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Communication

Antibodies against the Calcium-Binding Protein

Calsequestrin from *Streptanthus tortuosus* (Brassicaceae)

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ABSTRACT

Plant microsomes contain a protein clearly related to a calcium-binding protein, calsequestrin, originally found in the sarcoplasmic reticulum of muscle cells, responsible for the rapid release and uptake of Ca^{2+} within the cells. The location and role of calsequestrin in plant cells is unknown. To generate monoclonal antibodies specific to plant calsequestrin, mice were immunized with a microsomal fraction from cultured cells of *Streptanthus tortuosus* (Brassicaceae). Two clones cross-reacted with one protein band with a molecular weight equal to that of calsequestrin (57 kilodaltons) by sodium dodecyi sulfate-polyacrylamide gel electrophoresis and immunoblotting. This band is able to bind $^{45}Ca^{2+}$ and can be recognized by a polyclonal antibody against the canine cardiac muscle calsequestrin. Rabbit skeletal muscle calsequestrin cross-reacted with the plant monoclonal antibodies. The plant monoclonal antibodies generated here are specific to calsequestrin protein.

Calsequestrin is located in the lumen of a smooth membrane system of muscle cells (for a review, see ref. 17), the SR.[4] Calcium is taken up or released from the SR membranes during cycles of muscle contraction and relaxation. Calsequestrin functions to regulate calcium in the SR. Calsequestrin has also been identified in smooth muscle (3) and cells (18), where it is thought to be located in small vesicles termed calciosomes (7, 22). Recently, we showed (13) that calsequestrin is also present in plant cells and is enriched in microsomal membrane fractions. The function and localization of plant calsequestrin is not yet known. However, calcium is now generally known to play a central role in the regulation of plant cell metabolism (9, 12) and is also thought to be important in determining plant cell responses to pathogens (1). The regulation of these responses is dependent on the maintenance of a low cytosolic Ca^{2+} concentration, usually less than 1 μM. In order to maintain this low calcium level, plant cells are thought to sequester Ca^{2+} in their vacuoles and in the ER lumen. ATP-dependent Ca^{2+} uptake and inositol 1,4,5-triphosphate-induced Ca^{2+} release by microsomal membrane fractions from several plant tissues have been reported (4, 6, 16, 19). Nevertheless, little is known about the intracellular organelles and proteins that participate in this regulation in plants. Since calsequestrin has been linked to the uptake and release of Ca^{2+} by sarcoplasmic reticulum membranes in muscles (17) and by calciosomes in nonmuscle cells (22), the discovery of this protein in plants suggests that it may play a similar role in plant cells as well. To carry out further investigations on the function and localization of calsequestrin in plant cells, we have produced monoclonal antibodies against plant calsequestrin.

MATERIALS AND METHODS

Preparation of Microsomal Membrane Fractions

Streptanthus tortuosus callus cultures were grown as previously described (13). One hundred g of the cultured cells were homogenized in a medium containing 150 mM Tricine buffer (pH 7.5), 10 mM KCl, 1 mM EDTA, 1 mM $MgCl_2$, and 12% sucrose using a Polytron and centrifuged at 10,000,? for 10 min to get a postmitochondrial supernatant. The supernatant was applied to a sucrose density gradient as previously described (13). The microsomal fraction collected from the gradient was pelleted at 30,000 rpm in a Ti 50 rotor for 30 min and suspended in Tricine buffer at a protein concentration of 1 mg/mL.

Monoclonal Antibody Production

Five-to-six-week-old female BALB/c mice were immunized intraperitoneally with 200 μg microsomal proteins emulsified in Freund's complete adjuvant. The immunization was repeated four times at 2-week intervals with the same amount of protein in Freund's incomplete adjuvant. Hybridoma cells were produced by fusion of spleen cells with NS-1 myeloma cells. Hybridoma supernatants were screened against postmitochondrial fractions, microsomal fractions, a crude calsequestrin fraction, and partially purified calsequestrin using an immunodot assay (8) on Millititer plates (Millipore). Calsequestrin proteins were exposed within the microsomal vesicles by using digitonin at a concentration of 0.02%. Positive clones

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[4]Abbreviation: SR, sarcoplasmic reticulum.

were defined as those producing antibodies against all four protein preparations and having the strongest reaction with the partially purified calsequestrin. Positive hybridomas were grown, dilution-cloned, and passaged in RPMI 1640 medium supplemented with 10% fetal bovine serum. The supematants of the positive clones were rescreened by immunoblot assay against postmitochondrial proteins and microsomal proteins to get two monoclones. Ascites fluid was produced by injecting 5×10^6 hybridoma cells intraperitoneally into Pristane-primed BALB/c mice. The ascites fluid was delipidated with Seroclear reagent (CalBiochem).

SDS-PAGE Analysis of Proteins

The analysis of proteins was performed by using the discontinuous buffer system of Laemmli (14) in 1.5 mm thick polyacrylamide gradient gels (5-16%).

Immunoblot Assay

Proteins were separated on a 5 to 16% gradient gel and transferred to nitrocellulose membranes using a modification of the procedure of Towbin *et al*. (21). BLOTTO (Bovine Lacto Transfer Technique Optimizer/PBS/5% nonfat dry milk) (10) was used for blocking the nitrocellulose transfers and dilution of antibodies. Nitrocellulose blots were first incubated with primary antibodies (1:500 dilution) and then incubated with goat anti-mouse (for the plant monoclonal) or goat anti-rabbit (for the animal polyclonal) IgG peroxidase-linked secondary antibodies (Cooper Biomedical, Inc.) at 1:1000 dilution.

$^{45}Ca^{2+}$-Overlay

Proteins were transferred electrophoretically onto nitrocellulose membranes as described above. The transfers were blocked with PBS containing 0.05% Tween-20, and incubated for 1 h in three changes of a buffer containing 60 mM KCl, 5 mM $MgCl_2$, and 10 mM imidazole-HCl (pH 6.8), and then incubated in 1 μCi/mL of $^{45}Ca^{2+}$ with the same buffer for 20 min. The transfers were washed with distilled water for 5 min, and, if the background was high, also washed with 50% ethanol for 5 min or longer (11).

RESULTS AND DISCUSSION

We identified a plant calsequestrin protein from *Streptanthus tortuosus* cultured cells (13) using four criteria: (a) blue band on a Stains-all staining gel, (b) enrichment in microsomal membrane fractions, (c) cross-reaction with a polyclonal antibody against calsequestrin from canine cardiac muscle, and (d) $^{45}Ca^{2+}$ binding using a $^{45}Ca^{2+}$-overlay assay. Here, we generated monoclonal antibodies against this protein from the same cultured cells.

Mice were immunized with plant microsomal membrane proteins and then screened for monoclonal antibodies against calsequestrin. Twelve out of 960 clones showed positive reactions in immunodot assays, and two of them cross-reacted with only one protein band corresponding to a mol wt of 57 kD (Fig. 1C, shows only one clone) by immunoblotting of plant postmitochondrial proteins (Fig. 1B). A polyclonal antibody raised against canine cardiac muscle calsequestrin can also recognize this band (Fig. 1D). Furthermore, a $^{45}Ca^{2+}$-overlay technique showed that this protein band could bind $^{45}Ca^{2+}$ (Fig. 1E).

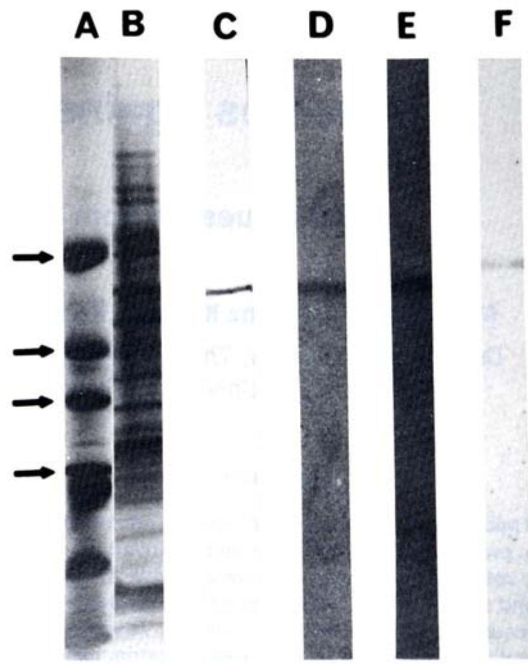

Figure 1. Identification of plant calsequestrin. A, Mol wt marker: 66, 45, 36, 29 kD. B, Postmitochondrial proteins from cultured plant cells stained with Coomassie blue. C-E, Postmitochondrial proteins on nitrocellulose papers; C, cross-reacted with plant monoclonal antibody; D, cross-reacted with canine cardiac calsequestrin polyclonal antibody; E, $^{45}Ca^{2+}$ binding. F, Rabbit skeletal muscle microsomal membrane proteins on nitrocellulose paper cross-reacted with plant monoclonal antibody.

The protein-calcium-binding of calcium binding proteins is electrostatic in nature and is based on ligands provided by the oxygens of carboxyl groups from amino acid residues. The proteins form calcium ligands in several configurations. Some calcium binding proteins have donor groups from residues in different regions of the primary structure constrained by disulfide bridges; other calcium binding proteins have closely sequential ligands on a relatively mobile framework, forming a so-called “EF hand” (15). Since the secondary and tertiary structures of these proteins are important for their calcium binding ability, these proteins are less likely to bind $^{45}Ca^{2+}$ on nitrocellulose paper after SDS-PAGE. However, calsequestrin uses a different feature to bind calcium. The complete amino acid sequences of canine cardiac (20) and rabbit skeletal muscle (5) calsequestrin deduced from cDNA reveal that they are highly acidic proteins with clustered acidic amino acid residues and that they lack the EF hand calcium-binding structures. Calsequestrin probably binds calcium by acting as a charged surface on the primary structure rather than by presenting multiple discrete calcium binding sites. It is for this reason that calsequestrin can bind $^{45}Ca^{2+}$ in the denatured state, after SDS-PAGE, as shown in Figure 1E.

Animal calsequestrin from microsomal membrane proteins of rabbit skeletal muscle stains as a blue band in a Stains-all staining gel (data not shown) and has a previously published mol wt of 63 kD (2). This animal protein also cross-reacted with the plant monoclonal antibodies (Fig. IV). In summary, we have used four criteria to characterize these monoclonal antibodies: first, the specificity was shown by immunoblotting; second, the protein recognized by the monoclonal antibodies can bind $^{45}Ca^{2+}$ on the nitrocellulose paper; third, the protein recognized by the plant monoclonal antibodies can also be recognized by an animal calsequestrin antibody; fourth, the animal calsequestrin from rabbit skeletal muscle microsomal membrane fractions can be recognized by the plant monoclonal antibodies. Based on these criteria, we believe that the plant monoclonal antibodies generated here are specific to calsequestrin proteins. These two hybridoma clones have already been used to raise additional amounts of monoclonal antibodies by generating ascites in primed mice.

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