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Plant Cells Contain Calsequestrin*

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Calsequestrin is a high capacity low affinity Ca^{2+}-binding protein thought to be essential for the function of the intracellular rapid releasable Ca^{2+} pool of a variety of animal cells. Here we show that two types of plant tissues, cultured *Streptanthus tortuosus* cells and spinach leaves, contain a form of calsequestrin. In subcellular fractions of *S. tortuosus* cells, Stains-all staining reveals a metachromatically blue-staining 56,000-Da protein enriched in the microsomal fraction. This protein shares several biochemical characteristics with animal calsequestrin: 1) it changes its apparent molecular weight with the pH; 2) it is able to bind $^{45}Ca^{2+}$ on nitrocellulose transfers; and 3) it is recognized by antibodies against canine cardiac calsequestrin. Calsequestrin was also identified in spinach leaves using a direct extraction procedure that was developed for muscle calsequestrin. Thus, our results demonstrate that plant cells contain calsequestrin within a subcellular membrane fraction. These results also suggest that calsequestrin is an ubiquitous protein rather than being limited only to animal cells.

Calsequestrin is a Ca^{2+}-binding protein, first identified in muscle cells of various animal species (1–5). It is located in the lumen of the terminal cisternae of the sarcoplasmic reticulum (6–8), where it serves as a Ca^{2+} storage protein (1–5). The primary structure of skeletal muscle and cardiac calsequestrin has been deduced by cDNA cloning (9, 10). Both the cardiac and the skeletal forms of calsequestrin are enriched in acidic residues and do not contain EF hand Ca^{2+}-binding structures.

Recently, calsequestrin has been found in various nonmuscle cells (11–14), where it is located in a sarcoplasmic reticulum-like organelle, named the calciosome (12). Both sarcoplasmic reticulum and calciosomes are thought to be responsible for rapid Ca^{2+} uptake from and Ca^{2+} release to the cytosol and therefore crucial in the regulation of Ca^{2+}-dependent cellular functions. Ca^{2+} storage by calsequestrin is considered to play a central role in the function of these organelles.

In plant cells, Ca^{2+} is now generally recognized as an important intracellular messenger (for review see Refs. 15 and 16). However, little is known about the regulation of the cytosolic free Ca^{2+} concentration by intracellular organelles. ATP-dependent Ca^{2+} uptake and inositol 1,4,5-trisphosphate-induced Ca^{2+} release by microsomal fractions of plant tissue has been described (17–19) and tentatively attributed to the endoplasmic reticulum. Given the importance of calsequestrin in Ca^{2+} handling by intracellular organelles of animal cells, we performed the present studies to determine if plant cells contain a form of calsequestrin. We have demonstrated the presence of calsequestrin in subcellular fractions of cultured cells of the Californian shield leaf, *Streptanthus tortuosus* (Brassicaceae), and in whole spinach leaves.

EXPERIMENTAL PROCEDURES

Preparation of Subcellular Fractions of S. tortuosus—Callus cultures, obtained from the Californian shield leaf *S. tortuosus* (*Brassicaceae*), were grown as previously described (20, 21). Fifty to eighty grams of 14-day-old cells from *S. tortuosus* callus cultures were homogenized (mortar and pestle homogenizer) in a buffer containing 150 mM Tricine,[1] pH 7.5, 10 mM KCl, 1 mM EDTA, 1 mM $MgCl_2$, and 12% (w/w) sucrose. The crude homogenate was filtered through four layers of cheesecloth. This entire procedure was repeated with the residue. The filtered homogenate was centrifuged at 10,000 × *g* for 10 min. The supernatant (postmitochondrial supernatant) was used for experiments or further separated on a 30–60% sucrose density gradient formed between cushions of 60 and 20% sucrose. All sucrose solutions contained 1 mM EDTA, pH 7.5 Gradients were centrifuged at 83,000 × *g* for 180 min. The microsomal fraction was collected at the 20/30% interface, washed by dilution in a 150 mM Tricine pH 7.5 buffer, centrifuged at 150,000 × *g* for 30 min, and rediluted in the Tricine buffer at a protein concentration of approximately 1 mg/ml (22). All steps were performed at 4 °C.

Electron Microscopy of S. tortuosus Subcellular Fractions—The pelleted microsomal fraction was fixed with 2.5% glutaraldehyde, 1% osmium, and embedded in plastic for conventional electron microscopy.

Identification of Calsequestrin in Spinach—Whole spinach leaves were homogenized in a Waring blender (3 × 30 s) using a buffer that contained 0.1 M KH_2PO_4, pH 7.1, 2.66 M $(NH_4)_2SO_4$, 1 mM EDTA, 0.05 mM benzamidine, 0.05 mM phenylmethylsulfonyl fluoride, and centrifuged at 14,300 × *g* for 30 min. The supernatant was first filtered through cheesecloth, ammonium sulfate, 150 g/liter, was then added, and the pH was lowered to 4.7 with phosphoric acid. The solution was stirred for 150 min and centrifuged at 14,300 × *g* for 30 min. The pellet was dissolved in 0.1 M KH_2PO_4, 1 mM EDTA, pH 7.1, and dialyzed in 50 mM NaCl, 0.1 M KH_2PO_4, 1 mM EDTA, pH 7.1, overnight, followed by a buffer change the next morning. The dialyzed sample was centrifuged at 14,300 × *g* for 10 min and applied to a DEAE-cellulose column. The column was washed with 3 volumes of a 200 mM NaCl buffer and eluted with a 200–400 mM NaCl gradient. Washing and elution buffers contained 10 mM Tris-HCl, pH 7.5, 0.05 mM benzamidine, and 0.05 mM phenylmethylsulfonyl fluoride. All steps were performed at 4 °C.

SDS-Polyacrylamide Gel Electrophoresis Analysis of Plant Calsequestrin—The analysis of proteins by SDS-polyacrylamide gel electrophoresis was performed using the discontinuous buffer system of Laemmli (23) in 1.5-mm-thick gradient gels (5–16% acrylamide). Gels were stained with Coomassie Blue and destained with a solution of 10% acetic acid and 5% methanol. Staining with the cationic carbocyanine dye Stains-all was carried out as described (24). Two-dimensional gel electrophoresis was carried out as previously described (25).

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[1] The abbreviations used are: Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; SDS, sodium dodecyl sulfate; TEMED, *N,N,N′,N′*-tetramethylethylenediamine.

Apparent molecular weights were calculated from a graph of relative mobilities *versus* log molecular weight for standard proteins. The following molecular weight standards (native or prestained) were used: myosin (205–206 kDa), β-galactosidase (116 kDa), phosphorylase *b* (100 kDa), albumin (66–68 kDa), ovalbumin (42–45 kDa), α-chymotrypsinogen (25–26 kDa), soybean trypsin inhibitor (19 kDa), β-lactoglobulin (18 kDa), and lysozyme (15 kDa).

Immunochemical Analysis of Plant Calsequestrin—Proteins were separated by SDS-polyacrylamide gel electrophoresis using the discontinuous buffer system of Laemmli (23) in 1.5-mm-thick gradient gels (5–16% acrylamide) and transferred electrophoretically onto nitrocellulose membranes according to Towbin *et al.* (26). For *$^{45}Ca^{2+}$ overlay* the transfers were blocked with phosphate-buffered saline containing 0.05% Tween 20 and incubated with $^{45}Ca^{2+}$ as described (27). For *immunoblots*, the transfers were blocked with TBST (10 mM Tris/HCl, pH 7.0, 500 mM NaCl, 0.05% Tween 20) containing 0.5% nonfat dry milk before incubation with the antibody. Polyclonal antibodies to canine cardiac calsequestrin were prepared in rabbits as previously described (7). Secondary antibodies were coupled to alkaline phosphatase, and blots were developed using 5-bromo-4-chloro-3-indoyl phosphate and nitro blue tetrazolium as described (28).

Materials—SDS, acrylamide, *N,N'*-methylenebisacrylamide, 2-mercaptoethanol, and TEMED were purchased from Bio-Rad. The cationic carbocyanine dye Stains-all (1-ethyl-2-[3-(1-ethylnaphtho [1,2d]thiazolin-2-ylidene)-2-methylpropenyl]naptho[1,2d]thiazolium bromide) was obtained from Eastman Organic Chemicals and prepared as a 0.1% stock solution in formamide. Alkaline phosphatase-coupled secondary antibodies, 5-bromo-4-chloro-3-indoyl phosphate and nitro blue tetrazolium, were purchased from Sigma. All other chemicals were reagent grade or the highest purity available.

RESULTS AND DISCUSSION

Ca^{2+} uptake by subcellular organelles was found in microsomal fractions of various plant tissues (17–19). Accordingly we used two subcellular fractions of *S. tortuosus* cells in this study: a postmitochondrial supernatant, as a crude mixture of various plant organelles, and a microsomal fraction, which was obtained by further purification of the postmitochondrial supernatant on a sucrose gradient. Fig. 1*A* is an electron micrograph of the microsomal fraction. The preparation is devoid of mitochondria, nuclei, and plastids; however, a variety of microsomal organelles, such as endoplasmic reticulum and Golgi stacks, can be seen.

The subcellular fractions were analyzed by SDS-polyacrylamide gel electrophoresis. Gels were stained with Coomassie Blue (Fig. 1*B*) or Stains-all (Fig. 1*C*). The Coomassie Blue staining shows a multitude of proteins in both subcellular fractions. The microsomal fraction seems to be depleted of a variety of low molecular weight proteins, while it is enriched in several proteins of higher molecular weight. The Stains-all staining revealed a metachromatically blue staining band with an apparent molecular mass of 56 kDa. This protein was greatly enriched in the microsomal fraction. Calsequestrin from animal muscle is recognized in Stains-all-stained Laemmli-type gels as a metachromatically blue staining band with an apparent molecular mass between 50 and 65 kDa, depending on muscle type and animal species used (1–5). The presence of a metachromatically blue-staining 56-kDa band in *S. tortuosus* cells suggests that these plant cells may contain a form of calsequestrin.

Calsequestrin from muscle cells has been shown to change its apparent molecular weight with pH (25). Thus, in a two-dimensional gel system with a first separation step at a pH of 7.0 (Weber-Osborn-type gel, Ref. 29) and a second separation step at a pH of 8.7 (Laemmli-type gel, Ref. 23), calsequestrin falls off the diagonal formed by the majority of the proteins. Fig. 2 shows a Stains-all-stained two-dimensional gel of the postmitochondrial supernatant of *S. tortuosus* cells. The metachromatically staining 56-kDa band behaved similar to calsequestrin of animal muscle, exhibiting a lower apparent molecular weight in the pH 7.0 gel system (run from *left* to *right*) when compared to other proteins of a similar apparent molecular weight in the pH 8.7 gel system (run from *top* to *bottom*).

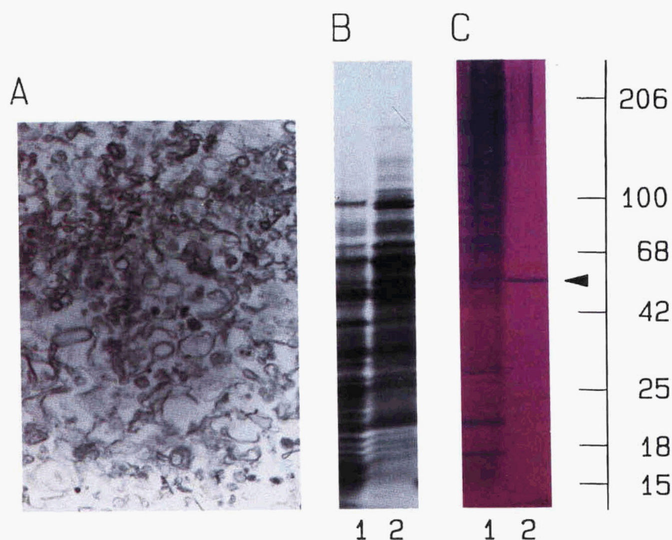

FIG. 1. **Electron micrograph and SDS-polyacrylamide gel electrophoresis of subcellular fractions of *S. tortuosus*.** Subcellular fractions of homogenates of *S. tortuosus* were analyzed by transmission electron microscopy (*panel A*, microsomal fraction only, magnification, × 30,000) or by SDS-polyacrylamide electrophoresis (5–16% gradient gels) and stained with Coomassie Blue (*panel B*) or Stains-all (*panel C*) as described under "Experimental Procedures." *Lane 1*, postmitochondrial supernatant (100 μg), supernatant of centrifugation of *S. tortuosus* homogenate at 10,000 × *g* for 10 min; *lane 2*, microsomal fraction (100 μg) obtained by purification of the postmitochondrial supernatant by a sucrose gradient. The *arrowhead* indicates calsequestrin. Several *blue-staining bands* with an apparent molecular mass above 100 kDa that do not have a corresponding band in the Coomassie Blue-stained gel can be observed in the postmitochondrial supernatant (*panels B* and *C*, *lane 1*). These bands do not necessarily correspond to proteins but possibly to polysaccharides.

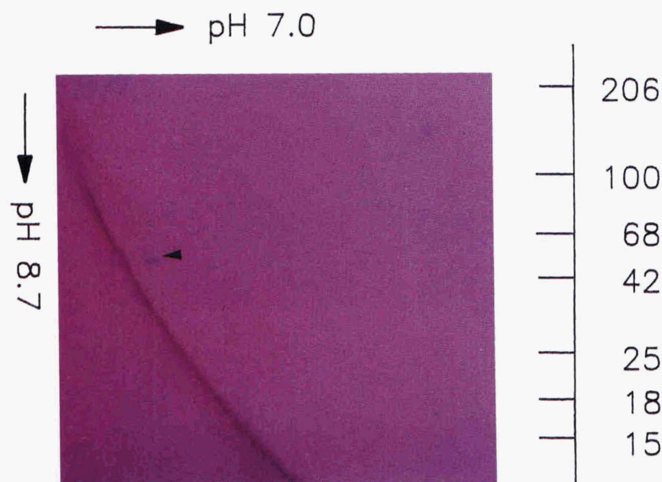

FIG. 2. **Stains-all-stained two-dimensional gel electrophoresis of proteins of *S. tortuosus*.** Postmitochondrial supernatant (100 μg) was separated by a Weber-Osborn-type gel (12% SDS gel, pH 7.0). A lane was cut from the Weber-Osborn gel and applied to a Laemmli-type gel (5–16% gradient gel, pH 8.7) which was stained with Stains-all as previously described. The *arrowhead* indicates calsequestrin.

Calsequestrin of animal muscle can be recognized on nitrocellulose transfers by its high capacity to bind $^{45}Ca^{2+}$. Autoradiography of $^{45}Ca^{2+}$-bathed nitrocellulose transfers of sub-

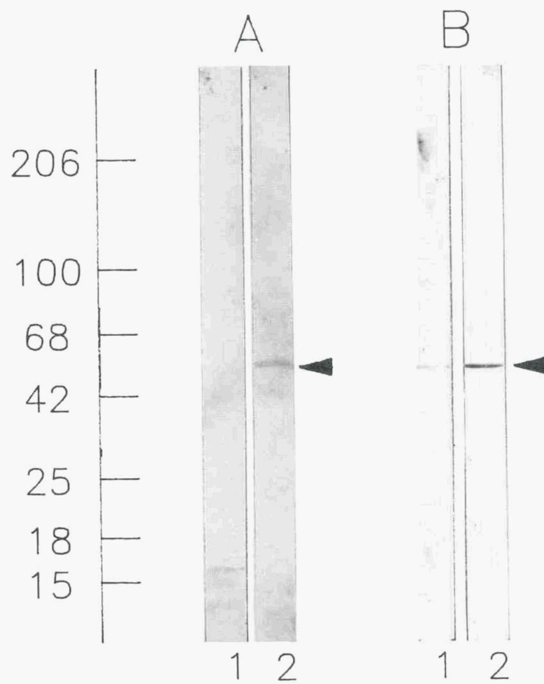

FIG. 3. **$^{45}Ca^{2+}$ overlay and anti-calsequestrin immunostaining of *S. tortuosus* proteins.** Subcellular fractions of homogenates of *S. tortuosus* were analyzed by SDS-polyacrylamide electrophoresis (5–16% gradient gels) and transferred to nitrocellulose as described under "Experimental Procedures." The transfers were blocked and incubated with $^{45}Ca^{2+}$ (*panel A*) or polyclonal antibodies against canine cardiac calsequestrin (*panel B*). *Lane 1*, postmitochondrial supernatant (100 μg); *lane 2*, microsomal fraction (100 μg). The *arrowhead* indicates calsequestrin.

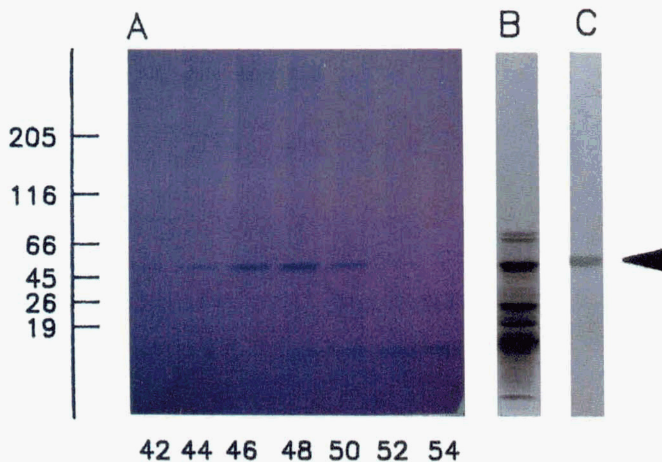

FIG. 4. **Partial purification of a plant calsequestrin from spinach.** Spinach leaves were homogenized, and a fraction enriched in calsequestrin was obtained by ammonium sulfate solubilization and acid precipitation. The enriched fraction was loaded on a DEAE-cellulose column and eluted with a 200–400 mM NaCl gradient. *Panel A* shows Stains-all-stained SDS gels of fractions 42–54 (fraction volume 2.5 ml, applied volume 100 μl) collected from this gradient. The peak elution of spinach calsequestrin was calculated to be at 325 mM NaCl. *Panel B* shows a Coomassie blue-stained SDS-polyacrylamide gel and *panel C* a $^{45}Ca^{2+}$ overlay of the pooled fraction (30 μg/lane). The *arrowhead* indicates calsequestrin.

cellular fractions of *S. tortuosus* cells revealed a 56-kDa band (Fig. 3*A*). This protein was greatly enriched in the microsomal fraction.

Polyclonal and monoclonal antibodies have been raised against animal muscle calsequestrin and have proven to be useful in identifying calsequestrin in biochemical and morphological studies (6–8, 12). Fig. 3*B* shows an immunoblot of the two subcellular fractions of *S. tortuosus* cells stained with polyclonal antibodies against canine cardiac calsequestrin. A 56-kDa protein, greatly enriched in the microsomal fraction, was recognized by the antibody, demonstrating that the plant form of calsequestrin is immunologically related to the animal form. Polyclonal antibodies against skeletal muscle calsequestrin only reacted weakly with the protein, while monoclonal antibodies did not react (not shown). Thus, based on these criteria: Stains-all staining, pH-dependent shift of molecular weight, $^{45}Ca^{2+}$ overlay, and immunological cross-reactivity, *S. tortuosus* cells contain a form of calsequestrin.

A method to isolate calsequestrin directly from muscle which is based upon the physical and chemical properties of calsequestrin has been developed in our laboratory. In order to test whether plant calsequestrin fractionates in the same manner, we have applied this method to whole spinach leaves. Fig. 4*A* shows Stains-all-stained SDS gels of fractions 42–54 of the DEAE column. A metachromatically blue-staining protein with the apparent molecular mass of around 56 kDa was eluted. The maximum of the elution was calculated to be around 325 mM NaCl. Fig. 4*B* shows a Coomassie Blue-stained gel and Fig. 4*C* a $^{45}Ca^{2+}$ overlay of the pooled calsequestrin fractions, demonstrating that the partially purified protein is able to bind Ca^{2+}. The partially purified protein is also recognized by antibodies against skeletal muscle calsequestrin (not shown). These results show that plant calsequestrin not only shares various properties with animal calsequestrin but can also be partially purified by a method that was developed for animal muscle calsequestrin.

The results obtained in this study are, to the best of our knowledge, the first demonstration of calsequestrin in plant tissue. This represents the continuation of an amazing change in our perception of calsequestrin. Originally thought to be a specific animal muscle protein, calsequestrin has been recognized in a variety of nonmuscle animal cells only during the last year (11–14). The discovery of calsequestrin in plants extends these observations. Calsequestrin might now be considered as an ubiquitous protein. The immunological similarity of the calsequestrins in so distant cells as canine cardiac muscle and *S. tortuosus* cells was surprising. It demonstrates that at least a portion of the protein is highly conserved during phylogenesis.

The widespread occurrence and phylogenetical conservation of calsequestrin suggest that it plays an important role for all types of cells. In animal cells, both muscle and nonmuscle, calsequestrin is thought to be the Ca^{2+} storage protein of the rapidly releasable intracellular Ca^{2+} pool. Functional studies with purified muscle calsequestrin show that it binds Ca^{2+} with high capacity and low affinity (4). These properties make it ideally suited for its role in storing large amounts of rapidly releasable Ca^{2+}. The Ca^{2+}-binding properties of purified plant calsequestrin will have to be studied further in order to understand if it can play a similar role in plant cells.

In both muscle and nonmuscle animal cells, calsequestrin can be used in morphological and subcellular fractionation studies as a marker protein of the organelle which is thought to be responsible for rapid uptake and release of intracellular Ca^{2+} (1–8, 12). In plant cells this organelle was hypothesized to be the endoplasmic reticulum (18), as previously thought for animal nonmuscle cells (30). However, smooth microsomal membranes, morphologically distinct from the typical endoplasmic reticulum, with a hitherto unknown function have been described in *S. tortuosus* (21) and other plant cells (31, 32). Thus, the investigation of the subcellular localization of

calsequestrin will be an important tool for the identification of the intracellular store of rapidly releasable Ca^{2+} in plant cells.

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