ kDa (Fig. 1, lanes 2–4). The specificity of the reaction with the rice protein is more apparent when it is considered that the $M_r \sim 230$ kDa band was a small proportion of the total protein added to the lane. The appearance and mobilities of the plasma membrane polypeptides were influenced by the treatment of the sample during preparation for electrophoresis. A strong and consistent immunoreaction was obtained only when plasma membrane aliquots were heated to 40°C for several min prior to the addition of sample preparation buffer (Fig. 1, lane 3). More conventional treatments of the sample during preparation (Fig. 1, lanes 2 and 4) consistently resulted in less intense antibody reactions. The gel banding pattern and blot intensity were not altered by the inclusion of additional protease inhibitors (16 μg/ml antipain, 1 μg/ml leupeptin, 1 μg/ml pepstatin) to the homogenization, resuspension, dilution, and sample preparation steps. Bands were not observed on control blots.

4. DISCUSSION

This study presents evidence that high molecular mass and spectrin-like polypeptides are associated with

Table I

Effect of inhibitors on ATPase activity associated with microsomal and U_4U_4' phase-partitioned (plasma membrane) fractions from rice roots

Treatment	ATPase specific activity (μmol Pi/mg·min)	
	Microsomal	U_4U_4'
Control pH 6.5	1.195 ± 0.080	1.319 ± 0.082
Δ vanadate (0.5 mM)	0.909 ± 0.055	1.269 ± 0.081
Control pH 8.0	0.768 ± 0.054	0.297 ± 0.015
ΔKNO_3 (50 mM)	0.451 ± 0.044	0.099 ± 0.009
ΔNaN_3 (1 mM)	0.516 ± 0.037	0.052 ± 0.014
ΔNa_2MoO_4 (1 mM)	0.086 ± 0.008	0.059 ± 0.009

ATPase activity was assayed at pH 6.5 and pH 8.0 in the presence of 5 mM $MgSO_4$, 50 mM KCl, 0.01% (w/v) Brij 58, and 4 mM Na_2ATP at 30°C. Inhibitor-sensitive activities (Δ inhibitor) are calculated as the difference in activity measured in the presence and absence of the inhibitor. Activities are presented as the mean ± the standard error of five separate isolations and assays

the plasma membrane of higher plants. This evidence was obtained by electrophoretic and western blot analysis following solubilization of rice root plasma membrane fractions of high purity.

The plasma membrane fraction obtained from rice roots for this study was comparable in markers for purity and contamination (Table I) to fractions previously obtained by phase partitioning from rice [15,16] and other plant root material [9].

High molecular mass polypeptides are among the most well-known constituents of the plasma membranes of animal systems (e.g. [17]). In contrast, this complement of polypeptides has not been characterized in plants. While sometimes observed in electrophoretic analysis, high molecular mass polypeptides of plant plasma membranes have only in rare cases (e.g. [18]) been the focus of study. In the present study, we executed sample preparation for electrophoretic analysis with the consideration that incomplete solubilization, aggregation, and protease activity may account for the lack of high molecular mass polypeptide detection in most previous studies. We performed sample preparation by many methods that did not result in consistent low mobility banding or immunoreactions. Consistent observations were obtained when the final plasma membrane fraction was resuspended in a phosphate buffer and heated to 40°C prior to solubilization and denaturation with SDS. While this treatment may seem unconventional for sample preparation for SDS-PAGE of membrane proteins, it is consistent with the well-established methods for the solubilization of spectrin from erythrocyte membranes [19,20].

Proteins of the spectrin family apparently play an integral role in the continuity of protein linkage between the cytoskeleton and the plasma membrane [1–4]. The identification of a spectrin-like polypeptide in association with a purified plasma membrane fraction from plants raises the question of how the spectrin-like protein is associated with the membrane. In previously characterized systems, spectrin and nonerythroid spectrin (fodrin) are often linked to the plasma membrane by an association with a form of ankyrin, and ankyrin has most often been found to associate with the plasma membrane by direct binding to ion transport proteins [1–4]. A characterization of spectrin-like protein associations in plants could lead to the identification of specialized transport domains and aid in the identification and characterization of ion transport proteins of plant plasma membranes.

The isolation and biochemical characterization of specialized membrane domains has contributed to the current understanding of cytoskeleton–plasma membrane interactions in animal systems [2]. Thus, plasma membranes purified by aqueous polymer two-phase partitioning may serve as appropriate starting material for the study of these interactions as they occur in plants.

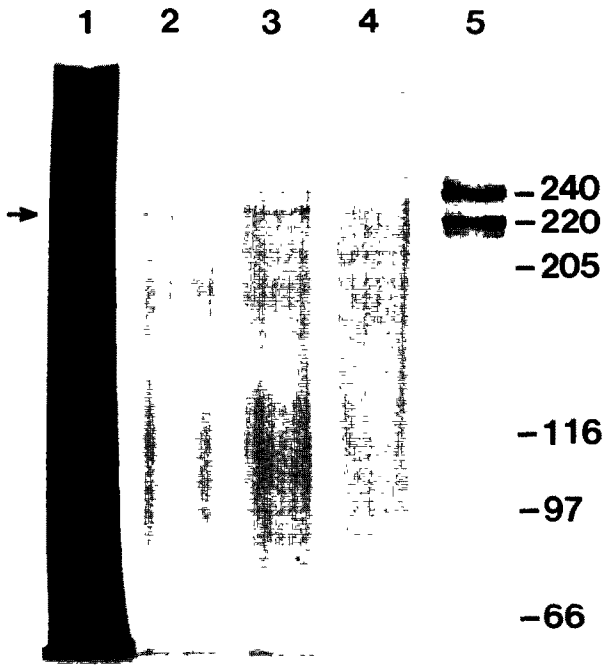

Fig. 1. SDS-PAGE and anti-spectrin immunoblot of rice root plasma membrane polypeptides. Lane 1: U_4U_4' fraction polypeptides were silver-stained to visualize the $M_r \sim 230$ kDa polypeptide. Lanes 2–5: anti-spectrin immunoblots of U_4U_4' fraction polypeptides (lanes 2–4) and spectrin standard (lane 5). Equal aliquots of membrane were solubilized by three sample preparation treatments: incubation in sample preparation buffer at 100°C for 30 s (lane 2); pretreatment at 40°C for 4 min followed by incubation in sample preparation buffer at 100°C for 30 s (Lane 3); incubation in sample preparation buffer at 75°C for 10 min (lane 4). Lane 5: human erythrocyte spectrin standard (0.625 μg). 11 μg total protein was applied to each of lanes 1–4. Gels were of 5% acrylamide. Numbers to the right represent apparent molecular mass (kDa). An arrow indicates the high molecular mass polypeptide recognized by anti-human erythrocyte spectrin.

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