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Purification, Calcium Binding Properties, and Ultrastructural Localization of the 53,000- and 160,000 (Sarcalumenin)-Dalton Glycoproteins of the Sarcoplasmic Reticulum*

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Ekkehard Leberer‡§, Barry G. Timms¶‖, Kevin P. Campbell ‡‡, and David H. MacLennan‡**

*From the ‡Banting and Best Department of Medical Research, Charles H. Best Institute, University of Toronto, Toronto, Ontario M5G 1L6, Canada and ¶the Central Electron Microscope Facility and the Department of Anatomy, College of Medicine, and the **Howard Hughes Medical Institute and the Department of Physiology and Biophysics, University of Iowa, Iowa City, Iowa 52242*

The 53-kDa glycoprotein and sarcalumenin (160-kDa glycoprotein) were extracted from rabbit skeletal muscle sarcoplasmic reticulum with EGTA and purified by fractionation on DEAE-Sephadex A-25 and lentil lectin-Sepharose 4B. Sarcalumenin was shown to bind up to 400 nmol of Ca^{2+}/mg of protein at pH 7.5, which is equivalent to binding of ~35 mol of Ca^{2+}/mol of protein. The apparent dissociation constant was 300 μM in the presence of 20 mM KCl and 600 μM in 150 mM KCl. The 53-kDa glycoprotein did not bind any Ca^{2+} under the conditions examined.

Immunoblot analysis of isolated sarcoplasmic reticulum subfractions demonstrated the presence of the two glycoproteins in both the longitudinal sarcoplasmic reticulum and the terminal cisternae. Their concentrations were higher, however, in the longitudinal sarcoplasmic reticulum vesicles. Comparative immunoelectron microscopic studies using monoclonal antibodies revealed a codistribution of the 53-kDa glycoprotein with the Ca^{2+}-ATPase in all regions of the free sarcoplasmic reticulum. A similar distribution was found for sarcalumenin, although immunolabeling was much weaker. The colocalization of the 53-kDa glycoprotein and sarcalumenin with the Ca^{2+}-ATPase and the Ca^{2+} binding properties of sarcalumenin suggest that the glycoproteins may be involved in the sequestration of Ca^{2+} in the nonjunctional regions of the sarcoplasmic reticulum.

Muscle cells contain an extensive intracellular membrane system, the sarcoplasmic reticulum, that is responsible for the control of cytosolic Ca^{2+} concentrations. Excitation of muscle cells leads to the release of Ca^{2+} from luminal spaces of the sarcoplasmic reticulum, thereby triggering muscle contraction. Muscle relaxation is achieved by the rapid reuptake of Ca^{2+} from the cytosol into the lumen of the sarcoplasmic reticulum through the action of the Ca^{2+}-ATPase.

Two structurally and functionally different regions of the sarcoplasmic reticulum can be distinguished (1, 2). The longitudinal or "free" sarcoplasmic reticulum surrounds the myofibrils and does not form junctions with other membrane systems. The Ca^{2+}-ATPase is uniformly distributed along the membrane of the longitudinal sarcoplasmic reticulum and the nonjunctional membranes of the terminal cisternae (3, 4) so that the major function of these regions appears to be the uptake of Ca^{2+} to allow muscle relaxation. The junctional membrane of the terminal cisternae forms connections with the transverse tubules, which are invaginations of the surface membrane (1, 2, 5). The terminal cisternae are believed to be the major site of Ca^{2+} storage during relaxation and the site of Ca^{2+} release following depolarization of the transverse tubular system (6–9). The low affinity, high capacity, Ca^{2+}-sequestering protein calsequestrin (10) is located in the lumen of the terminal cisternae (11, 12).

The sarcoplasmic reticulum contains two immunochemically related glycoproteins of unknown function with apparent molecular masses of 53 and 160 kDa (13–15). It has been proposed that the 53-kDa glycoprotein may function in the regulation of Ca^{2+} transport (16–18). The 160-kDa glycoprotein has been identified, by the use of a gel overlay assay, as a Ca^{2+}-binding protein located in the lumen of the sarcoplasmic reticulum; and the name sarcalumenin has been proposed for it (15). In previous studies (13–15), the purification of these glycoproteins was achieved only after their denaturation, and it was not possible to perform quantitative Ca^{2+} binding measurements on these denatured proteins. The availability of the purified nondenatured glycoproteins is a prerequisite for functional studies.

The molecular cloning of cDNAs encoding sarcalumenin and the 53-kDa glycoprotein (14, 15) has shown that they are derived from the same gene by alternative splicing, and the primary sequence of the COOH-terminal half of sarcalumenin has been found to be identical to the complete primary sequence of the mature 53-kDa glycoprotein. Analysis of the primary structures failed to yield clues that would clearly elucidate the functions of the two glycoproteins, but the sequence of sarcalumenin contains a highly acidic region in its NH_2-terminal half (15). This region is characterized by juxtapositions of negatively charged residues similar to those found in the primary structures of the low affinity, high capacity, Ca^{2+}-binding proteins calsequestrin (19) and chromogranin A (20).

In this study, we describe the isolation of sarcalumenin and the 53-kDa glycoprotein under nondenaturing conditions and show that sarcalumenin is a low affinity, high capacity, Ca^{2+}-

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§ Present address: Genetic Engineering Section, NRC Biotechnology Research Inst., Montreal, Quebec H4P 2R2, Canada.

‖ Present address: Dept. of Anatomy, University of South Dakota, Vermillion, SD 57069.

‡‡ Investigator of the Howard Hughes Medical Institute.

binding protein with Ca^{2+} binding characteristics similar to those of calsequestrin. Ultrastructural localization demonstrates that the two glycoproteins are colocalized with the Ca^{2+}-ATPase throughout the longitudinal sarcoplasmic reticulum and the nonjunctional membranes of the terminal cisternae. We suggest that sarcalumenin and, possibly, the 53-kDa glycoprotein are involved in the transfer of Ca^{2+} from the Ca^{2+} transport sites in the nonjunctional regions of the sarcoplasmic reticulum to the sequestering sites localized in the terminal cisternae.

EXPERIMENTAL PROCEDURES

Materials—Acrylamide, *N,N'*-methylenebisacrylamide, 2-mercaptoethanol, SDS,[1] and molecular mass standards were purchased from Bio-Rad. DEAE-Sephadex A-25 was obtained from Pharmacia LKB Biotechnology Inc. Lentil lectin-Sepharose 4B, α-methylmannoside, bovine serum albumin, Endo H (*Streptomyces griseus*), EGTA, dithiothreitol, PMSF, Trasylol, benzamidine, iodoacetamide, leupeptin, pepstatin, and Coomassie Brilliant Blue were obtained from Sigma. Nitrocellulose membrane filters (BA85) were from Schleicher & Schuell. Horseradish peroxidase-conjugated rabbit anti-sheep IgG was obtained from Jackson Immuno Research Laboratories, and horseradish peroxidase-conjugated goat anti-mouse IgG was from Cappel Laboratories. Gold particle-coupled goat anti-mouse IgG was purchased from Janssen Pharmaceutica. LR White resin was obtained from London Resin Company. $^{45}CaCl_2$ was received from Amersham Corp. All other reagents were of the highest purity grade commercially available.

Purification of Sarcalumenin and 53-kDa Glycoprotein—The heavy sarcoplasmic reticulum (1 g of protein) was prepared from rabbit fast-twitch skeletal muscle in a solution of 10 mM Tris-HCl, 1 mM histidine, pH 8.0, 0.25 M sucrose, and 0.1 mM PMSF as described previously (13). The vesicles were resuspended in 10 mM Tris-HCl, pH 8.5, 2 mM dithiothreitol, and 0.1 mM PMSF at a protein concentration of 5 mg/ml and gently stirred for 30 min at 4 °C. After centrifugation at 100,000 × *g* for 45 min, insoluble protein was resuspended at 5 mg of protein/ml in 10 mM Tris-HCl, pH 8.5, 1 mM EGTA, 2 mM dithiothreitol, and 0.1 mM PMSF (extraction buffer) and gently stirred for 30 min at 4 °C. The supernatant solution, after centrifugation at 100,000 × *g* for 45 min, was saved; and extraction of the insoluble protein with EGTA was repeated in extraction buffer. Both supernatant solutions were combined (EGTA extract) and concentrated under nitrogen pressure to 5 mg of protein/ml using an Amicon PM-10 membrane. The concentrated EGTA extract was passed through a 1.5 × 30-cm column of DEAE-Sephadex A-25 equilibrated with 10 mM Tris-HCl, pH 8.5, 2 mM dithiothreitol, and 0.1 mM PMSF (equilibration buffer A). After washing the resin with 100 ml of equilibration buffer A, proteins were eluted with a linear gradient of 0–0.6 M NaCl in 400 ml of equilibration buffer A. Fractions containing the 53-kDa glycoprotein and sarcalumenin were detected by SDS-polyacrylamide gel electrophoresis. The 53-kDa glycoprotein was identified in the fractions eluted between 0.1 and 0.2 M NaCl, whereas sarcalumenin was detected in the fractions eluted between 0.4 and 0.5 M NaCl. These fractions were combined into two pools; concentrated by ultrafiltration under nitrogen pressure to 2 mg of protein/ml using Amicon PM-10 membranes, and dialyzed overnight against 10 mM Tris-HCl, pH 8.5, 100 mM NaCl, 2 mM dithiothreitol, and 0.1 mM PMSF (equilibration buffer B). The protein solutions were applied to 0.9 × 15-cm columns of lentil lectin-Sepharose 4B equilibrated with equilibration buffer B. After washing the columns with 100 ml of equilibration buffer B, the electrophoretically homogeneous 53-kDa glycoprotein and sarcalumenin were eluted from their respective columns with equilibration buffer B containing 250 mM α-methylmannoside. The purified glycoproteins were concentrated to 1 mg of protein/ml using Amicon Centricon-30 microconcentrators; dialyzed overnight against 10 mM Tris-HCl, pH 7.5, and 0.1 mM PMSF; frozen in liquid nitrogen; and stored at −70 °C for further analysis.

Preparation of Sarcoplasmic Reticulum Subfractions—The longitudinal sarcoplasmic reticulum and the terminal cisternae were prepared from rabbit fast-twitch skeletal muscle according to the method of Saito *et al.* (4) in the presence of the protease inhibitors proposed by Imagawa *et al.* (21): 125 kallikrein-inhibitory units/ml of Trasylol, 1 mM benzamidine, 1 mM iodoacetamide, 1 μM leupeptin, 1 μM pepstatin, and 0.1 mM PMSF. The vesicles were resuspended in 5 mM imidazole, pH 7.4, 0.3 M sucrose, 1 mM benzamidine, 1 mM iodoacetamide, and 0.1 mM PMSF at a protein concentration of 20 mg/ml; frozen in liquid nitrogen; and stored at −70 °C.

Ca^{2+} Binding to Sarcalumenin and 53-kDa Glycoprotein—Quantitation of Ca^{2+} binding was achieved by equilibrium dialysis (10) in 10 mM Tris-HCl, pH 7.5, containing either 20 or 150 mM KCl and variable amounts of $^{45}CaCl_2$. Concentrations of free Ca^{2+} were achieved by buffering the solutions with EGTA using the calculations of Bulos and Sacktor (22).

Antibodies—The previously characterized monoclonal antibody, G10, raised against the 53-kDa glycoprotein (14) was also used in this study. Monoclonal antibodies $XB5_2$ (anti-53-kDa glycoprotein), VIE8 (anti-Ca^{2+}-ATPase), and VIE12 (anti-sarcalumenin) were prepared and purified as described by Campbell and co-workers (18, 23–25). Western blot analyses (26) were performed using horseradish peroxidase-conjugated secondary antibodies.

Immunoelectron Microscopy—Short lengths of myofibers were separated from rabbit psoas muscle and tied to applicator sticks prior to dissection. The muscle tissue was fixed for 3 h at room temperature in a mixture of freshly prepared 3% paraformaldehyde and 0.5% glutaraldehyde in 0.1 M sodium cacodylate, pH 7.2, containing 4.5

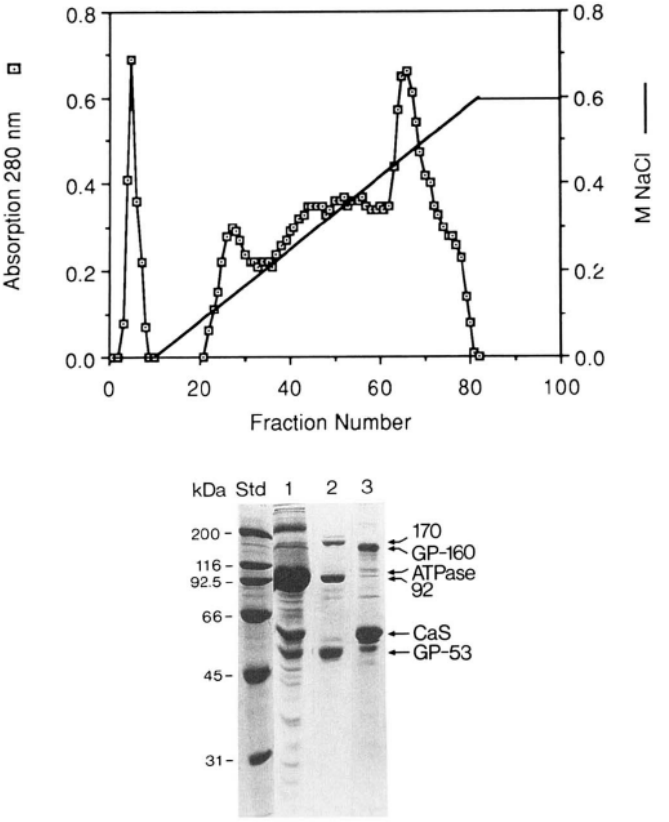

FIG. 1. **Ion-exchange chromatography of EGTA-solubilized sarcoplasmic reticulum proteins.** *Upper,* an EGTA extract of the heavy sarcoplasmic reticulum was fractionated on a 1.5 × 30-cm DEAE-Sephadex A-25 column as described under "Experimental Procedures." The fraction size was 3 ml. *Lower,* SDS-polyacrylamide gel electrophoresis (10% acrylamide) of standard proteins (*lane Std*), the heavy sarcoplasmic reticulum (*lane 1*), and combined fractions 22–35 (*lane 2*) and 62–78 (*lane 3*) from the DEAE-Sephadex A-25 column. Protein amounts were 30 μg in *lane 1* and 15 μg in *lanes 2* and *3*. Molecular masses of standard proteins are indicated on the left side of the gel. Note that the 53-kDa glycoprotein eluted with two major proteins with apparent molecular masses of 92 and 170 kDa (*lane 2*) and that sarcalumenin eluted with calsequestrin (*lane 3*). *GP-53,* 53-kDa glycoprotein; *CaS,* calsequestrin; *ATPase,* Ca^{2+}-ATPase; *GP-160,* sarcalumenin (160-kDa glycoprotein); *92,* 92-kDa protein; *170,* 170-kDa protein.

[1] The abbreviations used are: SDS, sodium dodecyl sulfate; Endo H, endo-β-*N*-acetylglucosaminidase H; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; PMSF, phenylmethylsulfonyl fluoride.

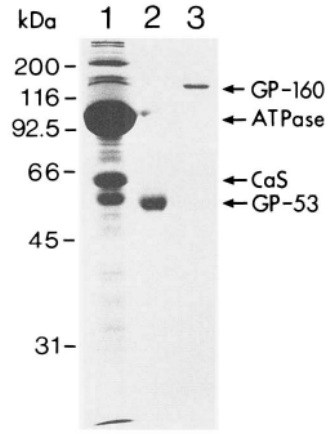

FIG. 2. **Purification of 53-kDa glycoprotein and sarcalumenin from rabbit skeletal muscle sarcoplasmic reticulum.** SDS-polyacrylamide gel electrophoresis (10% acrylamide) is shown of the heavy sarcoplasmic reticulum (*lane 1*), the 53-kDa glycoprotein (*lane 2*), and sarcalumenin (*lane 3*). The 53-kDa glycoprotein and sarcalumenin were purified by ion-exchange chromatography and lentil lectin affinity chromatography as described under "Experimental Procedures." Protein amounts were 30 μg in *lane 1*, 2 μg in *lane 2*, and 1 μg in *lane 3*. The positions of marker proteins are indicated on the left side of the gel.

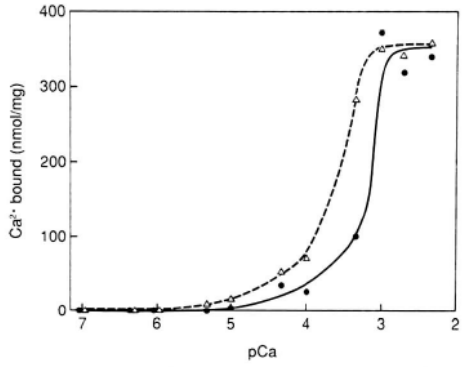

FIG. 3. **Binding of Ca^{2+} to sarcalumenin.** $^{45}Ca^{2+}$ binding was measured by equilibrium dialysis in 10 mM Tris-HCl, pH 7.5, and 20 mM (△) or 150 (●) mM KCl.

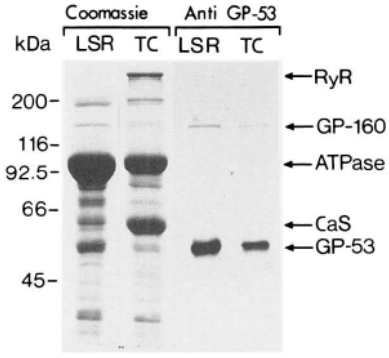

FIG. 4. **Distribution of 53-kDa glycoprotein and sarcalumenin in isolated sarcoplasmic reticulum subfractions.** The longitudinal sarcoplasmic reticulum (*lanes LSR*) and the terminal cisternae (*lanes TC*) were subjected to SDS-polyacrylamide gel electrophoresis in 10% gels and transferred electrophoretically to nitrocellulose. *Left*, Coomassie Brilliant Blue-stained gel; *right*, immunoperoxidase staining of the nitrocellulose membrane with anti-53-kDa glycoprotein monoclonal antibody G10. Note that the antibody recognizes both the 53-kDa glycoprotein and sarcalumenin. Protein amounts were 30 μg in each lane. The positions of marker proteins are indicated on the left side of the gel. *RyR*, ryanodine receptor.

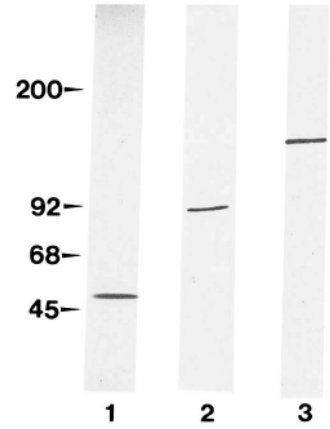

FIG. 5. **Immunoblot staining of rabbit** psoas **muscle extracts using monoclonal antibodies.** Psoas muscle extracts were separated by SDS-polyacrylamide gel electrophoresis (5–16% gradient gels); transferred electrophoretically to nitrocellulose paper; and immunoblotted with anti-53-kDa glycoprotein monoclonal antibody $XB5_2$ (*lane 1*), anti-Ca^{2+}-ATPase monoclonal antibody VIE8 (*lane 2*), and anti-sarcalumenin monoclonal antibody VIE12 (*lane 3*). Protein amounts were 50 μg in *lane 1*, 40 μg in *lane 2*, and 60 μg in *lane 3*. The positions of marker proteins (in kilodaltons) are indicated on the left side of the gel.

mM $CaCl_2$ (3). Small pieces of the fixed muscle tissue were also processed and embedded in LR White resin as described previously (28). Ultrathin sections were cut, transferred to grids, blocked for 10 min in buffer C (10 mM sodium phosphate, pH 7.4, containing 150 mM NaCl and 1% bovine serum albumin), and incubated with monoclonal antibodies in buffer C for 1–2 h at a final protein concentration of 0.1 mg/ml. Sections were washed extensively in buffer D (10 mM sodium phosphate, pH 7.4, containing 150 mM NaCl), blocked in buffer C, and incubated with goat anti-mouse IgG coupled to 5- or 10-nm gold particles diluted 1:10 in buffer C. Diluted gold-conjugated secondary antibodies were centrifuged at 1000 × *g* for 5 min prior to use to remove any large aggregates. Sections were washed extensively in buffer D and then in distilled water before counterstaining with aqueous uranyl acetate and lead citrate. For control sections, the primary antibody was substituted with preimmune mouse serum. Double labeling was carried out using opposite sides of the section faces (29). Sections were examined in a Hitachi H600 electron microscope.

Miscellaneous—Polyacrylamide gel electrophoresis in the presence of SDS was performed according to the method of Laemmli (30), and gels were stained with Coomassie Brilliant Blue. Protein was determined according to Lowry *et al.* (31) using bovine serum albumin as a standard. Oligosaccharide chains were removed from glycoproteins by incubation with Endo H at a concentration of 0.1 unit/mg of protein in 50 mM sodium citrate, pH 5.5, 0.1% SDS, and 0.1 mM PMSF as described by Trimble and Maley (32).

RESULTS

Purification of Sarcalumenin and 53-kDa Glycoprotein of Sarcoplasmic Reticulum—Heavy sarcoplasmic reticulum vesicles from rabbit fast-twitch skeletal muscle were washed under hypotonic conditions, and solubilized proteins were separated by ultracentrifugation as described previously (14, 15). The insoluble material was further incubated with 1 mM EGTA under hypotonic conditions. After a second ultracentrifugation, the EGTA-solubilized proteins were passed through a DEAE-Sephadex A-25 column, and proteins were eluted with a gradient of 0–0.6 M NaCl (Fig. 1, *upper*). As shown by SDS-polyacrylamide gel electrophoresis, the 53-kDa glycoprotein was eluted between 0.1 and 0.2 M NaCl together with two major proteins with molecular masses of 170 and 92 kDa, whereas sarcalumenin was eluted with calsequestrin between 0.4 and 0.5 M NaCl (Fig. 1, *lower*). Fractions containing the separated glycoproteins were combined

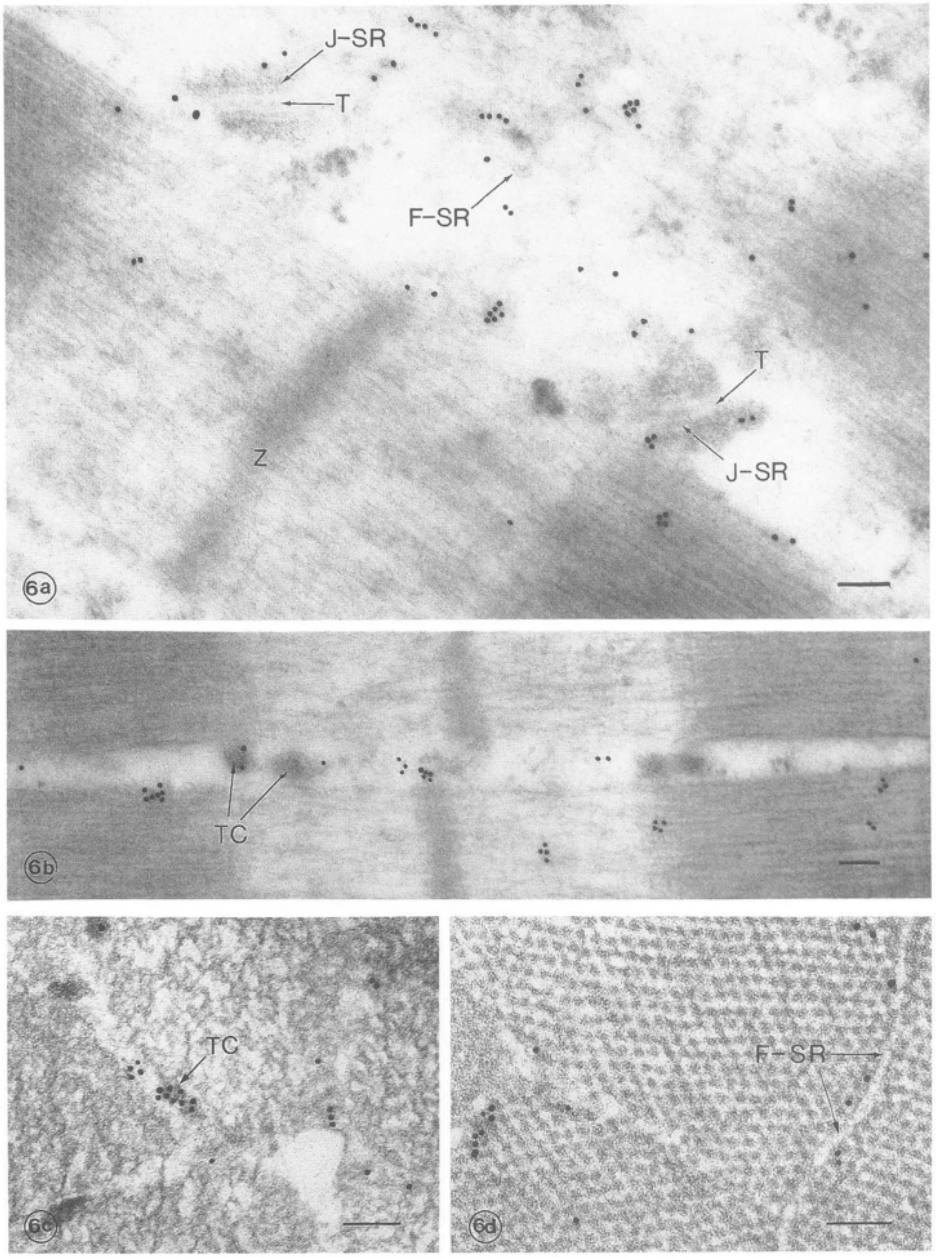

FIG. 6. **Ultrastructural localization of 53-kDa glycoprotein in rabbit skeletal muscle.** Electron micrographs are shown of longitudinal (*a* and *b*) and transverse (*c*, I-band region; and *d*, A-band region) thin resin sections of psoas muscle labeled with anti-53-kDa glycoprotein monoclonal antibody $XB5_2$. Most of the gold particles were associated with the free sarcoplasmic reticulum (*F-SR*). Nonjunctional portions of the terminal cisternae (*TC*) were also labeled (*b* and *c*), whereas junctional sarcoplasmic reticulum (*J-SR*) and transverse tubules (*T*) were labeled only at background levels (*a*). *Z*, Z line. *Bar*, 0.1 μm. Magnification × 90,000 (*a*), × 75,000 (*b*), × 105,000 (*c*), and × 120,000 (*d*).

into two pools and passed through lentil lectin affinity columns. The electrophoretically homogeneous 53-kDa glycoprotein (Fig. 2, *lane 2*) and sarcalumenin (*lane 3*) were eluted with 250 mM α-methylmannoside.

Ca^{2+} Binding to Sarcalumenin and 53-kDa Glycoprotein—The Ca^{2+} binding properties of sarcalumenin and the 53-kDa glycoprotein were studied by equilibrium dialysis at various concentrations of free Ca^{2+} in the presence of 20 or 150 mM KCl. The 53-kDa glycoprotein did not bind Ca^{2+} under any of the conditions examined (0.1 μM to 15 mM free Ca^{2+}, 20 or 150 mM KCl). Sarcalumenin, however, bound Ca^{2+} with a maximum of ~320–370 nmol of Ca^{2+}/mg of protein in the presence of both 20 and 150 mM KCl (Fig. 3). This corresponded to ~30–35 mol of Ca^{2+} bound per mol of protein on the basis of a molecular mass of 95,710 Da for sarcalumenin, as deduced from the sequence of its cDNA (15). The concentration of Ca^{2+} required for half-maximal saturation was dependent on the salt concentration. At 20 mM KCl, half-maximal saturation was achieved at a Ca^{2+} concentration of 300 μM, whereas at the more physiological KCl concentration of 150 mM, half-maximal saturation occurred at 600 μM free Ca^{2+} (Fig. 3).

Subcellular Localization of Sarcalumenin and 53-kDa Glycoprotein—Monoclonal antibody G10, raised against the 53-kDa glycoprotein (14), was used to study the distribution of the 53-kDa glycoprotein and sarcalumenin within isolated sarcoplasmic reticulum subfractions. As shown in a previous study (14), this antibody reacted with both glycoproteins equally well. As illustrated by immunoblot analysis (Fig. 4), the 53-kDa glycoprotein and sarcalumenin were present at a constant ratio both in isolated longitudinal sarcoplasmic reticulum vesicles and in vesicles derived from the terminal cisternae. In both subfractions, the relative amount of the 53-kDa glycoprotein was higher than that of sarcalumenin, but both glycoproteins occurred in a constant ratio with the Ca^{2+}-ATPase. Like the Ca^{2+}-ATPase, both glycoproteins were found to be more concentrated in the longitudinal sarcoplasmic reticulum fraction than in the terminal cisternae.

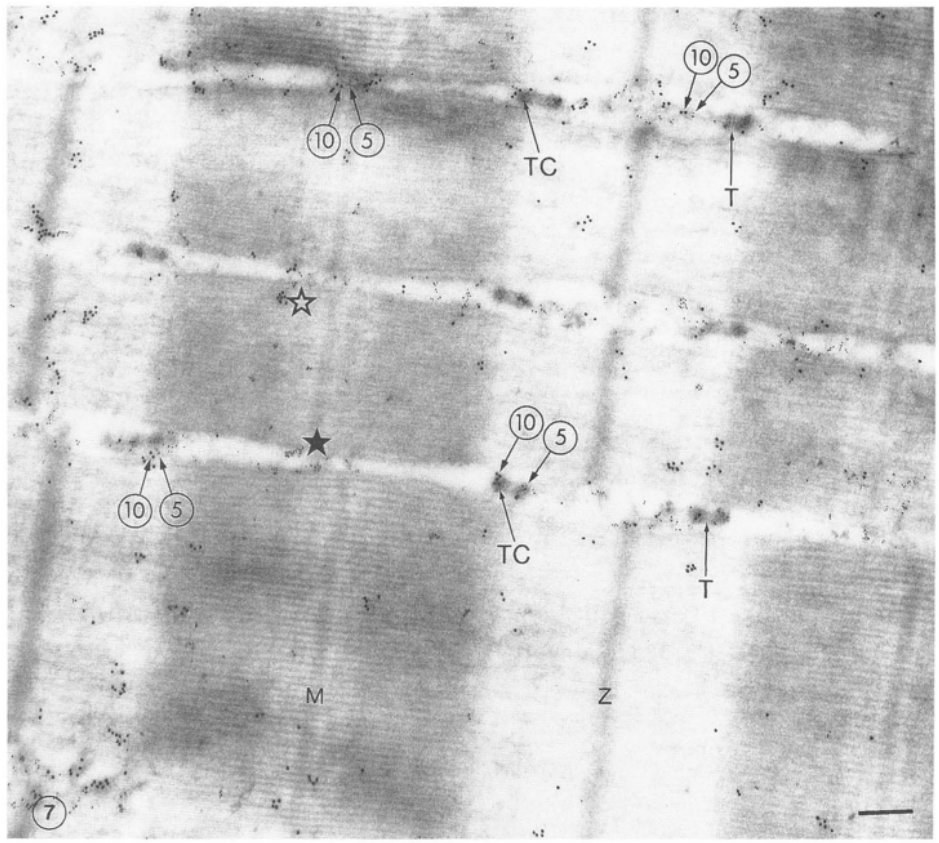

FIG. 7. **Ultrastructural localization of Ca^{2+}-ATPase and 53-kDa glycoprotein in rabbit skeletal muscle by double labeling of ultrathin resin sections with monoclonal antibodies.** Electron micrograph is shown of a longitudinal thin resin section of psoas muscle labeled first with anti-Ca^{2+}-ATPase monoclonal antibody VIE8 and goat anti-mouse IgG conjugated to 5-nm gold particles and then, on the reverse side, with anti-53-kDa glycoprotein monoclonal antibody $XB5_2$ and goat anti-mouse IgG conjugated to 10-nm gold particles. Many similar areas of the free sarcoplasmic reticulum were clearly labeled with gold particles for both the Ca^{2+}-ATPase and the 53-kDa glycoprotein. Those regions of the sarcoplasmic reticulum membrane labeled with only one particle size (*black star*) probably represent inaccessible antigen sites to the other antibody since the same cellular region also demonstrated double labeling (white star). *Bar*, 0.2 μm. Magnification × 45,000.

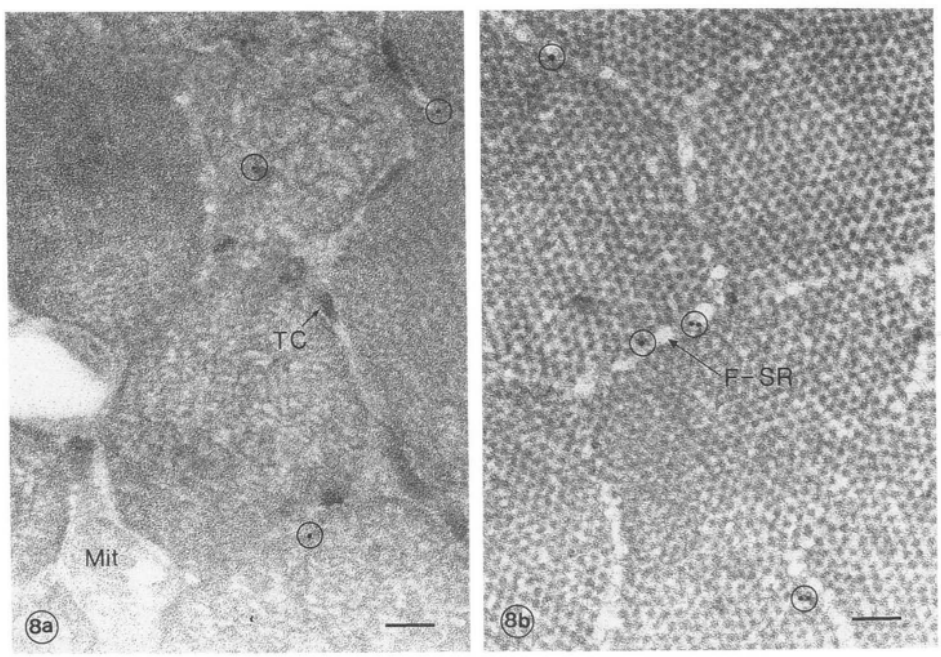

FIG. 8. **Ultrastructural localization of sarcalumenin in rabbit skeletal muscle.** Electron micrographs are shown of transverse thin resin sections through the I-band (*a*) and the A-band (*b*) regions of psoas muscle fibers labeled with anti-sarcalumenin monoclonal antibody VIE12. Although labeling is minimally above background levels, it is specifically associated with regions of the nonjunctional sarcoplasmic reticulum. *Mit*, mitochondrion. *Bars*, 0.1 μm. Magnification × 90,000.

This subcellular distribution was the inverse of that of calsequestrin and the ryanodine receptor, which were both found at the highest concentrations in the isolated terminal cisternae (Fig. 4).

The locations of the two glycoproteins and, for purpose of comparison, of the Ca^{2+}-ATPase were determined by immunoelectron microscopy using monoclonal antibodies. Most of the monoclonal antibodies that we obtained against the 53-kDa glycoprotein also recognized sarcalumenin. We found, however, that monoclonal antibody $XB5_2$ reacted solely with the 53-kDa glycoprotein, whereas VIE12 reacted solely with sarcalumenin, as shown by immunoblot analyses of rabbit psoas muscle extracts (Fig. 5, *lanes 1* and *3*, respectively). Monoclonal antibody VIE8 recognized only the Ca^{2+}-ATPase (*lane 2*). Monoclonal antibodies $XB5_2$ and VIE12 were also shown, by immunoblot analysis of sarcoplasmic reticulum vesicles after Endo H treatment, to recognize the protein portions of the glycoproteins (data not shown).

To determine the location of the proteins by electron microscopy, ultrathin sections were prepared from formalde-

hyde-fixed rabbit psoas muscle that was either frozen or embedded in resin. The sections were first incubated with the respective primary antibody and then incubated with goat anti-mouse IgG coupled to gold particles. As controls, the primary antibodies were replaced by preimmune mouse serum. The nonimmune antibodies gave no specific immunogold labeling (data not shown). Comparison of immunogold labeling patterns obtained from ultrathin cryosections or from ultrathin sections of resin-embedded tissue showed no difference in labeling specificity and intensity (data not shown). The morphological resolution, however, was enhanced by labeling of resin-embedded sections, and observations on larger areas of skeletal muscle were also possible by this technique.

Labeling of the 53-kDa glycoprotein was found in all of the free or nonjunctional portions of the sarcoplasmic reticulum (Fig. 6). Examination of transversely sectioned profiles of the muscle fibers in either the I-band (Fig. 6*c*) or A-band (Fig. 6*d*) regions further demonstrated the specificity of labeling for the free sarcoplasmic reticulum regions. The localization of the 53-kDa glycoprotein was compared with that of the Ca^{2+}-ATPase by double labeling of resin sections. One side of the section was stained with monoclonal antibody VIE8 to Ca^{2+}-ATPase and visualized with goat anti-mouse IgG coupled to 5-nm gold particles, whereas the reverse side was indirectly immunolabeled with antibodies to the 53-kDa glycoprotein and then with goat anti-mouse IgG coupled to 10-nm gold particles. As illustrated in Fig. 7, the pattern of Ca^{2+}-ATPase labeling was very similar to that observed with the 53-kDa glycoprotein in that the particle distribution closely followed the membrane profiles of the free or nonjunctional sarcoplasmic reticulum, in agreement with a previous report on rat skeletal muscle (3). Areas of the free sarcoplasmic reticulum associated with Ca^{2+}-ATPase staining were also associated with 53-kDa glycoprotein staining. In many regions of the sarcoplasmic reticulum, the 5- and 10-nm gold particles were associated with the same portion of the sarcoplasmic reticulum membrane. Ca^{2+}-ATPase labeling was not coincident with 53-kDa glycoprotein staining in some sections where the sarcoplasmic reticulum had been tangentially sectioned.

Immunogold labeling of sarcalumenin with monoclonal antibody VIE12 was only minimally above that of the background level of staining (Fig. 8, *a* and *b*), but the pattern of staining observed was consistent with the association of sarcalumenin with the free sarcoplasmic reticulum and similar to that observed with the localization of the 53-kDa glycoprotein and the Ca^{2+}-ATPase.

DISCUSSION

In this paper, we describe a procedure for the isolation of sarcalumenin (160-kDa glycoprotein) and the 53-kDa glycoprotein of the sarcoplasmic reticulum from rabbit fast-twitch skeletal muscle under nondenaturing conditions and in a form suitable for functional studies. The purification protocol makes use of the previous finding (14, 15) that both glycoproteins are eluted from sarcoplasmic reticulum vesicles by extraction with EGTA. The use of EGTA as a solubilization reagent avoids the aggregation of the glycoproteins with the Ca^{2+}-ATPase that is usually observed in detergent solutions (13). It also allows the separation of the glycoproteins by ion-exchange chromatography (Fig. 1), which was not feasible after solubilization in detergents.[2]

The availability of the purified proteins has allowed us to measure their Ca^{2+} binding properties by equilibrium dialysis. We have demonstrated that sarcalumenin has Ca^{2+} binding characteristics (Fig. 3) similar to those of calsequestrin (10, 33): a high capacity and a moderate affinity for Ca^{2+} binding (~35 Ca^{2+}-binding sites/molecule). Although the complete primary structure of the 53-kDa glycoprotein is identical to the COOH-terminal half of sarcalumenin (15), equilibrium dialysis failed to show any Ca^{2+} binding to the 53-kDa glycoprotein, in agreement with our previous findings using the $^{45}Ca^{2+}$ gel overlay technique (15). This confirms our previous assumption (15) that the highly acidic NH_2 terminus of sarcalumenin is responsible for Ca^{2+} binding.

Most monoclonal antibodies that we have obtained against the 53-kDa glycoprotein also recognize sarcalumenin. This result is not surprising in view of the finding that the complete amino acid sequence of the 53-kDa glycoprotein is also present in the primary structure of sarcalumenin (15). It is interesting therefore that we have isolated one monoclonal antibody, $XB5_2$, which reacts solely with the protein core of the 53-kDa glycoprotein, as demonstrated by immunoblotting of psoas muscle extracts (Fig. 5) and Endo H-digested sarcoplasmic reticulum vesicles (data not shown). Through immunoblotting of the fusion protein expressed by a series of different cDNA clones in λ gt11, we have been able to show that the epitope for this antibody is located close to the COOH terminus of the 53-kDa glycoprotein.[2] Thus, it appears that this epitope is folded differently in the common regions of the two glycoproteins.

We have shown previously (14, 15) that sarcalumenin and the 53-kDa glycoprotein reside in the lumen of the sarcoplasmic reticulum and are bound to the inner side of the membrane through a Ca^{2+}-dependent mechanism. As shown by immunoblot analyses of isolated sarcoplasmic reticulum subfractions (Fig. 4) and by immunoelectron microscopy (Figs. 6–8), the two glycoproteins are localized to the longitudinal sarcoplasmic reticulum and the nonjunctional membranes of the terminal cisternae in association with the Ca^{2+}-ATPase. Although the function of the two glycoproteins is not yet known, their codistribution with the Ca^{2+}-ATPase strongly supports the possibility that they play a role in Ca^{2+} transport and Ca^{2+} sequestration. Sarcalumenin is clearly a member of a group of acidic Ca^{2+}-binding proteins that are present in the lumen of the sarcoplasmic reticulum. Additional members of this group are calsequestrin (10, 19), the high affinity Ca^{2+}-binding protein or calreticulin (34, 35), and the 165-kDa low density lipoprotein and Ca^{2+}-binding protein (36, 37). One can assume that an interplay of these Ca^{2+}-binding proteins is involved in the movement of Ca^{2+} from the Ca^{2+}-ATPase in the nonjunctional membranes to the Ca^{2+} release channels located in the junctional membranes of the terminal cisternae.

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