Purification and immunolocalization of an annexin-like protein in pea seedlings

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Abstract. As part of a study to identify potential targets of calcium action in plant cells, a 35-kDa, annexin-like protein was purified from pea (Pisum sativum L.) plumules by a method used to purify animal annexins. This protein, called p35, binds to a phosphatidylserine affinity column in a calcium-dependent manner and binds $^{45}$Ca$^{2+}$ in a dot-blot assay. Preliminary sequence data confirm a relationship for p35 with the annexin family of proteins. Polyclonal antibodies have been raised which recognize p35 in Western and dot blots. Immunofluorescence and immunogold techniques were used to study the distribution and subcellular localization of p35 in pea plumules and roots. The highest levels of immunostain were found in young developing vascular cells producing wall thickenings and in peripheral root-cap cells releasing slime. This localization in cells which are actively involved in secretion is of interest because one function suggested for the animal annexins is involvement in the mediation of exocytosis.

Key words: Annexin – Calcium binding – Golgi-mediated secretion – Pisum (annexin-like protein)

Introduction

Both animal and plant cells use Ca$^{2+}$ as a second messenger to mediate many different cellular responses to external stimuli. Calmodulin has been demonstrated to be a major receptor for the calcium signal in eukaryotic cells. However, in recent years the annexins, a family of at least eight distinct groups of calcium-binding proteins, have emerged as another potential target of calcium in animal cells (see, e.g., Dedman 1986). Although their biological roles are as yet undetermined, they are suggested to function in membrane-cytoskeletal linkage, membrane fusion events in exocytosis, and regulation of cell-surface receptors. Annexins are defined as calcium-dependent, phospholipid-binding proteins with conserved internal repeat sequences of about 70 amino acids which occur at least four times in the primary structure (Crompton et al. 1988). Recently, the existence of annexin-like proteins in tomato suspension cells was confirmed by sequence homology (Smallwood et al. 1990). In this paper we report on the purification and immunolocalization of a 35-kDa pea protein (referred to hereafter as p35), which shares several biochemical and localization characteristics with the annexins. Our results show, as far as we are aware, the first immunolocalization data of an annexin-like protein in plant cells. The apparent higher levels of immunostain found in secretory plant cell types coupled with the cellular localization on the plasma membrane and some association with the Golgi apparatus indicate this protein may be involved in vesicle-mediated secretion.

Material and methods

Plant material. Peas (Pisum sativum L. cv. Alaska; Esco Distributors, Pharr, Tex., USA) were germinated and grown on unbleached packing paper (Kimberly-Clark, Roswell, Ga., USA) in darkness for 7d at 25° C. For purification, plumules were used; for fixation, samples were taken from the plumule and from the root tip.

Extraction and purification. All of these steps were carried out at 4° C. The harvested plumules (100 g) were placed in cold diethyl ether for 4-5 min, then homogenized in Buffer A (1 M sucrose, 10 mM 4-(2-hydroxyethyl)-l-piperazineethanesulfonic acid (Hepes), pH 7.2, 5 mM MgCl$_2$, 1 mM ethylene glycol-bis(β-aminoethyl ether)-N,N',N'-tetraacetic acid (EGTA), 10 mM 2-mercaptoethanol, 0.1 mM phenylmethylsulphonyl fluoride (PMSF), 0.1% aprotinin (Sigma Chemical Co., St. Louis, Mo., USA) with a polytron PTA 20 ts (Brinkmann Instruments, Westbury, N.Y., USA) at a low-to-medium speed for 1 min. The homogenate was filtered through three layers of Miracloth (Calbiochem, La Jolla, Cal., USA) and two layers of nylon mesh (pore size, 88 μm and 30 μm), then centrifuged at 1900 $g$ for 10 min to remove unbroken cells and debris. An equal volume of saturated ammonium-sulfate solution was slowly added with constant stirring. After stirring for 30 min, the
mixture was centrifuged at 11 300 · g for 30 min and the pellet was discarded. To the supernatant an equal volume of saturated ammonium-sulfate solution was slowly added and the preparation was stirred for 30 min then centrifuged at 11 300 · g for 30 min. The pellet was resuspended in Buffer B (50 mM 2-amino-2-(hydroxy-methyl)-1,3-propanediol (Tris) pH 7.8, 50 mM NaCl, 0.1 mM EDTA, 25 mM Tris-HCl, pH 8.5) and centrifuged at 100 000 · g for 60 min. After addition of calcium stock (to make 1 mM) the supernatant was loaded onto a phosphatidylserine affinity column, which was made using phosphatidylserine (Avanti Polar Lipids, Alabaster, Ala., USA) according to the protocol of Uchida and Filburn (1984). The flow rate was maintained at 0.6 ml · min⁻¹ during the load. After the sample was loaded, the column was washed with 10 column volumes of Buffer C (50 mM Tris pH 7.8, 50 mM NaCl, 0.1 mM CaCl, 0.1 mM PMSF, 1 mM DTT, 0.1% aprotinin), then step-eluted with Buffer D (50 mM Tris pH 7.8, 50 mM NaCl, 2 mM EDTA, 0.1 mM PMSF, 1 mM DTT, 0.1% aprotinin) into fractions of 1.5 ml. Sample purity was initially estimated by judging the relative staining intensity of the 35-kDa band after 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and silver-staining by the method of Oakley (1988).

**Western and dot-blot analysis.** The protein was subjected to SDS-PAGE according to Laemmli (1970) using minilab gels (0.8 mm thick, 10 cm long, 7.5 cm wide) containing 12% acrylamide. After 12% SDS-PAGE the protein was electroblotted onto a nitrocellulose membrane (0.45 µm pore size; Schleicher & Schuell, Keene, N.H., USA), by using a semi-dry, rapid-transfer apparatus (JKA-Biotech, Bronshoj, Denmark), as described by Kyhe-Anderson (1984). For the 45Ca²⁺ binding, 1 µg of purified p35 was dotted onto nitrocellulose membrane and treated by the method of Maruyama et al. (1984). One microgram of spinach calmodulin (Sigma) and 1 µg of bovine serum albumin (Sigma) were used as controls.

**Two-dimensional gel electrophoresis.** Equilibrium isoelectric focusing was performed according to O’Farrell (1975) using 10 µg of purified p35. The first dimension was 6% acrylamide and the second dimension was 10% acrylamide. The protein spots were stained overnight using Coomassie blue.

**In-situ peptide mapping.** Stained bands of the pI ~6.8 and ~7.0 p35 isoforms were excised and placed in the sample wells of a 1.5-mm-thick, 7.5% SDS polyacrylamide gel with a 5-cm stacking gel. The gel slices were covered with 10 µl overlay buffer followed by 0.05 µg of V-8 protease (sequencing grade; Boehringer Mannheim, Indianapolis, Ind., USA). The samples were then run at a constant current of 20 mA until the marker dye reached the top of the separating gel when the current was increased to 40 mA. Peptides were then stained overnight using Coomassie blue (Cleveland et al. 1977).

**Molecular-sieve chromatography.** A highly concentrated purified p35 preparation (20 µg/20 µl) was applied to an Ultrasphere-SEC 3000 HPLC molecular-sieve column, 30 cm long, 7.5 mm diameter (Beckman, San Ramon, Calif., USA) equilibrated in 100 mM Na-phosphate buffer, pH 7.0, and chromatographed isocratically at room temperature. The fractions eluting from the column were monitored by absorbance at 280 nm. The molecular-weight standards used for this column were thyroglobulin, γ-globulin, bovine serum albumin, ovalbumin, myoglobin, and vitamin B₁₂.

**Purification and sequence analysis of p35 peptides.** One hundred micrograms of purified p35 was fractionated by SDS-PAGE on a 12% gel and stained with Coomassie blue. The 35-kDa protein band was excised from the gel, electroeluted from the gel slices using an Elutrap device (Schleicher & Schuell), and then precipitated from the eluate by 95% ethanol. The pellet was washed in 95% ethanol twice then dissolved in digestion buffer (1 M urea, 1 mM ethylenediaminetetraacetic acid (EDTA), 25 mM Tris-HCl, pH 8.5) and incubated with 1 µg of endoproteinase Lys-C (sequencing grade; Boehringer Mannheim) for 12 h. The p35 digest was separated into component peptides by high-pressure liquid chromatography (HPLC), using a Vydac C18 peptide column, 25 cm long, 4.6 mm diameter (The Separations Group, Hesperia, Cal., USA), and a 60-µm linear gradient from solution A (0.1% trifluoroacetic acid (TFA) in water) to solution B (0.1% TFA in 60% acetonitrile). Peptide peaks were detected by absorbance at 214 nm. Several of the peptides were sequenced on an Applied Biosystems Sequencer (Model 477A) at the Protein Sequencing Center of the University of Texas, Austin.

**Preparation of antiserum.** The protein eluted from the phosphatidylserine affinity column was subjected to electrophoresis on 12% SDS-PAGE and the 35-kDa band was stained by Coomassie blue and excised. Approximately 100 µg of the excised p35 band was sent to Pocono Rabbit Farm (Dutch Hill Rd., Canadensis, Penn., USA) where it was injected in four equal aliquots into two guinea pigs over a six-week period. The two immune sera, 392 and 393, were collected and characterized separately. The data in this paper were all obtained using serum 392. The crude antiserum bleed and pre-immune serum were then purified by a protein-A sepharose (Sigma Chemical Co.) column according to the protocol described by Martin (1982). Affinity purity of anti-p35 was achieved by Western transfer of purified p35 to nitrocellulose, incubation of the bound protein band with crude 392 antiserum, and then elution of the antibody from the bound p35 with glycine buffer (Lillie and Brown 1987). Immune sera thus purified will be referred to hereafter as affinity-purified anti-p35.

**Light microscopy.** Samples were fixed in 2.5-3% formaldehyde and 0.1 M Na-phosphate buffer, pH 7.5, for 4-6 h, washed well in running water, dehydrated in ethanol, and embedded in paraffin. Sections 6-8 µm thick were then de-paraffinized, hydrated and stained by standard techniques of fluorosecent immunocytochemistry. Samples were blocked with 3% BLOTTO (3% w/v commercial non-fat dry milk in PBS, pH 7.8) for 1 h, and incubated with protein-A-purified anti-p35 (3 µg/10 µl), or protein-A-purified pre-immune serum (2.9 µg/10 µl) at 1:50 dilution for 2 h at room temperature. Some samples were incubated with affinity-purified anti-p35 at a 1:3 dilution for 2 h at room temperature. After several washes with 3% BLOTTO the samples were incubated with rabbit anti-guinea-pig immunoglobulin G (IgG) TRITC-conjugated secondary antibody (Sigma) at a 1:150 dilution for 1 h. The samples were washed several times with 3% BLOTTO, once with distilled H₂O, and mounted with glycerol, pH 9.

**Electron microscopy.** The samples were fixed in 2.5-3% formaldehyde and 0.17% glutaraldehyde in 0.1 M Na-phosphate buffer, pH 7.5, for 1 h. They were then washed, dehydrated in ethanol, and embedded in Lowicryl K4M (Polysciences, Warrington, Penn., USA). Pale-gold sections were cut and picked up on Parlodion-coated nickel grids, and were blocked in 5% BLOTTO for 1 h. They were then incubated with a 1:100 dilution of affinity-purified anti-p35 for 2 h at room temperature. After several washes with 3% BLOTTO the samples were incubated with rabbit anti-guinea-pig immunoglobulin G (IgG) TRITC-conjugated goat anti-guinea-pig IgG secondary antibody (Janssen, Piscataway, N. J., USA) diluted 1:150 for 1 h. The samples were washed three or more times with 3% BLOTTO, once with distilled H₂O, and then dried and poststained with 5% saturated uranyl acetate for 30 min.

**Assessment of staining validity and tissue identification.** For each light-microscopic-staining experiment, four to ten sections were mounted on two separate areas and run as parallel samples. Each staining experiment included antibody staining samples and control samples. Staining experiments were performed three or more times for both pea roots and plumules. Staining patterns were assessed on all of the serial sections for validity and to discount any error from sectioning or from artificial precipitation of staining solutions. Controls were run in the same way. For each electron-microscopic-