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Purification and Characterization of Calsequestrin from Canine Cardiac Sarcoplasmic Reticulum and Identification of the 53,000 Dalton Glycoprotein*

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Cardiac calsequestrin was extracted from canine cardiac sarcoplasmic reticulum vesicles with Nonidet P-40 and purified by precipitation with calcium phosphate followed by fractionation on DEAE-cellulose. It bound 300 nmol of Ca^{2+}/mg of protein and glutamic and aspartic acid constituted approximately 32% of the amino acid residues in the protein. The apparent molecular weight of canine cardiac calsequestrin was 55,000 when measured in alkaline sodium dodecyl sulfate gels and 44,000 when measured in neutral sodium dodecyl sulfate gels. Cardiac calsequestrin, like skeletal muscle calsequestrin, stains blue with "Stains-all" and is therefore the 55,000 dalton protein which Jones and Cala ((1981) *J. Biol. Chem.* 256, 11809-11818) identified as a component of ryanodine-sensitive cardiac sarcoplasmic reticulum. Unlike skeletal muscle calsequestrin, cardiac calsequestrin was sensitive to endo-β-*N*-acetylglucosaminidase H, indicating that it contained a "high mannose" oligosaccharide.

Cardiac calsequestrin cross-reacted with an antiserum raised against skeletal muscle calsequestrin. Indirect immunofluorescent staining of rat cardiac muscle using the skeletal muscle calsequestrin antiserum revealed a polygonal pattern in transverse sections and I-band staining in longitudinal sections. A comparison between the localization of the (Ca^{2+} + Mg^{2+})ATPase and calsequestrin suggested that cardiac calsequestrin was confined to the regions of cardiac muscle cells where the sarcoplasmic reticulum was localized.

A 53,000 dalton, intrinsic glycoprotein was also identified in cardiac sarcoplasmic reticulum by endo-β-*N*-acetylglucosaminidase H digestion, ^{125}I-concanavalin A binding, and indirect antibody staining with skeletal muscle glycoprotein antiserum and ^{125}I-protein A. Its molecular weight of 53,000 was reduced to 49,000 by endo-β-*N*-acetylglucosaminidase H digestion. Cardiac sarcoplasmic reticulum also contained a Stains-all blue staining, 130,000 dalton glycoprotein, which was similar to the 160,000 dalton glycoprotein of skeletal muscle sarcoplasmic reticulum.

The sarcoplasmic reticulum membrane plays an essential role in the control of intracellular Ca^{2+} concentration in both skeletal and cardiac muscle (1-3). Sarcoplasmic reticulum vesicles isolated from rabbit skeletal muscle contain as major proteins, the (Ca^{2+} + Mg^{2+})ATPase (M_r = 105,000), calsequestrin (M_r = 63,000), and two intrinsic glycoproteins (M_r = 53,000 and 160,000) (4-6). The (Ca^{2+} + Mg^{2+})ATPase is an intrinsic membrane protein which is responsible for the active transport of Ca^{2+} across the sarcoplasmic reticulum membrane (7). The 53,000 dalton glycoprotein is a transmembrane protein, largely exposed on the cytoplasmic surface of the vesicles (6). It binds 8-N_3-ATP with high affinity.[1] Less is known about the 160,000 dalton glycoprotein, but it shares many properties with the 53,000 dalton glycoprotein. Calsequestrin is a very acidic protein which binds some 900 nmol of Ca^{2+}/mg of protein and is probably involved in Ca^{2+} storage within the lumen of the sarcoplasmic reticulum (5). Biochemical and morphological analysis of highly purified sarcoplasmic reticulum vesicles indicate that calsequestrin, possibly as a Ca^{2+}-calsequestrin complex, makes up the electron dense content in the terminal cisternae (8, 9). Immunofluorescence and immunoferritin localization studies in skeletal muscle *in situ* have shown that the (Ca^{2+} + Mg^{2+})ATPase is found throughout the sarcoplasmic reticulum, except for the junctional sarcoplasmic reticulum membrane, while calsequestrin and the 53,000 dalton glycoprotein are found in the region of the terminal cisternae (10-13). Similarly, the (Ca^{2+} + Mg^{2+})-ATPase in cardiac muscle is also rather uniformly distributed in the free sarcoplasmic reticulum and absent from both peripheral and interior junctional sarcoplasmic reticulum (14).

In cardiac muscle, unlike skeletal muscle, Ca^{2+} required for contraction comes both from the extracellular space and from the sarcoplasmic reticulum (15). Relaxation is initiated when Ca^{2+} is removed from the myofilament space by Ca^{2+} transport into the sarcoplasmic reticulum and by Ca^{2+} transport across the sarcolemma (15). Sarcoplasmic reticulum vesicles isolated from canine cardiac muscle actively transport Ca^{2+} but they

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[1] Campbell, K. P., and MacLennan, D. H. (1983) *J. Biol. Chem.*, in press.

accumulate less Ca^{2+} than skeletal muscle sarcoplasmic reticulum vesicles (16). Cardiac sarcoplasmic reticulum vesicles appear to contain a more complex protein composition than skeletal vesicles (17) and only the (Ca^{2+} + Mg^{2+})ATPase has been identified and purified from this source (18, 19). In a recent review, Tada and Katz (20) noted that calsequestrin has not been reported to be a component of cardiac sarcoplasmic reticulum.

There is circumstantial evidence that the junctional sarcoplasmic reticulum of cardiac muscle contains a protein with chemical, functional, and morphological properties like skeletal muscle calsequestrin. Junctional sarcoplasmic reticulum and peripheral, junctional sarcoplasmic reticulum membranes in cardiac muscle have an electron dense content similar to the electron dense content in skeletal muscle terminal cisternae (21). Ca^{2+} has been localized in the junctional sarcoplasmic reticulum in both skeletal and cardiac muscle (22, 23) and the presence of polyanionic material inside the junctional sarcoplasmic reticulum of cardiac and skeletal muscle has been suggested by cytochemical studies (21, 24). Jones *et al.* (17, 25) have shown that cardiac sarcoplasmic reticulum contains proteins which stain deep blue with Stains-all. We have recently found that calsequestrin is one of the few proteins in muscle that stain deep blue with Stains-all,[2] suggesting that one of the blue-staining bands in cardiac sarcoplasmic reticulum might be calsequestrin.

In this paper we describe the biochemical and immunological identification and the purification of calsequestrin from canine cardiac sarcoplasmic reticulum. We have also identified and characterized the intrinsic glycoproteins of 53,000 and 130,000 daltons in cardiac sarcoplasmic reticulum which are similar to the 53,000 and 160,000 dalton glycoproteins in rabbit skeletal muscle sarcoplasmic reticulum. It appears, therefore, that cardiac sarcoplasmic reticulum contains at least four of the major sarcoplasmic reticulum proteins which are found in skeletal muscle.

MATERIALS AND METHODS

Sodium dodecyl sulfate, acrylamide, *N,N'*-methylene bisacrylamide, 2-mercaptoethanol, and *N,N,N',N'*-tetramethylethylenediamine were purchased from BioRad. Endo *β-N*-acetylglucosaminidase H (*Streptomyces griseus*) was a product of Health Research Inc., Albany, NY. The cationic carbocyanine dye Stains-all (1-ethyl-2-[3-(1-ethylnaphthol[1, 2*d*]thiazolin-2-ylidene)-2-methylpropenyl] naphthol[1, 2d]thiazolium bromide) was obtained from Kodak Organic Chemicals. ^{125}I was obtained from Amersham.

Preparation of Cardiac Calsequestrin—Canine cardiac membrane vesicles were prepared according to the method of Jones *et al.* (17) and of Suko and Hasselbach (26). Sarcoplasmic reticulum vesicles were prepared from rabbit skeletal muscle according to the method of MacLennan (4) as modified by Campbell and MacLennan (6). Cardiac membrane vesicles (17) (usually 1 g) at a concentration of 5 mg/ml were extracted with Nonidet P-40 (1–2 mg/mg of protein) at 4 °C in a solution containing 20% glycerol (v/v), 1 mM phenylmethylsulfonyl fluoride, and 100 mM Tris-HCl, pH 8.5. After 15 min, an equal volume of 100 mM Tris-HCl, pH 8.5, was added and the mixture was centrifuged at 100,000 × *g* for 60 min. The supernatant was removed and solid calcium phosphate was added in a ratio of 3.5 g/liter. The slurry was stirred at 0 °C for 4 h and the insoluble calcium phosphate was collected by centrifugation at 5,000 × *g*. The pellet was washed by resuspension and centrifugation first with 200 ml of 1 mM Tris-HCl, pH 8.5, then with 200 ml of 10 mM sodium phosphate, pH 7.0, 200 ml of 25 mM sodium phosphate, pH 7.0, and, finally, calsequestrin was eluted with 100 ml of 100 mM sodium phosphate, pH 7.0. The solution containing the cardiac calsequestrin was then applied directly to a DEAE-cellulose column and eluted with a gradient of 0 to 0.6 M NaCl in 10 mM Tris-HCl, pH 7.5, and 0.1 mM EGTA.[3] The purification of cardiac calsequestrin was monitored by SDS-slab gel electrophoresis. Slab gels were stained selectively for calsequestrin using Stains-all.[2] Cardiac calsequestrin eluted between 0.35 and 0.45 M NaCl. Fractions containing purified cardiac calsequestrin were combined, concentrated, and dialyzed against 10 mM Tris-HCl, pH 7.5, and 0.1 mM EGTA. The yield of protein was approximately 1.0 mg/g of cardiac vesicles. Rabbit skeletal calsequestrin was also purified using the same procedure. Protein was determined by the method of Lowry *et al.* (27) using bovine serum albumin as a standard.

Amino Acid Analysis—Amino acid compositions were determined after hydrolysis of proteins in 6 N HCl for 24, 48 and 72 h in sealed, evacuated tubes at 110 °C (28). Values for serine and threonine were derived by extrapolation to zero time to correct for hydrolytic losses. Values for valine and isoleucine reached plateau values at 72 hr. Cysteine was determined as cysteic acid and methionine was determined as methionine sulfone after performic acid oxidation. Calsequestrin samples were extensively dialyzed against distilled, deionized H_2O prior to hydrolysis.

Ca^{2+} Binding to Calsequestrin—$^{45}Ca^{2+}$ binding to calsequestrin was measured by an equilibrium dialysis procedure (29) in the presence of 100 mM KCl, 5 mM Tris-HCl, pH 7.5, and various amounts of $CaCl_2$ containing ^{45}Ca. Calsequestrin samples were dialyzed extensively against 100 mM KCl, 5 mM Tris-HCL, pH 7.5, prior to $^{45}Ca^{2+}$ binding experiments.

Endo H Removal of Carbohydrate Chains—Oligosaccharide chains were removed from skeletal and cardiac sarcoplasmic reticulum glycoproteins with Endo H following extraction with deoxycholate (6). Control incubations were carried out in the absence of Endo H. Typically, 100 μg of cardiac sarcoplasmic reticulum proteins in 10 μl were solubilized using SDS so that the final protein to SDS ratio was 1:2 (w/w). The samples were heated at 100 °C for 1 min and then cooled and diluted with a solution of 50 mM sodium citrate, pH 5.5, to bring the SDS concentration to 0.2%. Phenylmethylsulfonyl fluoride was added to 1 mM and Endo H was added to 50 milliunits/ml. After 6 h at 37 °C the reaction was terminated by the addition of SDS sample buffer followed by incubation at 100 °C for 1 min (30).

Radioiodination of Sarcoplasmic Reticulum Proteins—Sarcoplasmic reticulum proteins were radioiodinated using Bio-Rad Enzymobeads which have lactoperoxidase and glucose oxidase covalently attached. The iodination of intact sarcoplasmic reticulum vesicles was carried out in the presence of 0.1 M sodium phosphate, pH 7.2, using 50 μl of Enzymobeads, 100 μg of sarcoplasmic reticulum proteins, and 50 μCi of ^{125}I. The reaction was initiated by the addition of 1% β-D-glucose and terminated after 30 min at room temperature by separation of the mixture on a Sephadex G-25 column in a 3-ml disposable syringe casing containing 3 ml of wet Sephadex beads. The syringe was centrifuged at 100 × *g* for 1 min and the iodinated protein was collected in the eluate.

Radioiodination of Protein A—Protein A was radioiodinated by the chloramine-T method (31) with minor modifications. Iodination was performed at room temperature in 50 μl of a solution of 150 mM NaCl and 10 mM sodium phosphate, pH 7.2 (buffer A) containing 10% dimethyl sulfoxide, 1–2 mCi of carrier-free $Na^{125}I$, and 0.05% chloramine-T. The reaction was stopped after 15 min by the addition of 5 μl of a 0.1% sodium metabisulfite solution. The radioiodinated protein A was separated from unreacted iodide by chromatography on two successive 4-ml Sephadex G-25 columns. The first column was equilibrated with buffer A; the second column was equilibrated with 0.05 M sodium phosphate, pH 4.0. The total volume of purified ^{125}I-protein A was adjusted to 10 ml in 0.03 M sodium phosphate, pH 4, containing 40% ethanol and the ^{125}I-protein A was stored at −20 °C. The specific activity was about 60,000 cpm/μl. Concanavalin A was also iodinated using chloramine-T as previously described (32).

Immunostaining of Western Blots—The indirect ^{125}I-protein A assay of Western blots was carried out as described by Towbin *et al.* (33). Protein samples were first separated by Laemmli slab gel electrophoresis and then transferred electrophoretically to nitrocellulose sheets. The nitrocellulose sheets were first incubated with 3% bovine serum albumin in a solution of 150 mM NaCl and 10 mM Tris-HCl, pH 7.2 (buffer B), for 1 h at 37 °C. The sheets were incubated for 5–16 h at room temperature with antiserum to calsequestrin or to the 53,000 dalton glycoprotein, both diluted 1:100 with 3% bovine serum

[2] Campbell, K. P., MacLennan, D. H., and Jorgensen, A. O. (1982) *Biophys. J.* **37**, 188a.

[3] The abbreviations used are: EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; SDS, sodium dodecyl sulfate; Endo H, endo-β-*N*-acetylglucosaminidase H.

albumin in buffer B. The sheets were subsequently washed in buffer A and then incubated for 5–16 h at room temperature with ^{125}I-protein A diluted to 10^6 cpm/ml with 3% bovine serum albumin in buffer B. Finally, the sheets were washed in buffer B, air-dried, and exposed to Kodak AR5 x-ray film.

Preparation and Characterization of Antisera to Sarcoplasmic Reticulum Proteins—Antisera to the purified 53,000 dalton glycoprotein (6) was prepared in sheep as previously described for other sarcoplasmic reticulum proteins (34). The antiserum to skeletal 53,000 glycoprotein was characterized by immunoprecipitation of radioiodinated sarcoplasmic reticulum proteins from cardiac and skeletal muscle. Antiserum to rat calsequestrin was prepared by injecting a rabbit at multiple sites with 3 mg of partially purified rat calsequestrin which was fixed with 0.2% glutaraldehyde and emulsified with an equal volume of Freund's complete adjuvant. Antiserum was collected after 21 days. The calsequestrin antiserum did not form an immunoprecipitate when challenged with skeletal or cardiac calsequestrin in a double diffusion test. However the antiserum and the affinity purified antibodies to calsequestrin bound specifically to cardiac and skeletal muscle calsequestrin when tested against a Western blot of cardiac and skeletal muscle sarcoplasmic reticulum proteins separated by two-dimensional gel electrophoresis.

Gel Electrophoresis—The analysis of proteins by SDS-polyacrylamide gel electrophoresis was performed using the discontinuous buffer system of Laemmli (35) in 1.5-mm thick, 7.5% acrylamide slab gels and gradient gels of 5–15% acrylamide. Electrophoresis was also carried out according to Weber and Osborn (36) on 7.5% polyacrylamide gels. Two-dimensional gel electrophoresis was carried out as previously described (32). Usually 50–100 μg of protein were applied to gel wells. Apparent M_r were calculated from a graph of relative mobilities *versus* log M_r of standard proteins. Staining with the cationic carbocyanine dye, Stains-all was carried out as previously described in Footnote 2.

We have previously shown[2] that almost all of the Coomassie blue-staining proteins in skeletal sarcoplasmic reticulum were visualized as red or pink bands and did not reproduce well in black and white photographs. This is also true for cardiac sarcoplasmic reticulum. Therefore, most red or pink bands in Stains-all staining gels are not seen in figures. Stains-all-stained gels were scanned at 625 nm which was the wavelength where the blue-staining protein bands absorbed maximally.

RESULTS

Biochemical Identification of Cardiac Calsequestrin—Skeletal muscle calsequestrin has several unique properties that distinguish it from other sarcoplasmic reticulum proteins and from other muscle proteins. We have used these properties to identify and purify the cardiac form of calsequestrin. We have previously shown that two-dimensional gel electrophoresis results in a unique purification of skeletal muscle calsequestrin since its mobility changes and it falls off of the diagonal (32) (Fig. 1*A*) and that skeletal muscle calsequestrin stains blue with the cationic carbocyanine dye Stains-all[2] (Fig. 2*A*). Under these conditions cardiac sarcoplasmic reticulum was found to contain a protein which fell off of the diagonal upon two-dimensional gel electrophoresis (Fig. 1*B*) and which stained blue with Stains-all (Fig. 2*B*). The M_r of this protein in the Laemmli (35) gel system was 55,000 and in the Weber and Osborn gel system (36) was 44,000. The protein, therefore, had two chemical properties in common with skeletal calsequestrin.

Skeletal muscle calsequestrin can be precipitated from extracts of sarcoplasmic reticulum with 4 mM Ca^{2+} (37). We investigated whether any cardiac protein would precipitate with Ca^{2+} using the same procedure. Fig. 3*A*, *Lane 3*, shows that Ca^{2+} precipitated the blue-staining protein from extracts of cardiac sarcoplasmic reticulum which ran with the same mobility as skeletal muscle calsequestrin (Fig. 3*A*, *Lane 2*) in the Weber and Osborn system. This indicates that the cardiac blue-staining protein binds large amounts of Ca^{2+}, a property shared with skeletal muscle calsequestrin. On the basis of these three properties common with skeletal muscle calsequestrin, we have called the protein cardiac calsequestrin.

Purification and Characterization of Cardiac Calsequestrin—We were able to monitor the purification of cardiac calsequestrin on the basis of its Stains-all staining property. Cardiac calsequestrin (M_r = 55,000) was extracted from canine cardiac membrane vesicles using the nonionic detergent Nonidet P-40 (Fig. 3*B*, *Lane 1*). It bound quantitatively to solid calcium phosphate during a 4-h incubation at 0 °C in the presence of 100 mM Tris-HCl, pH 8.5 (Fig. 3*B*, *Lane 2*). Longer incubations at 0 °C resulted in the binding of all the blue-staining proteins (M_r = 55,000, 60,000, 90,000, 130,000, 140,000) to calcium phosphate. Therefore, for the purification of cardiac calsequestrin, M_r = 55,000, 2–4-h incubations were used. The Nonidet P-40 extract (Fig. 4, *Lane 3*) was depleted of cardiac calsequestrin following removal of the calseques-

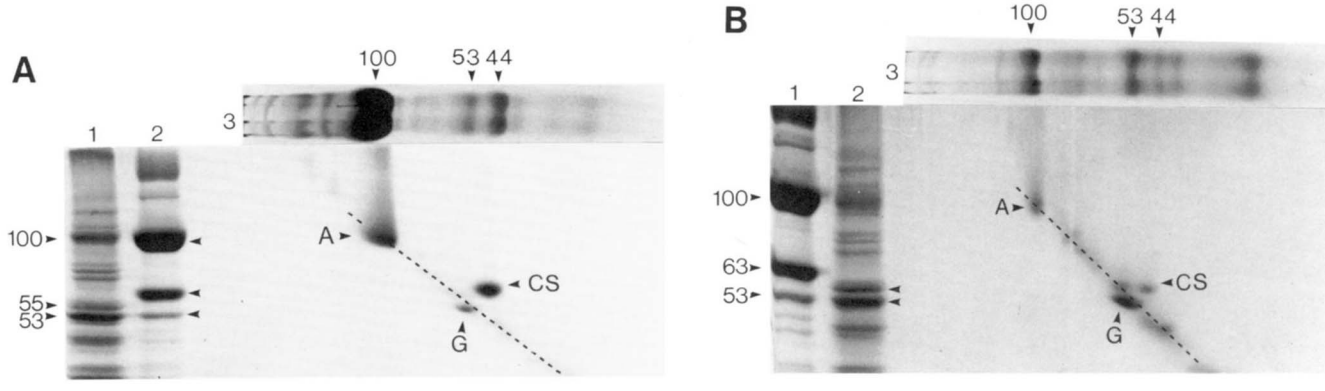

FIG. 1. **Two-dimensional gel electrophoresis of skeletal and cardiac muscle sarcoplasmic reticulum proteins.** Sarcoplasmic reticulum from rabbit skeletal (*A*) and canine cardiac muscle (*B*) were subjected to two-dimensional gel electrophoresis as described in Ref. 32 and stained with Coomassie blue. Skeletal muscle calsequestrin (*CS*) fell above the diagonal since its mobility differed in the two gel systems. The 53,000 dalton glycoprotein (*G*), by contrast, fell slightly below the diagonal. Cardiac sarcoplasmic reticulum also contained a protein (*CS*) which changed molecular weight depending on the gel system and it fell above the diagonal. The apparent M_r of cardiac calsequestrin was 55,000 on the Laemmli gel system and 44,000 on the Weber and Osborn gel system. The 53,000 dalton glycoprotein (*5G*) in cardiac sarcoplasmic reticulum fell slightly below the diagonal. The 100,000 dalton ATPase (*A*) fell on the diagonal in both skeletal and cardiac sarcoplasmic reticulum. Reference gels in *A* show cardiac sarcoplasmic reticulum (*Lane 1*), skeletal sarcoplasmic reticulum (*Lane 2*), and skeletal sarcoplasmic reticulum on a Weber and Osborn gel (*Lane 3*), and in *B* show skeletal sarcoplasmic reticulum (*Lane 1*), cardiac sarcoplasmic reticulum (*Lane 2*), and cardiac sarcoplasmic reticulum on a Weber and Osborn gel (*Lane 3*). *Numbers* on the *ordinate* and the *abscissa* represent $M_r \times 10^{-3}$.

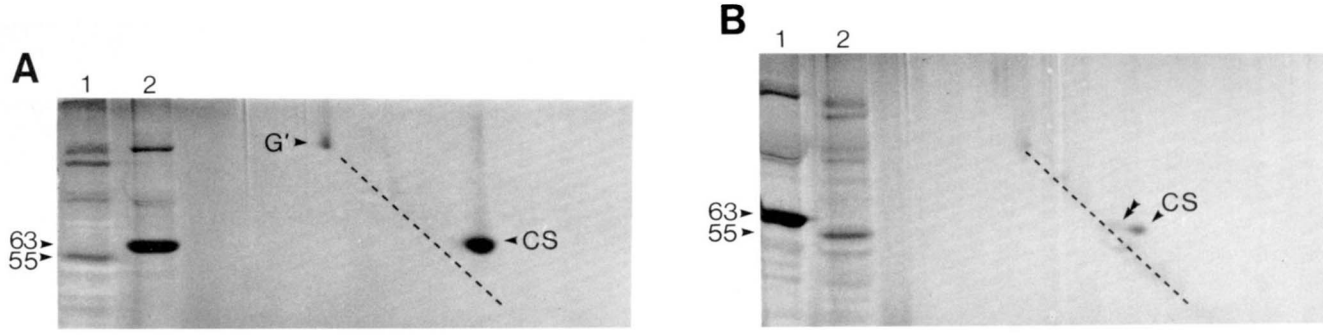

FIG. 2. **Stains-all staining of two-dimensional SDS-polyacrylamide gels of skeletal and cardiac sarcoplasmic reticulum proteins.** Sarcoplasmic reticulum from rabbit skeletal muscle (*A*) and canine cardiac muscle (*B*) were subjected to two-dimensional gel electrophoresis as described in Ref. 32 and then stained with Stains-all as described in Footnote 2. Skeletal calsequestrin (*CS*, M_r = 63,000) and cardiac calsequestrin (*CS*, M_r = 55,000) both fell off of the diagonal and stained blue with Stains-all. A minor blue-staining band (*double arrow*) in cardiac sarcoplasmic reticulum also fell off of the diagonal. Reference gels in *A* show cardiac sarcoplasmic reticulum (*Lane 1*) and skeletal sarcoplasmic reticulum (*Lane 2*) and in *B* show skeletal sarcoplasmic reticulum (*Lane 1*) and cardiac sarcoplasmic reticulum (*Lane 2*). In *A*, *G'* refers to the 160,000 dalton glycoprotein from skeletal sarcoplasmic reticulum that also stains blue with Stains-all.

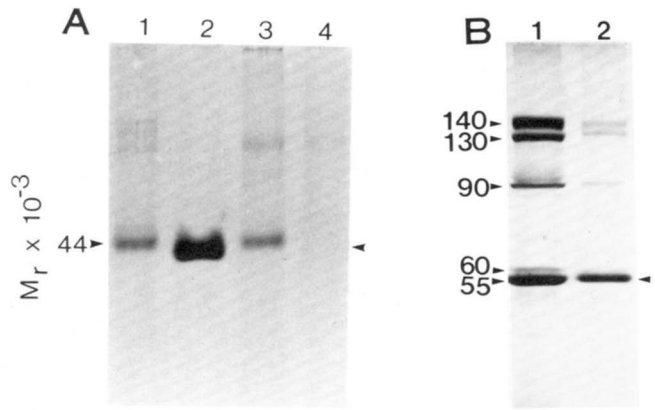

FIG. 3. **Calcium and calcium phosphate precipitation of cardiac calsequestrin.** *A*, canine cardiac sarcoplasmic reticulum (*Lane 1*) was solubilized with Triton X-100 and protein was precipitated with Ca^{2+} according to Ref. 37. The precipitate (*Lane 3*) and the clear supernatant (*Lane 4*) were separated by centrifugation. Samples were separated by SDS-polyacrylamide gel electrophoresis (Weber and Osborn system) and stained with Stains-all. The protein precipitated from cardiac sarcoplasmic reticulum had the same mobility and staining properties as skeletal muscle calsequestrin (*Lane 2*), while the supernatant contained no blue-staining proteins. It should be noted that under Weber and Osborn conditions of gel electrophoresis only cardiac calsequestrin could be seen after Stains-all staining. *B*, cardiac sarcoplasmic reticulum vesicles were extracted with Nonidet P-40 and cardiac calsequestrin was precipitated with calcium phosphate as described under "Materials and Methods." Nonidet P-40 extract of cardiac sarcoplasmic reticulum (*Lane 1*) and calcium phosphate precipitated protein (*Lane 2*) were analyzed on Laemmli slab gels which were stained with Stains-all. The Nonidet P-40 extract contained five blue-staining protein bands (M_r = 140,000, 130,000, 90,000, 60,000, and 55,000), while the protein precipitated with calcium phosphate was enriched in cardiac calsequestrin (M_r = 55,000). *Numbers* on the *ordinate* represent $M_r \times 10^{-3}$.

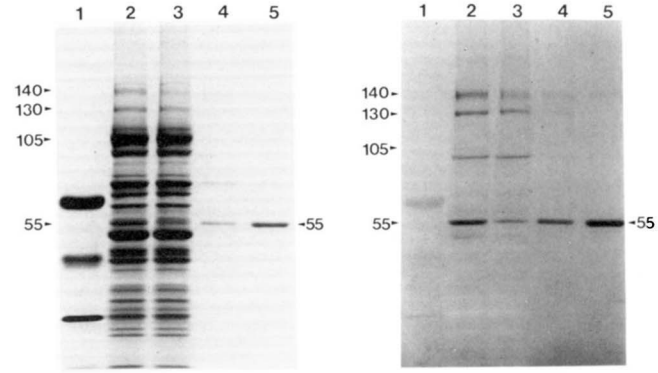

FIG. 4. **Purification of calsequestrin from canine cardiac sarcoplasmic reticulum.** Cardiac sarcoplasmic reticulum was extracted and solubilized with Nonidet P-40 and calsequestrin was precipitated with calcium phosphate as described under "Materials and Methods." Cardiac calsequestrin was batch eluted with 100 mM sodium phosphate, pH 7.0, and purified on DEAE-cellulose. Nonidet P-40 extracted cardiac sarcoplasmic reticulum (*Lane 2*), Nonidet P-40 extract following removal of calsequestrin-calcium phosphate complex (*Lane 3*), 100 mM sodium phosphate eluate (*Lane 4*), and DEAE-purified cardiac calsequestrin (*Lane 5*) were analyzed on Laemmli slab gels (5–15% gradient gels). Gel on the *left* is stained with Coomassie blue and gel on the *right* is stained with Stains-all. *Lane 1* has molecular weight standards: bovine serum albumin, M_r = 68,000; ovalbumin, M_r = 43,000; carbonic anhydrase, M_r = 30,000.

trin-calcium phosphate complex. The calsequestrin-calcium phosphate complex was recovered by centrifugation and washed with 1 mM Tris-HCl, pH 8.5, to remove any nonspecifically bound proteins. The complex was then washed sequentially with 10 mM sodium phosphate, 25 mM sodium phosphate, and 100 mM sodium phosphate, pH 7.0. Cardiac calsequestrin eluted from the calcium phosphate complex with 100 mM sodium phosphate (Fig. 4, *Lane 4*). The sample was then applied directly to DEAE-cellulose and the column was eluted with a gradient of 0 to 0.6 M NaCl. Fractions were analyzed for cardiac calsequestrin and selected fractions were combined, concentrated, and dialyzed against 10 mM Tris-HCl, pH 7.5, 0.1 mM EGTA. A sample of purified cardiac calsequestrin is shown in Fig. 4, *Lane 5*.

The binding of calcium to skeletal and cardiac calsequestrin is described in Table I. At a Ca^{2+} concentration of 5 mM where binding is maximal (5), cardiac calsequestrin bound some 300 nmol of Ca^{2+}/mg of protein, about one-third of that bound by skeletal muscle calsequestrin.

The amino acid composition of the purified cardiac calsequestrin is listed in Table II. Like calsequestrin, the cardiac protein was strongly acidic consisting of 32% aspartate and glutamate (assuming the aspartic and glutamic acid residues are not amidated) and about 11% lysine and arginine.

Immunochemical Characterization of Cardiac Calsequestrin—To determine whether cardiac calsequestrin was antigenically related to skeletal calsequestrin, affinity purified, rabbit anti-rat skeletal calsequestrin was used to label Western blots of partly purified canine cardiac calsequestrin by the indirect ^{125}I-protein A immunoassay (32). Fig. 5 shows that

TABLE I

Ca^{2+} bound by rabbit skeletal and canine cardiac calsequestrin

Source of calsequestrin	Ca^{2+} bound[a]
	nmol/mg protein
Rabbit skeletal muscle	633
Canine cardiac muscle	302

[a] Binding was measured by equilibrium dialysis. Protein samples (0.3 mg in 0.75 ml) were dialyzed for 36 h at 24 °C against 20 ml of solution of 5 mM $CaCl_2$, containing ($^{45}Ca^{2+}$), 5 mM Tris-HCl, pH 7.5, and 100 mM KCl.

TABLE II

Amino acid composition of calsequestrin from canine cardiac sarcoplasmic reticulum

Amino acid		
	mol %	*residues/mol*[a]
Cysteine[b]	0.47	2
Aspartic acid	15.85	78
Threonine[c]	3.63	18
Serine[c]	4.79	23
Glutamic acid	15.99	78
Proline	3.97	19
Glycine	6.79	33
Alanine	5.96	29
Valine[d]	6.98	34
Methionine[e]	0.85	4
Isoleucine[d]	4.82	24
Leucine	8.23	40
Tyrosine	2.95	14
Phenylalanine	5.12	25
Histidine	2.57	13
Lysine	7.56	37
Arginine	3.47	17

[a] Residues per polypeptide chain assuming a molecular weight of 55,000 derived from SDS gels. Values are rounded off to the nearest whole number.

[b] Determined as cysteic acid after performic acid oxidation.

[c] Estimated by extrapolation to zero time hydrolysis from 24-, 48-, and 72-h hydrolysis data.

[d] 72-h hydrolysis values.

[e] Determined as methionine sulfone after performic acid oxidation.

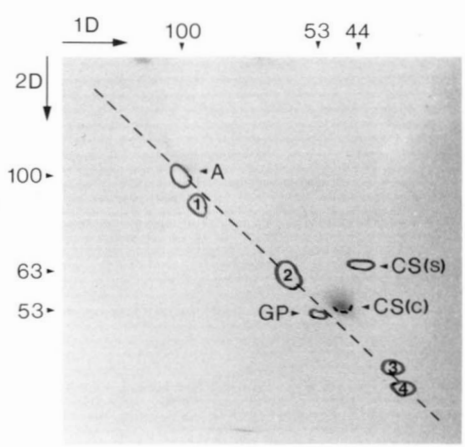

FIG. 5. **Western blot of a two-dimensional gel of canine cardiac sarcoplasmic reticulum extract.** Canine cardiac sarcoplasmic reticulum proteins were separated in the first dimension of a Weber and Osborn slab gel (7.5% polyacrylamide) and in the second dimension of a Laemmli slab gel (7.5% polyacrylamide gel) as previously described (32). The Western blot was first incubated with affinity purified antibodies to rat skeletal calsequestrin and subsequently ^{125}I-protein A. The autoradiograph shows that the canine cardiac calsequestrin was labeled by the antibodies to rat skeletal calsequestrin. The Coomassie blue-stained marker proteins are: *1*, phosphorylase (M_r = 94,000); *2*, bovine serum albumin (M_r = 68,000); *3*, ovalbumin (M_r = 44,000); and *4*, carbonic anhydrase (M_r = 30,000). *A*, (Ca^{2+} + Mg^{2+})ATPase; *CS(S)*, calsequestrin from rabbit skeletal muscle; *CS(C)*, calsequestrin from canine cardiac muscle; and *GP*, 53,000 dalton glycoprotein.

the antibodies bound to the spot identified as cardiac calsequestrin.

Localization of Cardiac Calsequestrin in Dog and Rat Cardiac Muscle—We have previously reported on the localization of the (Ca^{2+} + Mg^{2+})-dependent ATPase in adult rat papillary muscle at the light and electron microscopic level of resolution (14). The results obtained in that study suggested that the (Ca^{2+} + Mg^{2+})ATPase was rather uniformly distributed in the free sarcoplasmic reticulum but absent from both peripheral and interior junctional sarcoplasmic reticulum, transverse tubules, sarcolemma, and mitochondria. It was, therefore, of interest to determine whether we could detect cardiac calsequestrin in rat papillary muscle and whether we could localize the protein in particular regions of the muscle cells. We used the indirect immunofluorescence labeling techniques and compared the results with our previous findings on the localization of the (Ca^{2+} + Mg^{2+})ATPase in adult rat cardiac muscle.

The fluorescent staining pattern observed after labeling with antibodies to calsequestrin (Fig. 6*c*) was compared with the position of the A and I bands in the same field (Fig. 6*b*)

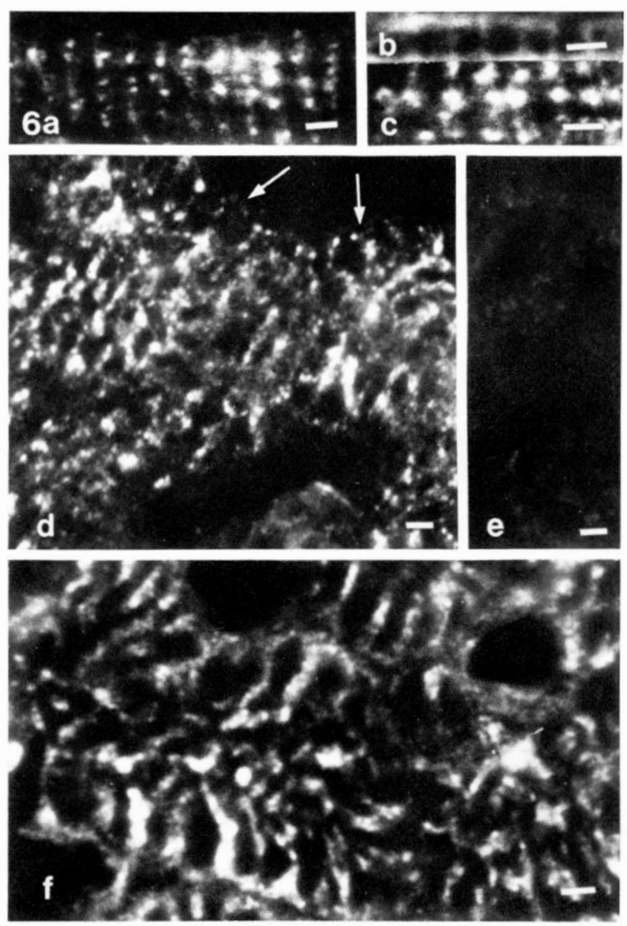

FIG. 6. **Immunofluorescent localization of cardiac calsequestrin.** Light micrographs of longitudinal (*a*, *b*, and *c*) and transverse (*d*, *e*, and *f*) sections of unfixed rat (*a*, *b*, *c*, *d*, and *e*) and canine (*f*) papillary muscle labeled with calsequestrin antibodies (*a*, *c*, *d*, and *f*) and calsequestrin antibodies absorbed with calsequestrin from rabbit skeletal muscle (*e*). The fluorescent staining pattern in *c* was compared with the position of the A and I bands in the same field as seen by phase-contrast microscopy in *b*. The fluorescent staining pattern observed after labeling with calsequestrin antibodies (*a* and *c*) corresponded to the I band region (only shown for *c*), while no regular staining pattern was observed in the A band region (*a*). A polygonal staining pattern was observed in the transversely sectioned myofibers (*d* and *f*). It should be noted that the labeling of the cell peripheries were discontinuous (*arrows*).

as determined by phase contrast microscopy in longitudinal 4 to 6 μm thick cryostat sections of adult rat cardiac myofibers. In fibers ranging in sarcomere length from 1.8 to 2.2 μm, intense, regular, fluorescent staining was present in the I band region (Fig. 6, *a* and *c*) while no regular staining pattern was observed in the A band region. It should be noted that intercalated discs remained unlabeled (not shown).

In transverse, 4-6-μm thick cryostat sections of rat (Fig. 6*d*) and dog (Fig. 6*f*) papillary muscle treated with antibodies to rat skeletal calsequestrin, a polygonal staining pattern was observed within each myofiber. Furthermore, a discontinuous labeling pattern was observed in the periphery of some of the myofibers (Fig. 6*d*). To demonstrate the specificity of the staining patterns observed in adult rat and dog papillary muscle we absorbed the antibodies to rat skeletal calsequestrin with rabbit skeletal calsequestrin. The supernatant was then used in the indirect immunofluorescent staining procedure. As shown in Fig. 6*e*, the staining pattern described above was greatly diminished. It should be noted that a faint nonspecific fluorescent labeling was observed on endothelial cells (Fig. 6*e*).

Identification of the Intrinsic 53,000 Dalton Glycoprotein in Cardiac Membranes—The effect of Endo H digestion on cardiac and skeletal muscle sarcoplasmic reticulum proteins solubilized with deoxycholate is shown in Fig. 7. As previously shown (6) skeletal muscle sarcoplasmic reticulum contained two predominant Endo H-sensitive proteins, the 53,000 dalton glycoprotein which was reduced to 49,000 daltons and the 160,000 dalton glycoprotein which was reduced to 155,000 daltons after Endo H digestion (Fig. 7*B*). Cardiac sarcoplasmic

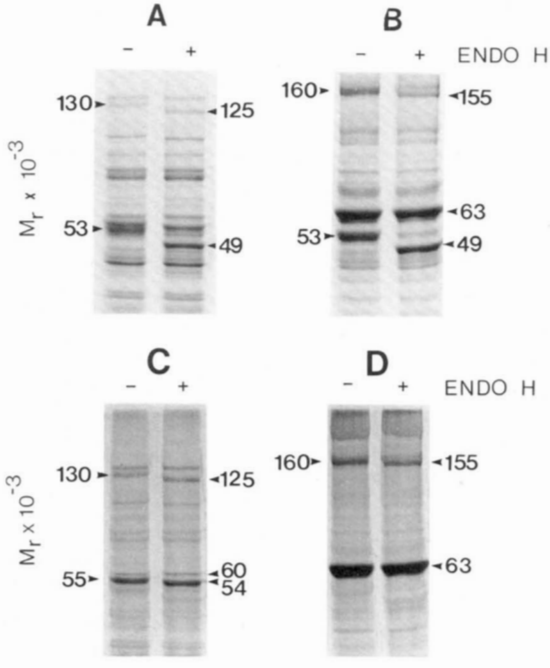

FIG. 7. **Endo H digestion of cardiac and skeletal muscle sarcoplasmic reticulum proteins.** Cardiac (*A* and *C*) and skeletal (*B* and *D*) sarcoplasmic reticulum deoxycholate extracts were solubilized in SDS and incubated in the presence (+) or absence (−) of Endo H according to "Materials and Methods." Samples were analyzed by SDS-polyacrylamide gel electrophoresis (Laemmli system) and stained with Coomassie blue (*A* and *B*) or Stains-all (*C* and *D*). Skeletal sarcoplasmic reticulum contained two blue-staining proteins (*D*) M_r = 160,000 and 63,000, while cardiac sarcoplasmic reticulum contained five blue-staining protein (*C*), M_r = 140,000, 130,000, 90,000, 60,000, and 55,000. Skeletal sarcoplasmic reticulum contained two Endo H-sensitive proteins of 53,000 and 160,000 daltons. Cardiac sarcoplasmic reticulum contained three Endo H-sensitive proteins of 130,000, 53,000, and 55,000 daltons (cardiac calsequestrin).

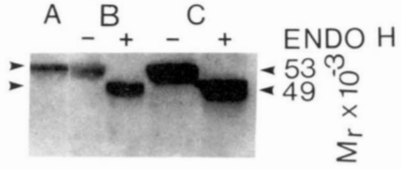

FIG. 8. **Indirect ^{125}protein A staining of the 53,000 dalton glycoprotein in skeletal and cardiac sarcoplasmic reticulum.** Skeletal (*A*), cardiac (*B*), and a deoxycholate extract of cardiac (*C*) sarcoplasmic reticulum were treated without (−) or with (+) Endo H and then subjected to indirect ^{125}I-protein A staining following electrophoresis and Western blotting as described under "Materials and Methods." Antiserum against the 53,000 dalton glycoprotein from skeletal muscle reacted with the 53,000 dalton glycoprotein from both cardiac and skeletal muscle sarcoplasmic reticulum. Following Endo H digestion the antiserum reacted with the sugar-free 49,000 dalton glycoprotein in skeletal and cardiac sarcoplasmic reticulum.

reticulum also contained a 53,000 dalton glycoprotein which was reduced to 49,000 daltons and a 130,000 dalton glycoprotein which was reduced to 125,000 daltons after Endo H digestion (Fig. 7*A*). Fig. 7*C* shows that the 130,000 dalton protein that stained blue with Stains-all was the 130,000 dalton Endo H-sensitive glycoprotein. Skeletal muscle calsequestrin was resistant to Endo H digestion (Fig. 7*D*) but the 55,000 dalton blue-staining cardiac protein which we identified as cardiac calsequestrin was sensitive to Endo H (Fig. 7*C*) and it was reduced in mass to 54,000 daltons. We have also observed that purified cardiac calsequestrin was reduced from 55,000 to 54,000 daltons after Endo H digestion (not shown) and that chicken skeletal and rat cardiac sarcoplasmic reticulum contained Endo H-sensitive forms of calsequestrin (not shown).

To determine whether any of the canine cardiac sarcoplasmic reticulum proteins were antigenically related to the 53,000 dalton glycoprotein of skeletal muscle sarcoplasmic reticulum, sheep antiserum to the 53,000 dalton glycoprotein from rabbit skeletal muscle was used to label Western blots by the ^{125}I-protein A immunoassay. Fig. 8 shows that the glycoprotein antiserum was bound to a single band at 53,000 daltons when tested against either sarcoplasmic reticulum or deoxycholate-extracted sarcoplasmic reticulum from canine myocardium. When either of these two samples were digested with Endo H before gel electrophoresis the antiserum was bound to a single band of 49,000 daltons. This change in molecular weight following Endo H treatment of sarcoplasmic reticulum was equivalent to the change in molecular weight of the glycoprotein from skeletal muscle sarcoplasmic reticulum following treatment with Endo H.

DISCUSSION

In this study we were able to identify and purify a cardiac form of calsequestrin from canine cardiac sarcoplasmic reticulum. The protein resembles skeletal muscle calsequestrin in several ways. Both proteins can be purified using the same procedure, which involves fractionation on calcium phosphate and DEAE-cellulose. Both bind large quantities of Ca^{2+} and are precipitated from solution in the presence of millimolar Ca^{2+}. Both are antigenically related as shown by their ability to bind antibodies to skeletal calsequestrin. They stain dark blue with Stains-all and their mobilities change in polyacrylamide gel electrophoresis according to pH so that their apparent molecular weights are greater in the Laemmli system than in the Weber and Osborn system. Thus they can be separated cleanly from other proteins since they fall off of the diagonal when subjected to two-dimensional gel electrophoresis at two different pH values. They are very acidic proteins, eluting from DEAE-cellulose with high concentrations of salt

and their amino acid compositions indicate that they both contain high concentrations of both glutamic and aspartic acid residues. Because of the low concentration of calsequestrin in cardiac sarcoplasmic reticulum we had to modify the purification scheme for calsequestrin to purify cardiac calsequestrin. We first found that Ca^{2+} precipitation followed by DEAE-cellulose chromatography resulted in about 90% purity. We then found that calcium phosphate precipitation followed by batch elution of calsequestrin from the calcium phosphate-calsequestrin complex led to a product that could be completely purified on DEAE-cellulose. This procedure is applicable to purification of calsequestrin from skeletal tissue. We have not tested whether it is applicable to other tissues such as porcine or bovine heart.

Cardiac calsequestrin differs from skeletal calsequestrin in several significant ways. Its mobility in gel systems suggests that it is somewhat smaller in size. The glutamic and aspartic acid content of cardiac calsequestrin is 32% whereas skeletal calsequestrin contains 37% glutamic and aspartic acid residues. This is probably why cardiac calsequestrin elutes slightly earlier than skeletal calsequestrin from DEAE-cellulose; and the smaller capacity for Ca^{2+} binding by cardiac calsequestrin might be due to its lower glutamic and aspartic acid content. We have not yet obtained sufficient material to determine whether the affinity for Ca^{2+} is the same as that of skeletal calsequestrin. Both cardiac and skeletal calsequestrin (10) are glycoproteins but the sugar composition may be somewhat different. Digestion of skeletal calsequestrin with Endo H does not alter its molecular weight but digestion of cardiac calsequestrin with Endo H reduces its molecular weight by some 1000 daltons suggesting that a high mannose carbohydrate chain is removed from cardiac calsequestrin under these conditions.

Comparison between the localization of the $(Ca^{2+} + Mg^{2+})$-ATPase and calsequestrin in cardiac muscle, as determined by the indirect immunofluorescence labeling technique suggests that cardiac calsequestrin is confined to those regions of the cardiac muscle cells where the sarcoplasmic reticulum is localized. Furthermore it appears that calsequestrin, unlike the $(Ca^{2+} + Mg^{2+})$ATPase, is absent from any sarcoplasmic reticulum localized in the A band region. This finding is in agreement with the idea that calsequestrin is localized in those regions of the sarcoplasmic reticulum which are in close apposition to the transverse tubules. Since considerable calsequestrin staining was also observed in the periphery of the cell, it is possible that calsequestrin located in this region might be present in the lumen of the peripheral, junctional sarcoplasmic reticulum. However, in order to determine more precisely the localization of calsequestrin in cardiac muscle it will be necessary to carry out immunocytochemical labeling of calsequestrin at the ultrastructural level of resolution.

Jones and Cala (25) have shown that cardiac vesicles can be separated, after Ca^{2+} loading, into ryanodine-sensitive and -insensitive populations. The ryanodine-sensitive population may originate from areas of Ca^{2+} release since ryanodine is believed to be an inhibitor of Ca^{2+} efflux (38). The ryanodine-sensitive population was enriched in the 55,000 dalton band that we have identified in this paper as cardiac calsequestrin. These observations suggest that cardiac calsequestrin in cardiac sarcoplasmic reticulum is located near sites of Ca^{2+} release, perhaps in those regions of cardiac sarcoplasmic reticulum that correspond to the terminal cisternae in skeletal muscle.

We have surveyed other tissues for the presence of calsequestrin using Stains-all and Endo H probes. We have noted that chicken skeletal muscle calsequestrin, which we previously isolated (39), is rather similar to cardiac calsequestrin. It binds less Ca^{2+} than rabbit skeletal muscle calsequestrin, it has a lower M_r in the Laemmli gel system and its M_r is reduced by Endo H digestion. A detailed comparison of chicken skeletal and canine cardiac calsequestrin would be of interest. Rabbit heart is a poor source of sarcoplasmic reticulum and we have not been able to compare rabbit cardiac and skeletal calsequestrin. We have, however, observed that rat cardiac and skeletal calsequestrins have molecular weights of 55,000 and 66,000, respectively, in the Laemmli gel system, suggesting that the differences in these proteins are tissue-specific rather than species-specific. Rat cardiac calsequestrin is also sensitive to Endo H digestion. Lobster sarcoplasmic reticulum has been reported to be devoid of calsequestrin (40). We have used Stains-all in an attempt to identify calsequestrin in this preparation. We did not see a band in the 50,000 dalton range but we did observe strong diffuse bands in the 20,000 dalton range.[4] We suspect that these represent proteolytic digests of a larger calsequestrin molecule. These observations would suggest that calsequestrin may be an invariant component of sarcoplasmic reticulum from all sources.

We have previously used ^{125}I-concanavalin A binding to analyze the glycoprotein composition of skeletal muscle sarcoplasmic reticulum (32). We found that the major concanavalin A binding protein is the 53,000 dalton glycoprotein. We have found that dog and rat cardiac sarcoplasmic reticulum membranes also contain a 53,000 dalton protein which binds ^{125}I-concanavalin A (not shown). The 53,000 dalton glycoprotein in cardiac sarcoplasmic reticulum is probably the same protein that Jones *et al.* (17) were able to stain with periodic acid-Schiff reagent in canine cardiac sarcoplasmic reticulum.

The 130,000 dalton cardiac glycoprotein shares properties with the 160,000 dalton glycoprotein of skeletal muscle and these two proteins, in turn, share several properties with the 53,000 dalton glycoprotein found in sarcoplasmic reticulum from both tissues. They copurify, eluting in the same peak from DEAE-cellulose, they bind ^{125}I-concanavalin A strongly and their molecular weights are reduced by Endo H. This suggests that the 160,000 or 130,000 dalton protein may share some common primary structure with the 53,000 dalton glycoprotein. Our comparisons of the 53,000 dalton glycoprotein from rabbit and rat skeletal and dog and rat cardiac muscle have not revealed any obvious structural differences. The proteins have the same M_r, the same sensitivity to Endo H, they all bind ^{125}I-concanavalin A, neither stains blue with Stains-all and they are antigenically related.

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