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Biochemistry

Molecular cloning and expression of cDNA encoding a lumenal calcium binding glycoprotein from sarcoplasmic reticulum

(160-kDa glycoprotein/Ca^{2+} binding protein/cDNA cloning/"sarcalumenin")

EKKEHARD LEBERER*, JEFFREY H. M. CHARUK†, N. MICHAEL GREEN‡, AND DAVID H. MACLENNAN*§

*Banting and Best Department of Medical Research, Charles H. Best Institute, University of Toronto, 112 College Street, Toronto, ON M5G 1L6, Canada; †Membrane Biology Group, Department of Medicine, University of Toronto, Toronto, ON M5S 1A8, Canada; and ‡National Institute for Medical Research, The Ridgeway, Mill Hill, London NW7 1AA, United Kingdom

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ABSTRACT **Antibody screening was used to isolate a cDNA encoding the 160-kDa glycoprotein of rabbit skeletal muscle sarcoplasmic reticulum. The cDNA is identical to that encoding the 53-kDa glycoprotein except that it contains an in-frame insertion of 1308 nucleotides near its 5′ end, apparently resulting from alternative splicing. The protein encoded by the cDNA would contain a 19-residue NH_2-terminal signal sequence and a 453-residue COOH-terminal sequence identical to the 53-kDa glycoprotein. It would also contain a 436-amino acid insert between these sequences. This insert would be highly acidic, suggesting that it might bind Ca^{2+}. The purified 160-kDa glycoprotein and the glycoprotein expressed in COS-1 cells transfected with cDNA encoding the 160-kDa glycoprotein were shown to bind $^{45}Ca^{2+}$ in a gel overlay assay. The protein was shown to be located in the lumen of the sarcoplasmic reticulum and to be associated through Ca^{2+} with the membrane. We propose that this lumenal Ca^{2+} binding glycoprotein of the sarcoplasmic reticulum be designated "sarcalumenin."**

Muscle contraction is triggered by the release of Ca^{2+} from the sarcoplasmic reticulum, whereas muscle relaxation is achieved by rapid reuptake of Ca^{2+} from the cytosol into the lumen of the sarcoplasmic reticulum. Sequestration of Ca^{2+} in the lumen of the sarcoplasmic reticulum is also an essential step in the overall contraction–relaxation cycle of muscle cells. The Ca^{2+} pump has been found to be distributed along the membranes of the longitudinal sarcoplasmic reticulum and the nonjunctional region of the terminal cisternae (1), whereas the Ca^{2+} release channel from sarcoplasmic reticulum has been localized in the junctional face of the terminal cisternae (1, 2). The low-affinity, high-capacity Ca^{2+} sequestering protein, calsequestrin, is located in the lumen of the terminal cisternae (3–5).

The sarcoplasmic reticulum also contains two immunochemically related glycoproteins of unknown function, with apparent molecular masses of 53 and 160 kDa (6, 7). It has been proposed (8, 9) that the glycoproteins are involved in the regulation of Ca^{2+} transport. We have cloned cDNA encoding the 53-kDa glycoprotein (7) but were unable to deduce its function from analysis of its primary structure. In this study we describe the cloning of cDNA encoding the 160-kDa glycoprotein.¶ Analysis of its deduced amino acid sequence and studies of its localization and Ca^{2+} binding properties lead us to conclude that it is a Ca^{2+} binding protein located in the lumen of the longitudinal sarcoplasmic reticulum.

MATERIALS AND METHODS

Isolation, Analysis, and Expression of cDNA Clones. A λgt11 cDNA expression library from rabbit fast-twitch skeletal muscle (a kind gift from M. Harpold, S. B. Ellis, and A. Schwartz) was screened with a mixture of polyclonal and monoclonal antibodies cross-reacting with the 53- and 160-kDa glycoproteins. This screening resulted in the isolation of a group of 47 immunopositive cDNA clones, as described (7). In 2 of these clones, the cDNA encoding the signal sequence of the 53-kDa glycoprotein (7) was replaced by an in-frame sequence of 381 nucleotides. A *Stu* I–*Stu* I fragment of this unique sequence was used as a hybridization probe to isolate a longer cDNA clone, pGPN1, from a rabbit neonatal skeletal muscle cDNA library constructed in the vector pcDX (10). All recombinant DNA techniques and isolation and analysis of mRNA were performed as described by Leberer *et al.* (7). Transfection of COS-1 cells with the cDNA clone pGPN1 in the vector pcDX, cell culture, isolation of microsomal fractions from COS-1 cells, and Western blot analysis of the expressed protein with the monoclonal antibody G10 were carried out as described by Maruyama and MacLennan (11) and by Leberer *et al.* (7).

Preparation of Sarcoplasmic Reticulum Membrane Fractions and Isolation of the 160-kDa Glycoprotein. Longitudinal sarcoplasmic reticulum and terminal cisternae were prepared from rabbit fast-twitch skeletal muscle according to the method of Saito *et al.* (1) in the presence of protease inhibitors, as proposed by Imagawa *et al.* (12). Sarcoplasmic reticulum vesicles were prepared from rabbit fast-twitch skeletal muscle as described by Campbell and MacLennan (6). The 160-kDa glycoprotein was purified from heavy sarcoplasmic reticulum according to the method of Leberer *et al.* (7). Differential extraction of sarcoplasmic reticulum was conducted by successive washing in 10 mM Tris·HCl, pH 8.5/2 mM dithiothreitol/0.1 mM phenylmethylsulfonyl fluoride (extraction buffer) and in extraction buffer containing 1 mM EGTA, as described by Leberer *et al.* (7). Extraction under isotonic conditions was performed in the presence of 250 mM sucrose (7). Endoglycosidase H and trypsin treatment of calcium oxalate-loaded heavy sarcoplasmic reticulum vesicles to determine the localization of the 160-kDa glycoprotein were carried out exactly as described (7).

Miscellaneous Assays. Protein was determined according to Lowry *et al.* (13) using bovine serum albumin as a standard. $NaDodSO_4$/PAGE was performed according to the method of Laemmli (14). Sarcoplasmic reticulum Ca^{2+} binding proteins were identified by $^{45}Ca^{2+}$ binding after $NaDodSO_4$/PAGE and transfer to a nitrocellulose membrane (BA-85, Schleicher & Schüll) according to the method of Maruyama *et al.* (15).

RESULTS

Isolation and Analysis of cDNA Encoding the 160-kDa Glycoprotein. In our previous study of cDNA encoding the 53-kDa glycoprotein (7), we noted that polyclonal and monoclonal

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§To whom reprint requests should be addressed.

¶The sequence reported in this paper has been deposited in the GenBank data base (accession no. M25750).

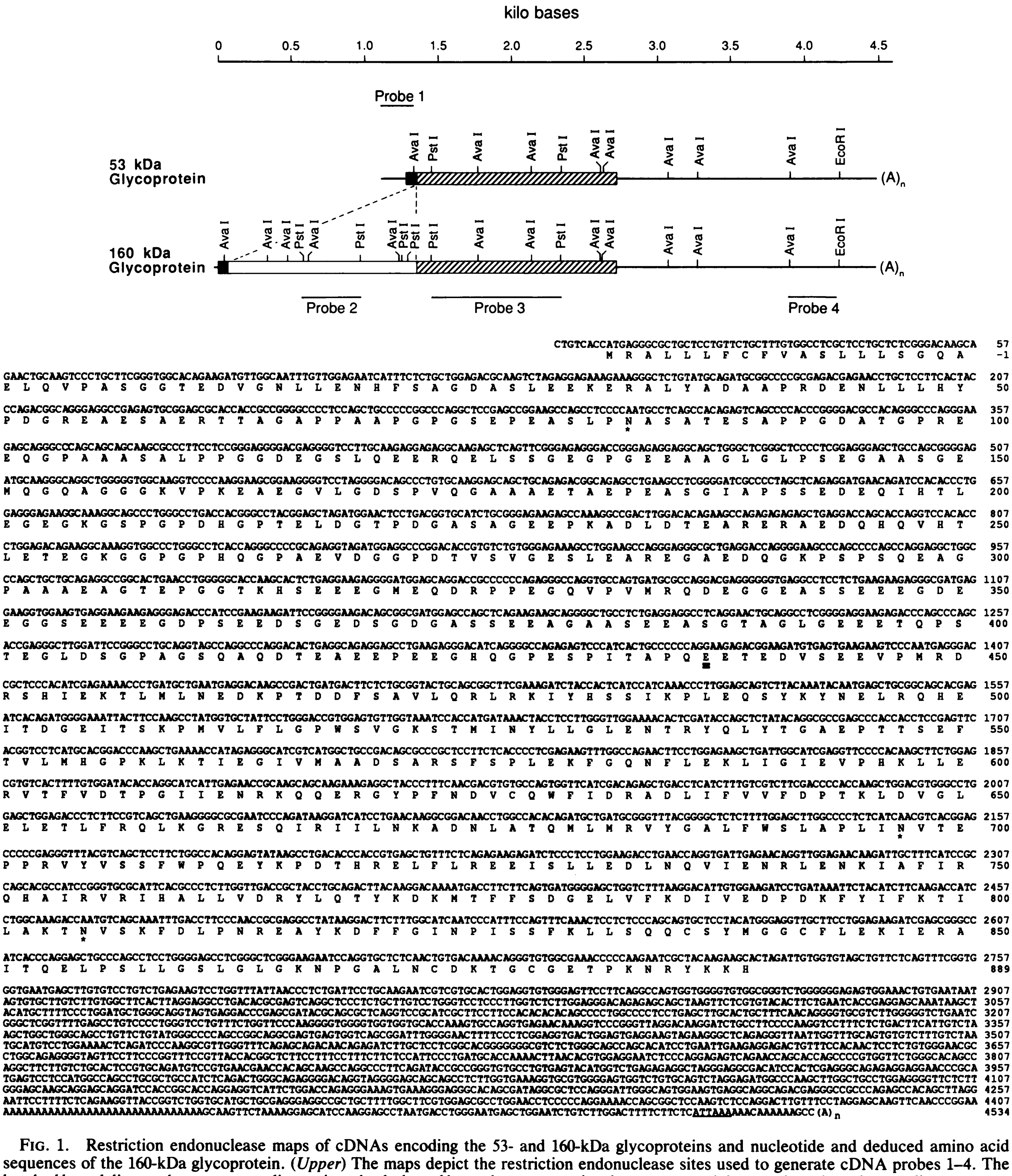

FIG. 1. Restriction endonuclease maps of cDNAs encoding the 53- and 160-kDa glycoproteins and nucleotide and deduced amino acid sequences of the 160-kDa glycoprotein. (*Upper*) The maps depict the restriction endonuclease sites used to generate cDNA probes 1–4. The hatched bars delineate the common coding region, the darkened bars, the common signal sequence, and the open bar, the unique coding sequence of the 160-kDa glycoprotein cDNA. (*Lower*) Amino acid residues are numbered negatively within the signal peptide, with amino acid residue 1 corresponding to the first residue of the mature, processed protein. The doubly underlined glutamate residue at position 437 corresponds to the NH_2 terminus of the mature, processed 53-kDa glycoprotein (7). The three putative N-glycosylation sites are indicated by stars. The poly(A) signal is underlined and $(A)_n$ denotes the poly(A) tail.

antibodies raised against this protein from rabbit fast-twitch skeletal muscle sarcoplasmic reticulum cross-reacted with the protein portion of the 160-kDa glycoprotein. We also noted that cDNA encoding the 53-kDa glycoprotein hybridized to two mRNAs of 3.5 and 5.0 kilobases (kb). These observations suggested that antibodies used to screen expression libraries should bind to the protein products of cDNA encoding the 53- and 160-kDa glycoproteins and, since the 53-kDa glycoprotein is encoded by the 3.5-kb mRNA (7), that the 160-kDa glycoprotein might be encoded by the 5.0-kb mRNA.

In our initial screening of an expression library (7) we isolated 47 clones that expressed immunopositive protein.

Restriction endonuclease mapping and partial nucleotide sequencing revealed that in 2 of these clones the cDNA encoding the signal sequence of the 53-kDa glycoprotein was replaced by an in-frame sequence of 381 nucleotides that encoded a rather acidic amino acid sequence at the NH_2 terminus of the 53-kDa glycoprotein. We used a fragment of this nucleotide sequence as a hybridization probe to screen a neonatal rabbit skeletal muscle cDNA library in the vector pcDX (10). A full-length cDNA clone designated pGPN1 was isolated from the library.

As illustrated in Fig. 1 *Upper*, the sequence of the cDNA in clone pGPN1 was identical to the sequence of the cDNA encoding the 53-kDa glycoprotein (7) except that it contained an in-frame insertion of 1308 nucleotides near its 5′ end. The deduced amino acid sequence of this cDNA (Fig. 1 *Lower*) begins with the same stretch of 19 hydrophobic residues that appear to constitute an NH_2-terminal signal sequence in the 53-kDa glycoprotein. The insertion, which begins precisely at the 3′ end of the codons for the signal sequence, encodes a unique sequence of 436 amino acids. This sequence is highly negatively charged, containing 24.8% acidic amino acids and only 4.3% basic residues. In 30 locations, two or more acidic residues are juxtaposed. In most cases, these acidic clusters are bounded by residues containing hydroxyl or amido groups in their side chains, sequences such as Ser-Glu-Glu-Glu or Gln-Glu-Glu-Thr-Glu-Asp being characteristic. These hydrophilic clusters are, in turn, interconnected by short hydrophobic sequences containing proline, leucine, or, most often, the short aliphatic residue alanine or glycine. The sequence from residues 316 to 445 is especially rich in these alternative hydrophilic and hydrophobic clusters. In this region glutamic and aspartic acids make up 36.9% of the total residues, threonine, serine, and glutamine make up 22.3%, and lysine and arginine constitute only 1.5%. The remainder of the cDNA encodes the precise sequence of the mature, processed 53-kDa glycoprotein (7). This common sequence is polar throughout, containing 14.1% acidic and 15.0% basic residues. It does not, therefore, contain any strong net charge.

These observations suggest that cDNAs encoding the 53- and 160-kDa glycoproteins are derived from the same gene by alternative splicing of a single transcript. This conclusion is supported by Northern blot analysis of mRNA from rabbit fast-twitch skeletal muscle. As shown in Fig. 2, cDNA probes from the region encoding the common sequence in the 53- and 160-kDa glycoprotein hybridized to two mRNA species of 3.5 and 5.0 kb. By contrast, a cDNA probe from the insert that encodes only the 160-kDa glycoprotein hybridized only to the 5.0-kb mRNA.

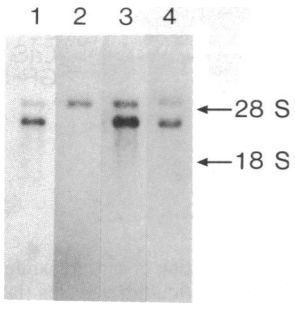

FIG. 2. Northern blot analysis of mRNA from rabbit skeletal muscle. Poly(A)$^+$ RNA from fast-twitch psoas muscle was fractionated on a formaldehyde gel and transferred to an Amersham Hybond nylon membrane. The membrane was hybridized successively with the individual ^{32}P-labeled cDNA probes 1–4, denoted in Fig. 1 *Upper*, and washed at high stringency (7). Positions of the 28S and 18S ribosomal subunits are depicted by arrows. Note that probes 1, 3, and 4 from the common region of the cDNAs (Fig. 1 *Upper*) hybridize to two mRNA species of 3.5 and 5.0 kb, whereas probe 2 from the unique sequence of the 160-kDa glycoprotein cDNA (Fig. 1 *Upper*) binds solely to the 5.0-kb mRNA.

The deduced amino acid sequence of the 160-kDa glycoprotein contains three potential N-glycosylation sites (Fig. 1 *Lower*), one within the unique COOH-terminal half at residue 83 (Asn-Ala-Ser) and two in the domain common with the 53-kDa glycoprotein at positions 697 (Asn-Val-Thr) and 805 (Asn-Val-Ser). A computer-assisted search for identity with other sequences present in the EMBL Gene Data Bank failed to find any significant matches.

Hydropathy plots of the predicted amino acid sequence were performed according to the method of Kyte and Doolittle (16). No segment, other than the NH_2-terminal hydrophobic signal sequence, was found to be hydrophobic enough and long enough to be considered as transmembrane sequence. We conclude, therefore, that the 160-kDa glycoprotein, like the 53-kDa glycoprotein, is localized entirely in the lumen of the sarcoplasmic reticulum.

Secondary structural analysis (17) of the NH_2-terminal half of the molecule shows low probability of β structure and a predominance of bends and short α-helices. Many of the predicted helices are atypical of the amphipathic helices of normal globular proteins in that they lack well-defined hydrophobic patches and carry a strong negative charge. These predicted helices may, however, be stabilized by cation binding. The bend sequences between the predicted helices are those rich in glycine, alanine, and proline.

If we assume that the NH_2-terminal signal sequence is cleaved from the 160-kDa glycoprotein as it is from the 53-kDa glycoprotein (7), then it can be deduced that the mature, processed protein would contain 889 amino acids with a molecular mass of 95,710 Da. The addition of three unprocessed carbohydrate chains [the two carbohydrate chains in the 53-kDa glycoprotein are unprocessed (6)] would add a further mass of 6000 Da to bring the total molecular mass of the mature protein to about 102,000.

Because of the large discrepancy in the molecular mass of 102,000 deduced from amino acid and carbohydrate content and the molecular mass of 160,000 deduced from the mobility of the glycoprotein in Laemmli NaDodSO$_4$ gels (6), we transfected the cDNA encoding the 160-kDa glycoprotein into COS-1 cells by the method of Maruyama and MacLennan (11) and measured the mobility of the expressed protein

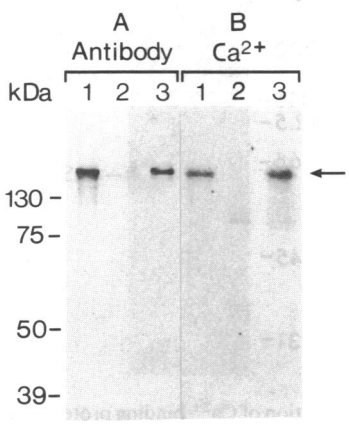

FIG. 3. Expression of the recombinant 160-kDa glycoprotein in COS-1 cells. (*A*) Western blot analysis with monoclonal anti-53-kDa glycoprotein antibody (7). (*B*) ^{45}Ca overlay. Lanes 1, purified 160-kDa glycoprotein; lanes 2, microsomes from nontransfected COS-1 cells; lanes 3, microsomes from COS-1 cells transfected with the vector pcDX containing cDNA encoding the 160-kDa glycoprotein. Proteins (1 μg in lanes 1, 10 μg in lanes 2 and 3 of *A*, and 20 μg in lanes 2 and 3 of *B*) were separated by NaDodSO$_4$/PAGE in 7.5% gels. Molecular masses of standard proteins are indicated on the left. The arrow points to the position of the 160-kDa glycoprotein.

in the Laemmli NaDodSO$_4$ gel system. As illustrated in Fig. 3, transfected COS-1 cells expressed an immunoreactive protein migrating in NaDodSO$_4$/PAGE with the purified 160-kDa glycoprotein. We conclude, therefore, that the cDNA clone that we have isolated encodes the complete sequence of the 160-kDa glycoprotein.

Identification of the 160-kDa Glycoprotein as a Ca^{2+} Binding Protein. Although we could not detect a high-affinity Ca^{2+} binding sequence in the primary sequence of the 160-kDa protein, we noted that juxtapositions of acidic amino acids and alternating hydrophobic/hydrophilic clusters are also found in the sequences of the low-affinity, high-capacity Ca^{2+} binding proteins calsequestrin (18) and chromogranin A (19). Therefore, we examined the Ca^{2+} binding properties of the 160-kDa glycoprotein and its localization in the sarcoplasmic reticulum membrane through the use of a Ca^{2+} binding overlay technique (15). It is apparent in Fig. 4 that the isolated 160-kDa glycoprotein binds Ca^{2+} strongly under the conditions of the Ca^{2+} overlay assay. In Fig. 3 we also demonstrate that the protein expressed in COS-1 cells transfected with cDNA from clone pGPN1 binds Ca^{2+} in the overlay assay. Lane 3 of Fig. 4 shows that calsequestrin is the predominant Ca^{2+} binding protein in the terminal cisternae of the sarcoplasmic reticulum and that no Ca^{2+} binding is detectable in the region where the 160-kDa glycoprotein would migrate. Other Ca^{2+} binding proteins with molecular masses of 170 and 200 kDa are observed in this preparation, however. By contrast, strong Ca^{2+} binding by a protein of the same mobility as the 160-kDa glycoprotein is observed in longitudinal sarcoplasmic reticulum, a preparation that is depleted of calsequestrin (Fig. 4; ref. 4).

The 160-kDa glycoprotein was shown to be located in the lumen of the sarcoplasmic reticulum by using the same criteria as were used previously for localization of the 53-kDa glycoprotein (7). The protein has an NH_2-terminal signal sequence; it has no convincing transmembrane sequences; and it was resistant to trypsin in intact vesicles (Fig. 5).

The mode of association of the 160-kDa Ca^{2+} binding protein with the sarcoplasmic reticulum membrane was studied by its differential extraction from sarcoplasmic reticulum vesicles. The 160-kDa glycoprotein, like the 53-kDa glycoprotein, remained membrane bound when vesicles were

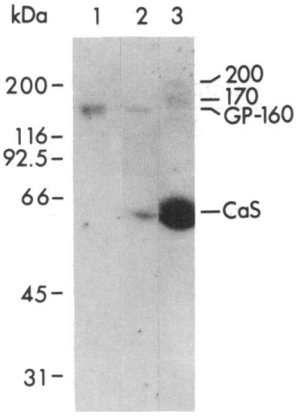

FIG. 4. Identification of Ca^{2+} binding proteins from sarcoplasmic reticulum. Purified 160-kDa glycoprotein (lane 1), longitudinal sarcoplasmic reticulum (lane 2), and terminal cisternae (lane 3) were subjected to NaDodSO$_4$/PAGE in 7.5% gels and transferred electrophoretically to a nitrocellulose membrane. The panel shows an autoradiograph of the membrane after incubation with $^{45}CaCl_2$. Molecular masses of standard proteins are given on the left. Positions of calsequestrin (CaS), the 160-kDa glycoprotein (GP-160), and the 170- and 200-kDa Ca^{2+} binding proteins are indicated. One microgram of purified 160-kDa glycoprotein was applied to lane 1, whereas 30 μg of longitudinal sarcoplasmic reticulum and terminal cisternae protein was applied to lanes 2 and 3, respectively.

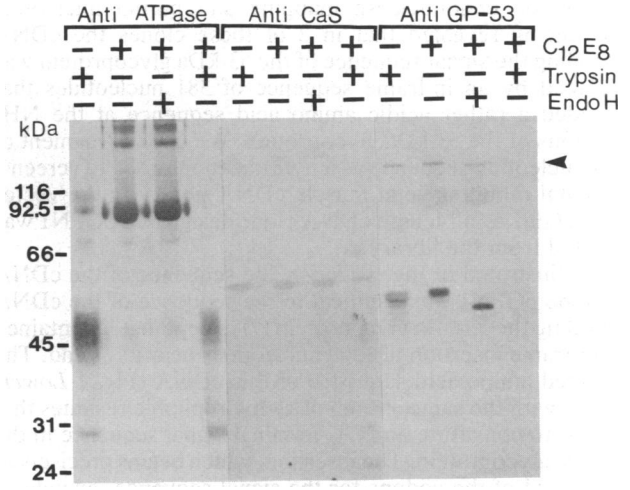

FIG. 5. Trypsin and endoglycosidase H (Endo H) treatment of calcium oxalate-loaded heavy sarcoplasmic reticulum. Digestions were carried out as described (7). It is clear that neither the 160-kDa glycoprotein nor calsequestrin was accessible to trypsin in intact vesicles, even though each was readily digested in detergent. The Ca^{2+}-ATPase, by contrast, was digested in both cases. GP-53, 53-kDa glycoprotein; CaS, calsequestrin; ATPase, Ca^{2+}-ATPase. Molecular masses of standard proteins are indicated on the left. The arrowhead indicates the position of the 160-kDa glycoprotein.

washed under hypotonic conditions that eluted large amounts of peripheral (e.g., phosphorylase) and lumenal (e.g., calsequestrin) proteins with a large range of molecular masses (Fig. 6, lane 2). The 160- and 53-kDa glycoproteins were extracted, however, when EGTA was included in the hypotonic extraction buffer (Fig. 6, lane 3). Both glycoproteins remained bound to the membrane when EGTA extraction was carried out under isotonic conditions that would keep the membrane intact (data not shown). These data suggest that the 160-kDa glycoprotein exists as a lumenal Ca^{2+} binding protein in the longitudinal sarcoplasmic reticulum and that it is attached to the inner side of these membranes through Ca^{2+} bridges.

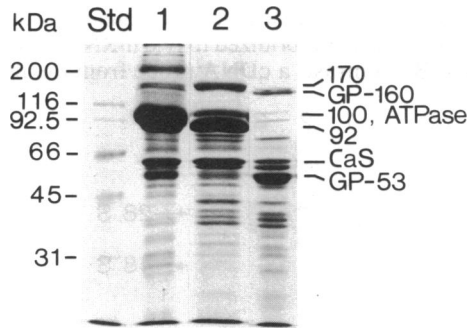

FIG. 6. Differential extraction of sarcoplasmic reticulum proteins with EGTA. Sarcoplasmic reticulum vesicles (lane 1) were washed under hypotonic conditions and solubilized proteins (lane 2) were separated by ultracentrifugation. The insoluble material was treated twice with 1 mM EGTA under hypotonic conditions and the eluted proteins (lane 3) were separated by ultracentrifugation. Proteins were analyzed by NaDodSO$_4$/PAGE in a 10% gel and stained with Coomassie blue. The amount of protein applied to each lane was 30 μg. Note that the 160- and 53-kDa glycoproteins were eluted specifically with EGTA. Molecular masses of standard proteins (Std) are depicted on the left. The positions of the 53-kDa glycoprotein (GP-53), calsequestrin (CaS), the Ca^{2+}-ATPase (ATPase), the 160-kDa glycoprotein (GP-160), and proteins with apparent molecular masses of 92, 100, and 170 kDa are denoted on the right.

DISCUSSION

The data presented in this paper show that the 53- and 160-kDa glycoproteins of the sarcoplasmic reticulum share, in part, the identical amino acid sequence, accounting for their immunological similarity and for some of their additional properties. It is probable that these proteins are expressed from alternatively spliced products of a single transcript. These alternative transcripts are observed in muscle mRNAs where they hybridize to probes from their common regions. The larger transcript encoding the 160-kDa glycoprotein can be distinguished in mRNA populations by its unique hybridization to probes unique to the cDNA encoding the 160-kDa glycoprotein.

The 160-kDa glycoprotein has long been suspected to be a Ca^{2+} binding protein. It stains blue with StainsAll (20), a feature common to acidic Ca^{2+} binding proteins; it is Ca^{2+} precipitable, like calsequestrin (21), and a band with a mobility corresponding to 160 kDa binds Ca^{2+} in a Ca^{2+} overlay (22). In this study, we have shown that the purified 160-kDa glycoprotein and the 160-kDa glycoprotein expressed in COS-1 cells bind Ca^{2+}, thus proving the identity of the glycoprotein as a Ca^{2+} binding protein.

The Ca^{2+} binding sites clearly lie in the NH_2-terminal half of the protein since no Ca^{2+} binding by the 53-kDa glycoprotein has been observed. This region is very acidic and contains an interesting motif of juxtaposed glutamic or aspartic acid residues, bounded by threonine, serine, or glutamine residues and interspersed with glycine, alanine, or proline residues. Thus the protein could have the character of short, Ca^{2+} binding helices separated by short loops. The sequence bears no identity to any other protein in the EMBL Gene Data Bank, but it is similar to calsequestrin (18) and chromogranin A (19) in containing juxtaposed acidic residues, alternating clusters of hydrophobic and hydrophilic amino acids, and high-capacity Ca^{2+} binding. It is of interest that it also shares with calsequestrin and chromogranin A the property of anomolous migration in alkaline $NaDodSO_4$ gels.

An outstanding question regarding the 160-kDa glycoprotein is its functional relationship to the 53-kDa glycoprotein. These proteins are both located in the lumen of the longitudinal sarcoplasmic reticulum and they are both released from this site following disruption of the membrane under hypotonic conditions and extraction with the Ca^{2+} chelator EGTA. Since the 53-kDa glycoprotein does not bind Ca^{2+}, it is possible that the 53- and 160-kDa glycoproteins form a Ca^{2+} binding complex in the lumen of the sarcoplasmic reticulum and that this complex is the functional form for both proteins. We have noted in our purification schemes (see also ref. 6) that these proteins aggregate, but we have not yet studied their ability to associate with each other. Earnshaw *et al.* (23) have suggested that protein–protein interactions among several nuclear proteins might occur through acidic domains neighbored by serine residues. In this respect the sequence Ser-Glu-Glu occurs repeatedly in the 160-kDa glycoprotein and once in the NH_2 terminus of the 53-kDa glycoprotein. Thus this could be a point of interactions between these proteins. It should also be noted that the sequence Ser-Glu-Glu-Glu occurs once in calsequestrin, which is self associating in the presence of Ca^{2+}, as well as in troponin C and calmodulin, which clearly interact with other proteins in the presence of Ca^{2+}.

A Ca^{2+} binding complex in the lumen of the longitudinal sarcoplasmic reticulum could act to reduce the Ca^{2+} gradient built up by the Ca^{2+} pump and consequently accelerate Ca^{2+} transport. This would explain the stimulatory effect of the glycoproteins on the efficiency of Ca^{2+} transport described by Chiesi and Carafoli (8) and Leonards and Kutchai (9). It is of interest that the Ca^{2+}-ATPase contains the acidic sequence Glu-Glu-Gly-Glu-Glu on its lumenal surfaces between transmembrane sequences 1 and 2 (10) and this would be an appropriate site for interaction, through Ca^{2+} bridging, of the 160-kDa glycoprotein with a protein in the sarcoplasmic reticulum membrane. This would also bring the two proteins into close contact for passage of Ca^{2+} from the pump to internal stores.

We do not yet know the function of the 160-kDa glycoprotein but we know that it is a Ca^{2+} binding protein localized in the lumen of the longitudinal sarcoplasmic reticulum. In the tradition of distinguishing Ca^{2+} binding proteins by unique names, we propose that the protein be designated "sarcalumenin."

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