Chapter 3

PROTEIN COMPONENTS AND THEIR ROLES IN SARCOPLASMIC RETICULUM FUNCTION

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I. INTRODUCTION

The structure and function of the sarcoplasmic reticulum have been studied for a period of about 40 years. The sarcoplasmic reticulum was first obtained as paniculate material from skeletal muscle by Kielley and Meyerhof.[1] Marsh[2] discovered that an extract of muscle tissue, when added to an actomyosin preparation, induced relaxation. Ebashi[3] showed that the relaxing factor was paniculate and that its relaxing activity was correlated with ATPase activity. Hasselbach and Makinose[4] showed that the sarcoplasmic reticulum was a vesicular membrane and the membrane possessed an ATPase system capable of reducing external Ca^{2+} to micromolar concentrations. These observations on the sarcoplasmic reticulum together with the observations on the Ca^{2+} sensitivity of the actomyosin system[5] firmly established the basis for Ca^{2+} control of muscle contraction and the importance of the sarcoplasmic reticulum in the regulation of muscle contraction.

The current understanding of excitation-contraction coupling in skeletal muscle is the following: the action potential spreads out along the surface membrane, and the depolarization from the action potential spreads inward along the transverse tubular network.[6] The depolarization of the transverse tubular system and accompanying membrane charge movement initiates the release of Ca^{2+} from the terminal cisternae of the sarcoplasmic reticulum.[7-9] Ca^{2+} ions then diffuse to the myofilaments and bind to the troponin-tropomyosin complex associated with the thin filaments.[10] This binding removes the inhibition on the actomyosin systems, which then reacts to produce tension.[10] Relaxation occurs when the bound Ca^{2+} is removed by the action of the $Ca^{2+} + Mg^{2+}$ ATPase in the sarcoplasmic reticulum. which lowers the free Ca2" concentration below that required for contraction.[11] Ca^{2+} is then stored in the terminal cisternae of the sarcoplasmic reticulum during relaxation.[12,13]

Since the identification of the sarcoplasmic reticulum as the ATP-dependent vesicular relaxing factor,[11] a wealth of information has been accumulated about the mechanism of Ca^{2+} transpon by the sarcoplasmic reticulum and the proteins responsible for Ca^{2+} transpon.[14-16] In contrast, comparatively little is known about the mechanism of Ca^{2+} release or the proteins involved in Ca^{2+} release from the sarcoplasmic reticulum. How the depolarization of the transverse tubular membrane triggers Ca^{2+} release from the sarcoplasmic reticulum is the least understood process in skeletal muscle excitation-contraction coupling. The membranes directly involved in excitation-contraction coupling and Ca^{2+} release in skeletal muscle are the transverse tubular membrane and the junctional sarcoplasmic reticulum membrane.[7-9] Transverse tubular membrane vesicles have only recently been isolated and relatively little is known about their protein composition.[17,18] The protein composition of the junctional sarcoplasmic reticulum membrane is also relatively unknown. It is likely that the junctional sarcoplasmic reticulum proteins that function directly or indirectly in excitation-contraction coupling and/or Ca^{2+} release are all minor protein components of isolated sarcoplasmic reticulum vesicles.

In this chapter we will discuss both the major and minor protein components of skeletal muscle sarcoplasmic reticulum and their role in Ca^{2+} transport, Ca^{2+} storage, or Ca^{2+} release from the sarcoplasmic reticulum of skeletal muscle.

II. SARCOPLASMIC RETICULUM MEMBRANE

The striated muscle cell consists of three membrane systems all of which are involved in the process of excitation-contraction coupling.[19] The outer surface membrane, called the sarcolemma, surrounds the entire muscle cell and is excited by the innervating nerve to propagate an action potential which initiates excitation-contraction coupling. The invaginations of the sarcolemma into the fiber's interior are called transverse tubules or T tubules.[20] The transverse tubular system is responsible for the spread of depolarization to the interior of the fiber[6] and accompanying membrane charge movement.[9]

The muscle cell has an extensive internal membrane system called the sarcoplasmic reticulum. which is a membranous network that surrounds each myofibril like a fenestrated water jacket around a cylinder.[20-22] The sarcoplasmic reticulum is responsible for sequestration of Ca^{2+} to allow relaxation, storage of Ca^{2+} during relaxation, and release of Ca^{2+} to initiate contraction.[7,8,11,14,15] In skeletal muscle, the sarcoplasmic reticulum is the sole source of Ca^{2+} during the activation of contraction. The sarcoplasmic reticulum is especially well developed in fast contracting muscles.[20,23] The rapid activation-inactivation cycle of the contractile system of these muscles depends on the sudden release of considerable quantities of Ca^{2+} followed by the complete removal of Ca^{2+} from the sarcoplasm.

The sarcoplasmic reticulum consists of longitudinal reticulum and terminal cisternae.[20,21] The longitudinal reticulum faces the myofibrils and is referred to as the free sarcoplasmic reticulum because it does not participate in the formation of any junctions with other membrane systems.[24] The terminal cisternae of the sarcoplasmic reticulum forms junctions with the transverse tubules. The combination of the central transverse tubule sandwiched between two terminal cisternae is called a triad.[21] Triads are located in a regular disposition either opposite the Z line (frog twitch fibers) or opposite the A-I junction (mammalian muscle).[24] The junctional sarcoplasmic reticulum membrane is that portion of the terminal cisternae which is in direct apposition to the transverse tubule membrane.[24] There is approximately ten times more free sarcoplasmic reticulum than junctional sarcoplasmic reticulum in frog twitch fibers.

The longitudinal reticulum or free sarcoplasmic reticulum is identical in appearance along the whole sarcomere and is relatively devoid of internal content.[20] Freeze-fracture of the longitudinal reticulum reveals a cytoplasmic fracture face that consists of closely packed panicles, approximately 8 nm in diameter.[24-28] The luminal fracture face of the longitudinal sarcoplasmic reticulum is smooth in appearance. The particles on the cytoplasmic fracture face are present at a density of several thousand particles per square micron. It has been confirmed that these particles of the sarcoplasmic reticulum represent the intramembranous hydrophobic portion of the Ca^{2+} + Mg^{2+} ATPase.[26] The major function of the longitudinal reticulum appears to be the sequestration of Ca^{2+} to allow relaxation. The longitudinal reticulum does not appear to be a major site of Ca^{2+} storage, nor does it delay the return of Ca^{2+} to the terminal cisternae.[13]

The structure of the terminal cisternae-transverse tubule junction has been extensively studied.[20-22,25,27-35] The terminal cisternae of the triad contains an electron-dense filamentous or granular material.[20] This electron-dense content within the terminal cisternae is arranged in longitudinal rows and appears to adhere to the junctional sarcoplasmic reticulum membrane.[32-36] In stimulated fibers, the amorphous granular material is denser throughout the terminal cisternae and particularly dense in a discontinuous plaque underlying the junctional sarcoplasmic reticulum membrane.[34]

The triad junction is quite unlike that of any of the known low resistance junctions found elsewhere. A comparative study of the triadic-junction in skeletal muscle fibers has shown that the membrane architecture of the junction is remarkably similar throughout the animal kingdom.[24] The transverse tubular and terminal cisternae membranes are separated by a distance of 10 to 20 nm, which is larger than a typical gap junction.[22] At periodic intervals of about 30 nm. the terminal cisternae membrane forms small projections that contact the transverse tubular membrane.[27] These projections have been called sarcoplasmic reticulum feet[22] or junctional feet.[27] The junctional feet cross the entire junctional gap, but they appear to be primarily attached to the junctional sarcoplasmic reticulum membrane rather than to the transverse tubule membrane. The space between the junctional feet has been shown to be accessible to ferritin molecules and therefore should be accessible to solutes diffusing from the sarcoplasm.[30] The junctional gap and feet are seen in freeze-dried muscle, indicating that the morphology of these structures is not due to fixation or dehydration.[32] In some

muscles, the feet exist extrajunctionally on the sarcoplasmic reticulum membrane which does not face towards the transverse tubule membrane.[36,37] The junctional feet have also been identified in isolated heavy sarcoplasmic reticulum vesicles.[38]

The structure of the sarcoplasmic reticulum feet has been described by several groups.[22,29,32,33,35,39] Junctional feet are arranged in two or more rows of varying length and contact both sarcoplasmic reticulum and transverse tubular membranes at tetragonally disposed sites. Each junctional foot has a less dense central core extending across the entire junctional gap. Junctional feet have lateral protrusions which extend in a plane parallel to the two junctional membranes and allow them to contact each other.[27] Franzini-Armstrong and Nunzi[27] have proposed that the junctional feet are composed of four subunits, but no direct evidence for such a structure has been obtained. The function of the sarcoplasmic reticulum feet seems to be the mechanical adhesion of the junctional sarcoplasmic reticulum to the transverse tubule membrane. The junctional feet are probably also directly or indirectly involved in the molecular mechanisms of excitation-contraction coupling, though no direct evidence for such involvement exists.

Freeze fracture replicas of the sarcoplasmic reticulum have revealed the asymmetry of the longitudinal and terminal cistemae membranes.[25,27,28,33] In the proximity of the triad, the sarcoplasmic reticulum membrane shows changes in both the cytoplasmic and luminal leaflets. The cytoplasmic leaflet changes from closely packed panicles to large panicles that are separated by smooth areas of membrane. The junctional sarcoplasmic reticulum which is in direct apposition to the transverse tubule membrane has large panicles and its panicles leave distinct pits on the luminal leaflet. The difference in structure between the longitudinal sarcoplasmic reticulum and the junctional sarcoplasmic reticulum may reflect their difference in function. There seems to be no correlation between the disposition of the panicles within the junctional membrane and that of the junctional feet on the membrane surface. Freeze fracture of the junctional transverse tubular membrane revealed large panicles with a maximum density of two per sarcoplasmic reticulum foot.[27] This ratio has been taken as a preliminary indication of anchoring of a component of each foot to the transverse tubular membrane.[27]

III. ISOLATED SARCOPLASMIC RETICULUM VESICLES

Sarcoplasmic reticulum vesicles can be easily isolated and purified in considerable quantities from rabbit skeletal muscle by differential centrifugation of muscle homogenates in either isotonic salt[40,41] or isotonic sucrose.[38,42] The major nonmembranous contaminant, actomyosin, can be removed by extracting the preparation of sarcoplasmic reticulum vesicles with 0.6 *M* KCl, which solubilizes actomyosin without disrupting the sarcoplasmic reticulum vesicles.[43]

Sarcoplasmic reticulum vesicles are identified by their ability to accumulate Ca^{2+} from ATP-containing solutions and by their Ca^{2+}-dependent ATPase activity. The homogenization and isolation do not give rise to randomized inversion of the membrane vesicles; therefore, they retain the proper orientation for Ca^{2+} transpon.[44] Ebashi[3] was the first to show that isolated sarcoplasmic reticulum vesicles accumulate Ca^{2+} with high affinity in the presence of ATP. Hasselbach[11] was the first to describe Ca^{2+} accumulation as active transpon against a Ca^{2+} gradient which derives its energy from the hydrolysis of ATP through an ATPase enzyme incorporated in the sarcoplasmic reticulum membrane. The existence of a phosphoenzyme intermediate acyl phosphate after incubation of the sarcoplasmic reticulum with gamma-^{32}P-ATP was first demonstrated by Manonosi.[45] The dependence upon the free Ca^{2+} concentration in the medium is the same for ATP hydrolysis, phosphorylated intermediate formation, and Ca^{2+} transpon by the sarcoplasmic reticulum vesicles.[14]

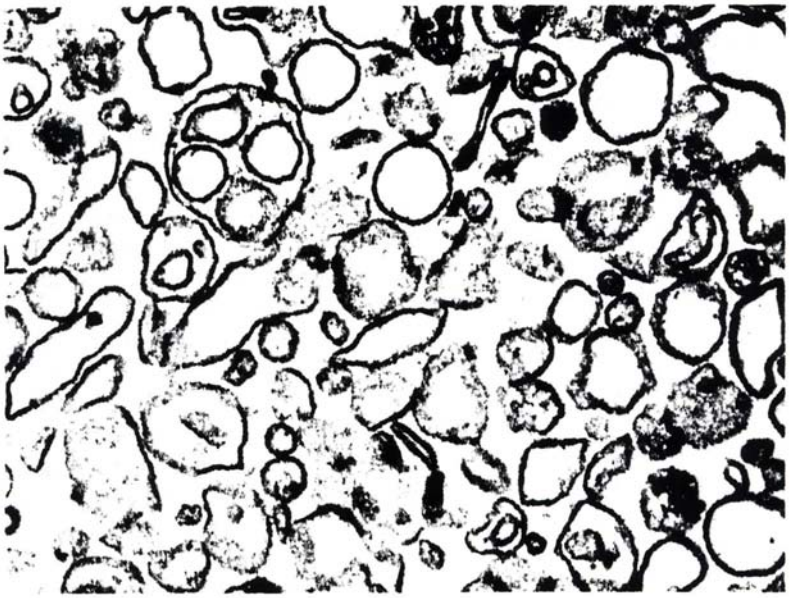
Ultrastructural characterization of isolated sarcoplasmic reticulum vesicles has shown that

the vesicles retain many of the morphological features of the sarcoplasmic reticulum *in situ* and are highly asymmetric and heterogeneous. Thin section electron microscopy of sarcoplasmic reticulum vesicles reveals closed spherical vesicles of approximately 0.1 μm with a single bilayer membrane.[46] Some vesicles are electron lucent, while some vesicles contain electron-dense material. Negatively or positively stained sarcoplasmic reticulum vesicles contain small panicles (3 to 4 nm) in or extruding from the cytoptasmic surface of the vesicles.[46,47] It has been shown that the ATPase is responsible for this surface particle and that it is removed upon tryptic digestion of the sarcoplasmic reticulum vesicles.[26,47] Saito et al.[48] have shown the asymmetry of isolated sarcoplasmic reticulum by staining thin sections of sarcoplasmic reticulum vesicles with tannic acid. Electron-dense stained material was localized only on the cytoplasmic surface of the isolated sarcoplasmic reticulum vesicles. while a symmetric appearance of electron-dense material was seen in reconstituted vesicles.[48]

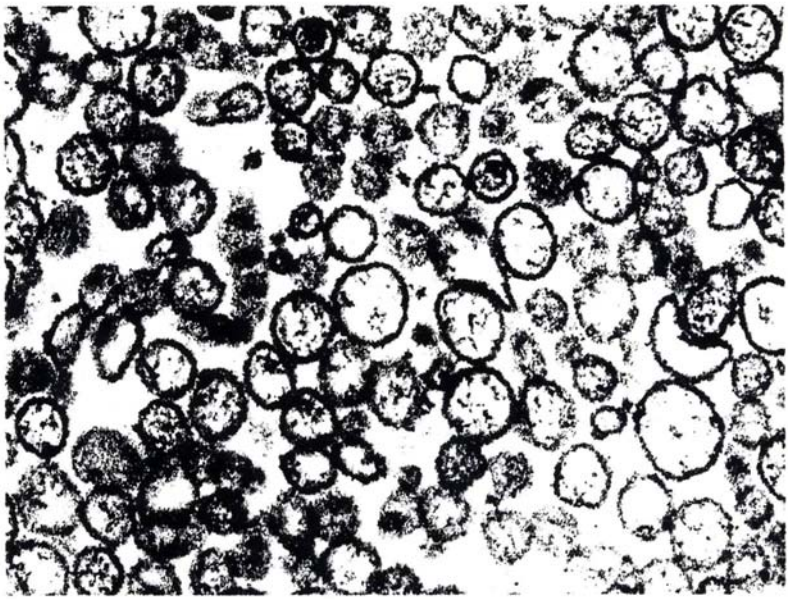
Freeze fracture profiles of isolated sarcoplasmic reticulum vesicles reveal an asymmetric arrangement of 8- to 9-nm globules in the hydrophobic interior of the membrane.[24] which corresponds to the Ca^{2+} + Mg^{2+} ATPase.[26] Jilka et al.[49] and Scales and Inesi[50] have shown that the number of negatively stained 4-nm surface particles exceeds the number of intramembranous 8- to 9-nm particles seen in freeze fracture of sarcoplasmic reticulum vesicles. Therefore, it is likely that the surface particle represents a single ATPase molecule, while the intramembranous particle is due to the formation of an ATPase oligomer that appears as a single freeze fracture particle.

Isolated sarcoplasmic reticulum vesicles from rabbit skeletal muscle have been found to be heterogeneous with respect to structure and function.[17,38,51-53] Meissner[51] was the first to purify skeletal sarcoplasmic reticulum by sucrose gradient centrifugation and to show that the isolated sarcoplasmic reticulum consisted of a heterogeneous population of vesicles. He found that sarcoplasmic reticulum vesicles with different buoyant densities (termed light and heavy sarcoplasmic reticulum vesicles) differed with respect to protein composition and electron-dense content. He suggested from his biochemical and morphological data that the heavy sarcoplasmic reticulum vesicles are derived from the terminal cisternae of the sarcoplasmic reticulum and that the light sarcoplasmic reticulum vesicles are derived from the longitudinal sarcoplasmic reticulum. Meissner[51] also suggested that the electron-dense material within the heavy vesicles was calsequestrin and/or a 55,000-dalton protein (M_{55}).

Campbell et al.[38] have also isolated light and heavy sarcoplasmic reticulum vesicles. Typical electron micrographs of light and heavy sarcoplasmic reticulum vesicles isolated according to Campbell et al.[38] are shown in Figures 1 and 2. The light sarcoplasmic reticulum vesicles are empty vesicles of various sizes and shapes (Figure 1A). Freeze fracture replicas of the light sarcoplasmic reticulum vesicles showed an asymmetric distribution of intramembranous particles similar to that seen in the longitudinal reticulum. The heavy sarcoplasmic reticulum vesicles appear as rounded vesicles of uniform size and shape, filled with electron-dense material similar to that found in the terminal cisternae. The electron-dense material in vesicles treated with KCl is dispersed throughout the vesicles (Figure 1B). In contrast, the heavy sarcoplasmic reticulum vesicles which are not treated with KCl contain electron-dense material that is localized in the junctional membrane region (Figure 1C). The heavy sarcoplasmic reticulum vesicles also have some material on the outer surface of the vesicle in the region of the localized electron-dense material. When heavy sarcoplasmic reticulum vesicles are fixed in cacodylate, the electron-dense material becomes dispersed. similar to that seen in vesicles treated with KCl (Figure 2). The material on the outer surface of the vesicles remains, and in certain vesicles this material has a periodicity and appearance of the junctional sarcoplasmic reticulum feet (Figure 2). Freeze fracture replicas of the heavy vesicles revealed an asymmetric distribution of particles which in some areas of the vesicle's surface were larger and less densely aggregated than those of the light vesicles. The luminal leaflet of the heavy vesicles in some regions showed evidence of pits. These structural details



A



B

FIGURE 1. (A) Thin section of light sarcoplasmic reticulum vesicles. The vesicles have variable size and shape and no visible content. Light vesicles often form invaginations producing the appearance of vesicles with a double wall. Small, flat profiles are vesicles which are probably derived from the transverse tubular system. (Magnification × 60,000.) (B) Thin section of heavy sarcoplasmic reticulum vesicles treated with KCl. The vesicles are round, of fairly uniform size, and with a visible electron-dense content. (Magnification × 60,000.) (C) Thin section of heavy sareoplasmic reticulum vesicles not treated with KCl. The electron-dense content is localized to the junctional sarcoplasmic reticulum membrane. The cytoplasmic surface is covered with some material. (Magnification × 60,000.)

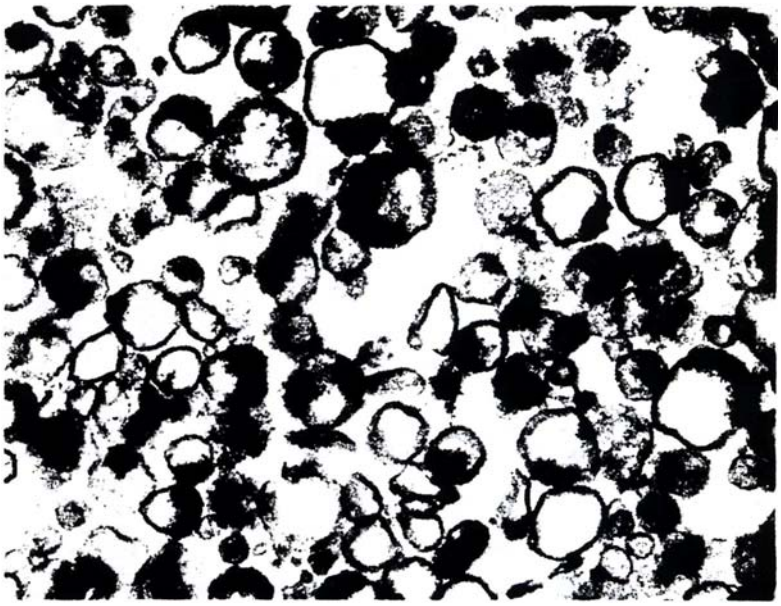


FIGURE 1C

are characteristic of the junctional sarcoplasmic reticulum membrane and support the idea that the heavy vesicles are derived from the terminal cisternae and contain junctional sarcoplasmic reticulum membrane.[38]

Campbell et al.[38] also performed a detailed characterization of the protein composition of the light and heavy sarcoplasmic reticulum vesicles using two high resolution gel electrophoresis systems. The electron-dense content in the heavy sarcoplasmic reticulum vesicles was identified as calsequestrin since calsequestrin was concentrated in the heavy vesicles, while several proteins of approximately 55,000 daltons were found in both light and heavy vesicles. Campbell et al.[38] suggested that the localized electron-dense material in the heavy vesicles was a Ca^{2+}-calsequestrin complex since the KCl treatment greatly reduced the Ca^{2+} content of the vesicles and dispersed the localized electron-dense material (compare Figure 1C to 1B). Heavy sarcoplasmic reticulum vesicles were also enriched in several proteins of molecular weights 30,000, 33,000, 34,000, and 38,000. The 34,000 and 38,000-dalton proteins were identified as components of the "junctional feet", since treatment of the heavy vesicles with KCl resulted in the loss of the 34,000- and 38,000-dalton proteins and in the disappearance of the visible portion of the junctional feet.

Caswell and co-workers[17,52-54] have also found the heterogeneous population of vesicles upon gradient centrifugation of skeletal sarcoplasmic reticulum vesicles. Their preparation of heavy vesicles consists of dyads or triads which they also found to be enriched in calsequestrin. They have used fractionation of the isolated triads or dyads to obtain transverse tubular membrane vesicles.[17] Caswell's group has performed detailed ultrastructural studies on fragmented junctions. They have identified sarcoplasmic reticulum feet in the junction of isolated dyads[53] and found the electron-dense material in the terminal cisternae to be localized to the junctional sarcoplasmic reticulum membrane. Cadwell and Caswell[54] have identified a pair of high molecular weight proteins (300,000 to 325,000 daltons) that are able to transfer from isolated transverse tubules to terminal cisternae vesicles during reformation and breakage of the triad junction. They proposed that these proteins possibly function in spanning the gap between the transverse tubule membrane and the junctional sarcoplasmic reticulum membrane.

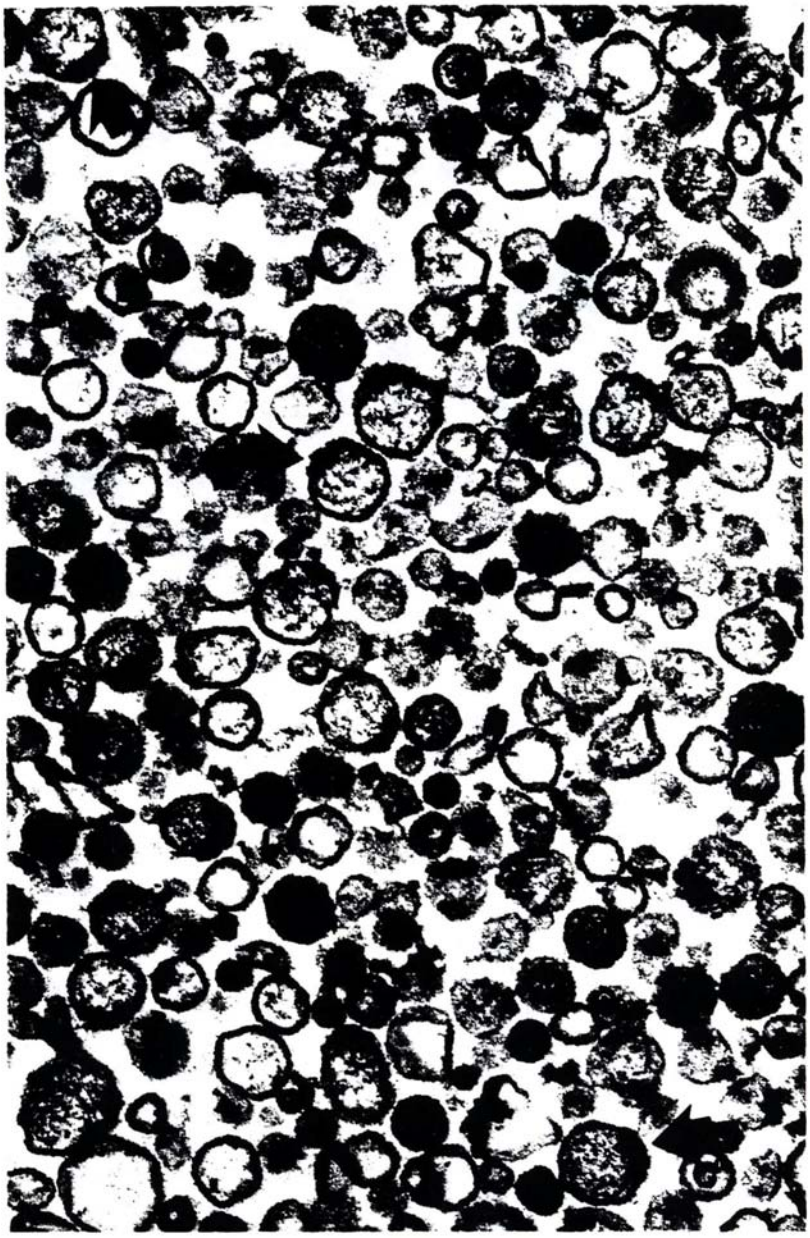


FIGURE 2. Thin section of heavy sarcoplasmic reticulum vesicles fixed in cacodylate. The electron-dense content of the vesicles is dispersed throughout the vesicle lumen. The cytoplasmic surface of the heavy vesicles is decorated by small projections. In certain vesicles (arrows) the projections have a periodicity and appearance of the junctional sarcoplasmic reticulum feet. (Magnification × 60,000.)

Fleischer and co-workers[55-57] have recently developed a procedure for the isolation of highly purified morphologically intact triads from rabbit skeletal muscle. The isolated triads consist of heavy sarcoplasmic reticulum vesicles and transverse tubule vesicles which have retained all the ultrastructural features observed in the intact muscle. This preparation of triads will be very useful in the in vitro study of Ca^{2+} release from the sarcoplasmic reticulum.

Jones and Cala[58] have shown that isolated sarcoplasmic reticulum vesicles from canine

Table 1
MAJOR SKELETAL SARCOPLASMIC RETICULUM PROTEINS

	Ca^{2+} + Mg^{2+} ATPase (Ca^{2+} pump)	Calsequestrin (Ca^{2+} binding protein)	Intrinsic glycoprotein (M_{55})
Function	Ca^{2+} transport	Ca^{2+} storage	N.K. (ATP binding site)
Molecular weight	105,000	63,000 (44,000)[a]	53,000
Percent of total protein	60–70	15–25	10–15
Ca^{2+} binding (mol/mol)	Yes (2)	Yes (40–50)	?
In situ sarcoplasmic reticulum localization	Free	Terminal cisternae	Free
In vitro localization	Light and heavy vesicles	Heavy vesicles	Light and heavy vesicles
Membrane structure	Intrinsic, transmembrane	Extrinsic, luminal	Intrinsic, transmembrane
Endo H sensitive	No	No	Yes (53,000 → 49,000)
Stains-all staining	Pink	Blue	Pink

Note: N.K., not known.
[a] Apparent molecular weight by neutral SDS gel electrophoresis

cardiac muscle are also heterogeneous with respect to structure and function. They have shown that cardiac sarcoplasmic reticulum vesicles can be separated after Ca^{2+} loading into subpopulations which appear to originate from free sarcoplasmic reticulum and junctional sarcoplasmic reticulum. The junctional sarcoplasmic reticulum vesicles have been identified by their high calsequestrin content and sensitivity to ryanodine. Recently, Jones' group[59] has also isolated junctional sarcoplasmic reticulum vesicles from skeletal muscle which are sensitive to ryanodine and enriched in calsequestrin. They have found that both preparations of junctional sarcoplasmic reticulum contain a unique set of high molecular weight proteins (290,000 to 350,000 daltons) which are not found in the free sarcoplasmic reticulum.

IV. PROTEIN COMPOSITION OF SARCOPLASMIC RETICULUM VESICLES

The protein composition of sarcoplasmic reticulum vesicles isolated from rabbit skeletal muscle is given in Tables 1 and 2. The major protein components of the skeletal sarcoplasmic reticulum vesicles (isolated according to Campbell and MacLennan[60]) are Ca^{2+} + Mg^{2+} ATPase[41] (105,000 daltons), calsequestrin[61] (63,000 daltons), and the 53,000-dafton glycoprotein[60] (Table 1 and Figure 3; see color plate). Ca^{2+} + Mg^{2+} ATPase accounts for approximately 60 to 70% of the total sarcoplasmic reticulum protein.[14] Calsequestrin and the 53,000-dalton glycoprotein account for 20 to 30% of the total protein, while minor protein components of the sarcoplasmic reticulum vesicles account for the remaining 5 to 20% of the total protein. The minor protein components of sarcoplasmic reticulum vesicles that have been clearly identified as originating from the skeletal sarcoplasmic reticulum membrane system are proteolipid (6,000 to 12,000),[62] calmodulin (17,000 daltons),[63-64] intrinsic 30,000-dalton protein,[38,65] extrinsic 34,000- and 38,000-dalton proteins.[38,65] high affinity Ca^{2+} binding protein,[66] 60,000-dalton phosphoprotein,[63,64] 160,000-dalton glycoprotein,[60] 170,000-dalton protein,[67] and 300,000- to 350,000-dalton protein[54,59] (Table 2). It should be noted that although these proteins are referred to as minor components of the sarcoplasmic reticulum vesicles, they are possibly very important to sarcoplasmic reticulum function. It is likely that the proteins which are responsible for excitation-contraction cou-

Table 2
MINOR SKELETAL SARCOPLASMIC RETICULUM PROTEINS

	Proteolipid	Calmodulin	Intrinsic 30,000 daltons	Extrinsic 34,000 and 38,000 daltons	High affinity Ca^{2+} binding protein	60,000 daltons phosphoprotein	160,000 daltons glycoprotein	170,000 daltons protein	300,000—350,000 daltons proteins
Function	N.K.	N.K.	N.K. (feet component?)	Feet components	N.K.	N.K. (Calmodulin–dependent phosphoprotein)	N.K. (ATP binding site)	N.K. (Ca^{2+} storage?)	Junctional component (calmodulin binding)
Molecular weight	6,000—12,000	17,000	30,000	34,000, 38,000	56,000 (55,000)[a]	60,000	160,000	170,000 (160,000)[a]	300,000—350,000
Ca^{2+} binding (mol/mol)	N.D.	Yes (4)	N.D.	N.D.	Yes (1)	N.D.	Yes	Yes	N.D.
In situ sarcoplasmic reticulum localization	N.D.	N.D. (junctional?)	N.D.	N.D.	N.D.	N.D.	Free	N.D.	N.D.
In vitro localization	Light and heavy vesicles	N.D.	Heavy vesicles	Heavy vesicles	Transverse tubular vesicles	N.D.	Light and heavy vesicles	Heavy vesicles	Heavy (junctional) vesicles
Membrane structure	Intrinsic	Extrinsic	Intrinsic	Extrinsic, cytoplasmic	Extrinsic	Intrinsic, cytoplasmic	Intrinsic, transmembrane	N.D. (Transmembrane?)	N.D. (transmembrane?)
Endo H sensitive	N.D.	No	No	No	No	No	Yes (160,000→155,000)	No	No
Stains-all staining	N.D.	Blue	Pink	Pink	Pink	N.D.	Blue	Purple	Pink

Note: N.D., not determined; N.K., not known.

[a] Apparent molecular weight by neutral SDS gel electrophoresis.

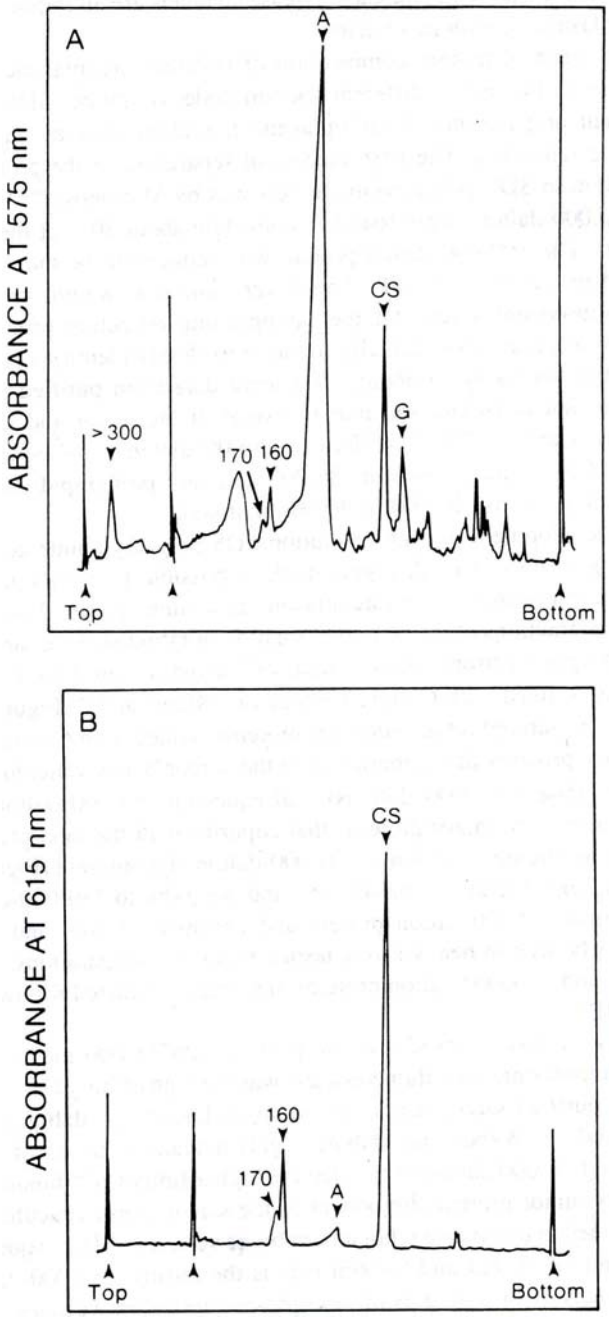


FIGURE 4. Densitometric scans of sarcoplasmic reticulum proteins stained with Coomassie blue (A) or "Stains-all"® (B) following separation by SDS gel electrophoresis (5 to 15% gradient of acrylamide). Scans were performed on a Gilford spectrophotomelerat 575 nm for Coomassie blue and 615 nm for "Stains-all"® stained gels. The top and bottom of the gel are indicated along with the top of the running gel (arrowhead) A. (Ca^{2+} + Mg^{2+} ATPase (105,000 daltons); CS. calsequestrin (63,000 daltons); G. glycoprotein (53,000 daltons). 160, 160,000-dalton glycoprotein; 170. 170,000-dalton protein; >300, 300,000- to 350,000-dalton proteins.

pling, junctional feet, and Ca^{2+} release channels are all minor protein components of isolated sarcoplasmic reticulum vesicles.

The reported protein composition of isolated sarcoplasmic reticulum vesicles has varied because of the use of different sodium dodecyi sulfate (SDS) gel electrophoresis systems, different preparations of sarcoplasmic reticulum vesicles, and different sources of sarcoplasmic reticulum. The first successful separation of the proteins of isolated sarcoplasmic reticulum in SDS polyacrylamide gels was by Martonosi.[68,69] Proteins of 100,000, 60,000, and 50,000 daltons were found to constitute about 50% of the total sarcoplasmic reticulum protein. The 100,000-dalton protein was deduced to be the ATPase since it was phosphorylated by gamma-^{32}P-ATP. The lower molecular weight proteins were not identified. In most subsequent studies of the sarcoplasmic reticulum protein composition, three major proteins were reported. Initially, in order to clearly identify a protein on SDS polyacrylamide gels, it is necessary to obtain correlative data from purified proteins. MacLennan[41,61,62,66] was the first to isolate and purify several of the major and minor sarcoplasmic reticulum proteins: Ca^{2+} + Mg^{2+} ATPase (105,000 daltons); calsequestrin (44,000 daltons); high affinity Ca^{2+} binding protein (56,000 daltons); proteolipid (6,000 to 12,000 daltons); and the acidic proteins (20,000 to 30,000 daltons).

The development of high resolution SDS polyacrylamide gel electrophoresis and the use of slab gels instead of disc gels made it possible to clearly identify the major and minor protein components of the sarcoplasmic reticulum. Figure 3 shows the protein composition of various fractions obtained in the isolation of rabbit skeletal sarcoplasmic reticulum vesicles using the gel electrophoresis system of Laemmli[70] on 5 to 15% gradient slab gels which have been stained with Coomassie blue or "Stains-all®". Figure 4 shows the densitometric scans of the sarcoplasmic reticulum proteins stained with Coomassie blue or "Stains-all®". The major proteins that copurify with the sarcoplasmic reticulum vesicles are the Ca^{2+} + Mg^{2+} ATPase (105,000 daltons), calsequestrin (63,000 daltons), and the 53,000-dalton glycoprotein. The minor proteins that copurify with the sarcoplasmic reticulum vesicles are calmodulin (Figure 3, bottom), 160,000-dalton glycoprotein (Figures 3 and 4A, B), 170,000-dalton protein (Figures 3 and 4A. B), and 300,000- to 350,000-dalton proteins (Figure 4A). The intrinsic 30,000-dalton protein and extrinsic 34,000- and 38,000-dalton proteins are more clearly seen in heavy sarcoplasmic reticulum preparations. Calsequestrin and both the 160,000- and 170,000-dalton proteins are better visualized following "Stains-all®" staining (Figure 4B).

Initially, it was believed that the protein with 55,000 mol wt, on Weber and Osbom[71] gels of sarcoplasmic reticulum vesicles, was the high affinity Ca^{2+} binding protein.[66] Analysis of highly purified sarcoplasmic reticulum on Laemmli[70] slab gels revealed that the 55,000-dalton band on Weber and Osbom[71] gels actually consists of three proteins of 53,000, 55,000, and 56,000 daltons[38] and that the high affinity Ca^{2+} binding protein (56,000 daltons) was a very minor protein component of the sarcoplasmic reticulum.[72] It actually was found to be enriched in transverse tubular membrane vesicles.[72] The major component of the 55,000-dalton band on Weber and Osbom gels is the intrinsic 53,000-dalton glycoprotein. Densitometric analysis of SDS gels of sarcoplasmic reticulum vesicles following endoglycosidase H (Endo H) digestion has shown that greater than 90% of the 55,000-dalton band is made up of the intrinsic 53,000-dalton glycoprotein.

Another fact that has led to confusion about sarcoplasmic reticulum protein composition is the apparent molecular weight of calsequestrin which is dependent on the pH of the gel electrophoresis system. Calsequestrin has an apparent 44,000[61] mol wt when determined by SDS gel electrophoresis at neutral pH (Weber and Osbom system), and an apparent 63,000[42] mol wt when determined at alkaline pH (Laemmli system). This property of calsequestrin has been used to develop a two-dimensional SDS gel electrophoresis system that can identify calsequestrin by its change in mobility and result in its purification.[72,73] Figure 5 shows the

two-dimensional analysis of the skeletal sarcoplasmic reticulum proteins that were first separated by the Weber and Osbom system[71] and then by the Laemmli system.[70] Calsequestrin (CS) can be seen to change its mobility and fall off the diagonal in both the Coomassie blue and "Stains-all®" stained gels.

The glycoprotein composition of skeletal sarcoplasmic reticulum vesicles has been analyzed using concanavalin A (^{125}I-Con-A) binding[72] and endoglycosidase H digestion.[60,73] Con-A binding to SDS slab gels has revealed four glycoproteins with apparent molecular weights of 53,000, 60,000, 63,000, and 160,000 in sarcoplasmic reticulum vesicles. The 53,000-dalton glycoprotein that bound the largest amount of ^{125}I-Con-A was found to be a major protein constituent of skeletal sarcoplasmic reticulum. The 63,000-dalton Con-A binding protein was identified as calsequestrin. The 160,000-dalton glycoprotein was identified as a minor protein component of the sarcoplasmic reticulum.

Endo-β-*N*-acetylglucosaminidase H (Endo H) digestion of sarcoplasmic reticulum proteins has been very useful in the identification and characterization of sarcoplasmic reticulum glycoproteins.[67,73] Endo H splits the chitobiosyi core of N-lined glycoproteins and has a specificity for high mannose sugar chains. The effect of Endo H digestion on skeletal muscle sarcoplasmic reticulum proteins, which had been solubilized with deoxycholate, is shown in Figure 6. The 53,000-dalton glycoprotein is reduced to 49,000 daltons after Endo H digestion (Figure 6A). The 160,000-dalton glycoprotein is reduced to 155,000 daltons after Endo H digestion (Figure 6A). "Stains-all®" staining of the Endo H-digested proteins shows that the 160,000-dalton protein, which stained blue with "Stains-all®", is the 160,000-dalton Endo H-sensitive glycoprotein (Figure 6B).

V. STRUCTURE AND FUNCTION OF SARCOPLASMIC RETICULUM PROTEINS

A. Major Protein Components

I. Ca^{2+} + Mg^{2+} ATPase

Ca^{2+} + Mg^{2+} ATPase consists of a single polypeptide that is able to couple the hydrolysis of one molecule of ATP to the active transport of two Ca^{2+} ions across the sarcoplasmic reticulum membrane,[14-16] It contains one active site, one phosphorylation site, and two high affinity Ca^{2+} binding sites per ATPase molecule.[14] IA The purified ATPase possesses all of the properties attributed to the Ca^{2+} transport system and when reconstituted into phospholipid vesicles it will transport Ca^{2+} against a concentration gradient.[74,75] Extensive studies have been carried out on the structure and function of the Ca^{2+} + Mg^{2+} ATPase. Recent reviews of the structure and function of the Ca^{2+} + Mg^{2+} ATPase have been written by Ikemoto,[16] deMeis and Vianna,[76] Hasselbach,[44] Tada et al.,[15] and MacLennan and Reithmeier.[77] Therefore, this chapter will not deal with a detailed description of the structure and function of the Ca^{2+} + Mg^{2+} ATPase.

MacLennan[41] was the first to successfully purify the Ca^{2+} + Mg^{2+} ATPase with high ATPase activity from rabbit skeletal muscle sarcoplasmic reticulum. He treated sarcoplasmic reticulum vesicles with a low concentration of deoxycholate and 1 M KCl in order to solubilize the extrinsic sarcoplasmic reticulum proteins without solubilizing the ATPase. The ATPase was then solubilized with a higher concentration of deoxycholate and then purified with ammonium acetate fractionation. Ca^{2+} + Mg^{2+} ATPase purified using the procedure of MacLennan[41] consists of one protein of approximately 105,000 daltons and has ATPase activities from 28 to 35 μmol/min/mg. The Ca^{2+} + Mg^{2+} ATPase has also been isolated using various other detergents.[75,78,79] Ikemoto[78] has used Triton® X-100 to solubilize the ATPase and gel filtration to purify the ATPase. Warren et al.[75] solubilized the ATPase with deoxycholate and then purified the ATPase by sucrose gradient centrifugation. Banerjee

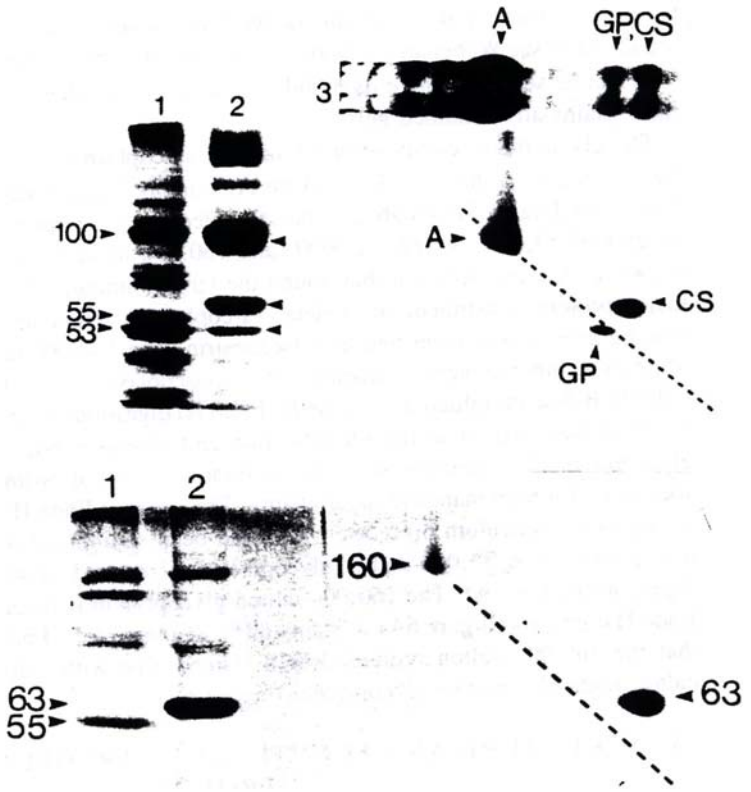


FIGURE 5. Two-dimensional gel electrophoresis of skeletal muscle sarcoplasmic reticulum proteins. Sareoplasmic reticulum from rabbit skeletal muscle was subjected to two-dimensional gel electrophoresis[72] and stained with Coomassie blue (top) or "Stains-all"® (bottom) Calsequestrin (CS) migrates off the diagonal (top), since it runs at 44,000 daltons in the horizontal dimension and 63,000 daltons in the vertical dimension. It also stained blue with "Stains-all"®® (bottom) The 53,000-dalton glycoprotein (GP). by contrast, fell slightly below the diagonal. The 105,000-dalton Ca^{2+} + Mg^{2+} ATPase (A) fell on the diagonal. Reference vertical gel lanes show cardiac sarcoplasmic reticulum (lane 1), skeletal sarcoplasmic reticulum (lane 2). and skeletal sarcoplasmic reticulum on a Weber and Osborn[71] gel (lane 3). Numbers on the ordinate and abscissa represent $M_r \times 10^{-3}$.

et al.[79] used octylglucoside in the presence of salt to isolate the ATPase from the sarcoplasmic reticulum.

Although the purified Ca^{2+} + Mg^{2+} ATPase of MacLennan[41] catalyzed Ca^{2+}-dependent ATP hydrolysis, phosphorylated intermediate formation, ATP-ADP exchange and ATP-^{32}P exchange, it was not able to sequester Ca^{2+} in an ATP-dependent manner. Packer[74] was first to develop a reconstitution procedure for the purified Ca^{2+} + Mg^{2+} ATPase His procedure involved the solubilization of the purified ATPase and exogenous phospholipid with cholate in the presence of 0.4 *M* phosphate. The detergent was slowly removed by dialysis and the ATPase and the phospholipid formed sealed vesicles containing phosphate The reconstituted ATPase was then able to sequester Ca^{2+} in an ATP-dependent manner. This procedure did require the presence of phosphate or another Ca^{2+} precipitating anion within the reconstituted vesicles. Warren et al.[75] were also able to reconstitute the purified ATPase even after the endogenous lipid of the ATPase was exchanged with synthetic lipid.

Reconstitution of the Ca^{2+} + Mg^{2+} ATPase without the addition of added phospholipid has been accomplished by Meissner and Fleischer- and Repke et al.[81] Isolated sarcoplasmic

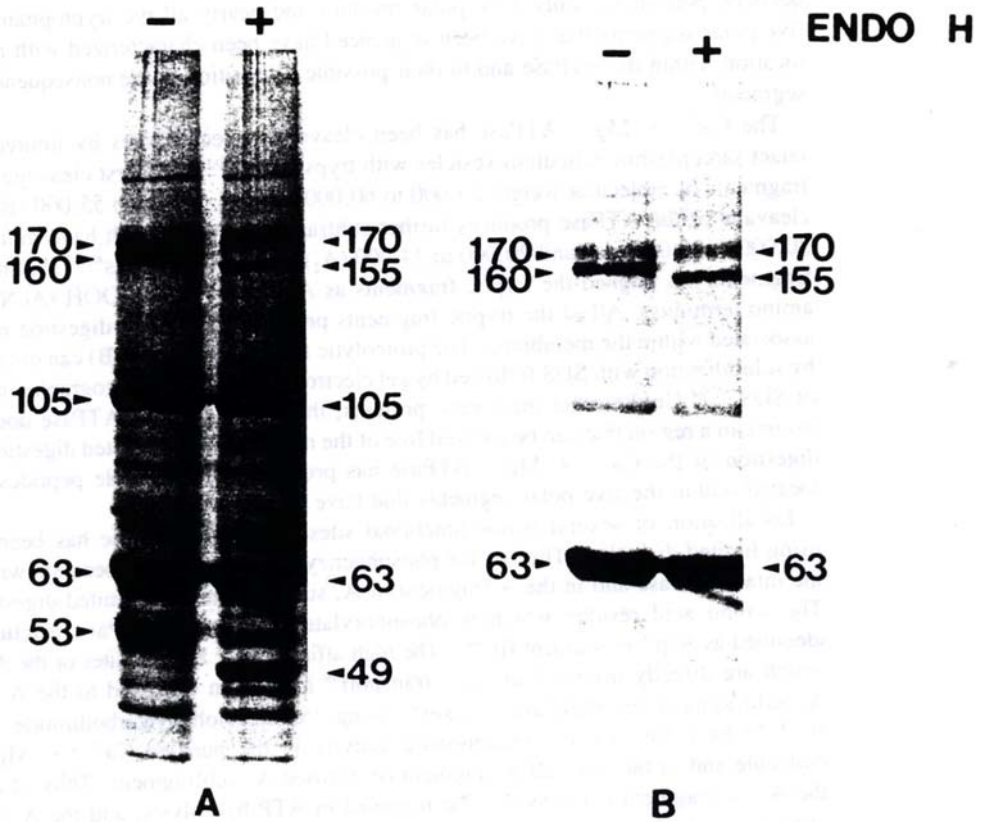


FIGURE 6. Endo H digestion of skeletal muscle sarcoplasmic reticulum proteins. Skeletal sarcoplasmic reticulum deoxycholate extracts were solubilized in 0.1% SDS and incubated in the presence (+) or absence (-) of Endo H (0.1 unit/mg) for 4 hr at 37°C.[60] Samples were analyzed by SDS-polyacrylamide gel electrophoresis and stained with Coomassie blue (A) or "Stains-all"® (B). Skeletal sarcoplasmic reticulum contained two glycoproteins of 53,000 and 160,000 daltons. which were reduced to 49,000 and 155,000 daltons respectively, following Endo H digestion.

reticulum vesicles were solubilized with detergent and then the detergent was removed by dialysis. In addition to the Ca^{2+} + Mg^{2+} ATPase. the reconstituted vesicles contained almost all of the 53,000- and 160,000-dalton glycoproteins that were present in the original sarcoplasmic reticulum. The reconstituted vesicles were able to transport and store Ca^{2+} in a similar manner as isolated sarcoplasmic reticulum vesicles in the presence or absence of a Ca^{2+} precipitating anion.

The structure of the Ca^{2+} + Mg^{2+} ATPase molecule has been extensively studied,[15,16,44,76,77] and information has been obtained about the primary structure of the ATPase and various functional sites within the ATPase. The Ca^{2+} + Mg^{2+} ATPase has an apparent molecular weight of 100,000 to 120,000 based on SDS-gel electrophoresis.[41,42,75,82] Us true molecular weight will probably not be known until it is fully sequenced. Approximately 43% of the amino acid residues within the ATPase molecule are polar.[42,82,83] This is consistent with more than half of the protein mass residing outside the sarcoplasmic reticulum membrane. Allen and Green[84-89] have sequenced five polar segments of the ATPase (segments I to V), which represents about 60% of the protein. The remaining 40% of the ATPase that has not been sequenced is most likely tightly associated with the membrane. The nonse-

quenced portion has only 18% polar residues and nearly all the tryptophan residues. The five polar segments that have been sequenced have been characterized with respect to their location within the ATPase and to their possible disposition to the nonsequenced membrane segments.

The Ca^{2+} + Mg^{2+} ATPase has been cleaved at specific sites by limited digestion of intact sarcoplasmic reticulum vesicles with trypsin.[26,82,90,91] The first cleavage produces two fragments of molecular weight 50,000 to 60,000 (A) and 45,000 to 55,000 (B) The second cleavage of the ATPase produces further subfragments of A which have molecular weight 30,000 to 33,000 (A_1) and 20,000 to 24,000 (A_2). Sequence analysis[89,92,91] of the proteolytic fragments has aligned the tryptic fragments as $AcNH_2$-A_2-A_1-B-COOH ($AcNH_2$-acetylated ammo terminus). All of the tryptic fragments produced by limited digestion remain tightly associated within the membrane. The proteolytic fragments (A_1, A_2, B) can only be separated by solubilization with SDS followed by gel electrophoresis or chromatography in the presence of SDS.[92,94] Unlike other membrane proteins, the Ca^{2+} + Mg^{2+} ATPase does not appear to contain a region that can be cleaved free of the membrane with limited digestion Extensive digestion of the Ca^{2+} + Mg^{2+} ATPase has produced water-soluble peptides[95] which are located within the five polar segments that have been sequenced.

Localization of several major functional sites within the ATPase has been determined using limited digestion. The site for phosphoenzyme formation has been shown to reside in the intact ATPase and in the A fragment or A_1 subfragment after limited digestion.[82,91,94,95] The ammo acid residue which is phosphorylated during the ATPase reaction has been identified as Asp^{26} in segment III.[89,92] The high affinity Ca^{2+} binding sites of the ATPase[42,96,97] which are directly involved in Ca^{2+} transport[98] have been localized to the A fragment or A_2 subfragment by pick and Racker[99] using ^{14}C-dicyclohexylcarbodiimide. Shamoo et al.[100-102] have found Ca^{2+} ionophoretic activity in the purified Ca^{2+} + Mg^{2+} ATPase molecule and in the purified A fragment or purified A_2 subfragment. Thus, it appears that the A_1 subfragment contains the site involved in ATP hydrolysis, and the A_2 subfragment contains the high affinity Ca^{2+} binding sites involved in Ca^{2+} transport.

The localization of the Ca^{2+} + Mg^{2+} ATPase in skeletal and cardiac muscle has been determmed by Jorgensen et al.[103-106] Indirect immunofluorescent staining has shown that the Ca^{2+} + Mg^{2+} ATPase was rather uniformly distributed throughout the sarcoplasmic reticulum. The ultrastructural localization of the Ca^{2+} + Mg^{2+} ATPase showed that the ATPase was concentrated in the longitudinal sarcoplasmic reticulum and in the nonjunctional regions of the terminal cistemae. The ATPase was absent from the junctional sarcoplasmic reticulum membrane as well as other membrane systems in skeletal muscle. The density of the Ca^{2+} + Mg^{2+} ATPase in the sarcoplasmic reticulum membrane in fast myofibers is approximately two times higher than in slow myofibers.

2. Calsequestrin

The second major protein constituent of the sarcoplasmic reticulum is calsequestrin Calsequestnn is a very acidic protein which binds some 900 nmol Ca^{2+} per milligram of protein (43 mol/mol), and therefore is probably responsible for Ca^{2+} storage within the sarcoplasmic reticulum. The structure and function of skeletal muscle calsequestrin have been recently reviewed by MacLennan et al.[107]

Calsequestrin was first isolated from skeletal muscle sarcoplasmic reticulum by MacLennan and Wong.[61] Extraction of sarcoplasmic reticulum vesicles with low concentrations ofdeoxycholate and 1 *M* KCl resulted in the solubilization of calsequestrin from the membrane. Calsequestrin was then purified by chromatography on DEAE cellulose, hydroxylapame- and Sephadex® G-200. Ikemoto et al.[108] also isolated calsequestrin using Triton® X-100 solubilization followed by Ca^{2+} precipitation. The amino acid composition of calsequestrin is extremely acidic.[61,109] It contains about 37% acidic amino acid residues (aspartic

and glutamic) and only 7% basic residues. Since only a small portion of the amino acid sequence of calsequestrin has been determined.[107] relatively little is known about the primary structure of the Ca^{2+} binding sites in calsequestrin.

The most accurate determination of the molecular weight of skeletal muscle calsequestrin has been made by Cozens and Reithmeier.[110] They subjected calsequestrin to physiochemical analysis using SDS gel electrophoresis, gel filtration, sedimentation, viscosity and circular dichroism techniques. They found that when calsequestrin was completely random coil in guanidine hydrochloride it had a molecular weight of 42,000 as determined by eel filtration Previous estimates of the molecular weight of calsequestrin[38,42,111-113] by gel filtration or SDS gel electrophoresis were inaccurate due to the extended structure of the protein. At alkaline pH, the extended structure of calsequestrin becomes even more asymmetric This is likely why calsequestrin has such a high apparent molecular weight on alkaline gel electrophoresis systems such as Laemmli.[70] Calsequestrin preparations from various muscle sources appear to have different molecular weights when determined by alkaline gel elec trophoresis, but their molecular weight is always approximately 44,000 when determined by neutral SDS gel electrophoresis system[73] (Figure 7).

The property of calsequestrin which allows its apparent molecular weight to be shifted by pH has been used to establish a two-dimensional gel electrophoresis system in which calsequestrin can be easily identified[72,73] (Figure 5). Another property ofcalsequestrin which allows it to be identified on SDS gels is its dark blue staining upon binding the cationic carbocyanine dye "Stains-all®"[67] (Figure 3). This property, when combined with the change in apparent molecular weight on two-dimensional gels, makes it possible to identify calsequestrin readily in a mixture of proteins[72,73] (Figure 5).

Ca^{2+} binding to skeletal muscle calsequestrin has been measured by MacLennan et al.,[61,109,110] Ikemoto et al.,[112,113] and Meissner et al.[42] The maximal amount of Ca^{2+} bound by calsequestrin was 900 to 1000 nmol Ca^{2+}/mg of protein. In the absence of salt calsequestrin binds Ca^{2+} with a dissociation constant between 4 to 40 μ*M*. while in the presence of 100 m*M* KCl, the dissociation constant for Ca^{2+} binding is shifted to 800 to 1300 μ*M* The specificity of Ca^{2+} binding to calsequestrin is rather low with most cations being able to compete for the Ca^{2+} binding sites. Ca^{2+} binding to calsequestrin results in extensive conformational changes.[110-113] These conformational changes in calsequestrin have been observed by various spectral measurements.[110-113] The protein appears to go from a highly extended structure (R_s = 45 Å) to a much more compact structure (R_s = 35 Å) upon Ca^{2+} binding.[110] This apparent change in the shape of calsequestrin has even been seen in the ultrastructure of isolated heavy sarcoplasmic reticulum vesicles.[38] The electron-dense content within heavy sarcoplasmic reticulum vesicles, which contain large quantities of endogenous Ca^{2+}, is compact and localized to the junctional membrane (Figure 1C). The electron-dense content becomes dispersed when the heavy vesicles are treated with KCl to remove the endogenous Ca^{2+} (Figure 1B). Even in the presence of SDS, Ca^{2+} can induce conformational changes in calsequestrin which result in a shift in the mobility of calsequestrin on SDS gels[67] (Figure 8). Similar changes are also seen in partial degraded calsequestrin or in the well-known calcium binding proteins calmodulin or troponin (Figure 8; see color plate). Finally at very high Ca^{2+} concentrations (greater than 1 m*M*), calsequestrin will precipitate in the form of a Ca^{2+}-calsequestrin complex.[61,108]

Cardiac sarcoplasmic reticulum vesicles appear to contain a more complex composition than skeletal sarcoplasmic reticulum vesicles and for several years calsequestrin was reported not to be a component of cardiac sarcoplasmic reticulum.[114] Campbell et al.[73] were the first to identify and punfy calsequestrin from canine cardiac sarcoplasmic reticulum 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. Cardiac calsequestrin bound approximately 300 nmol of Ca^{2+} per milligram

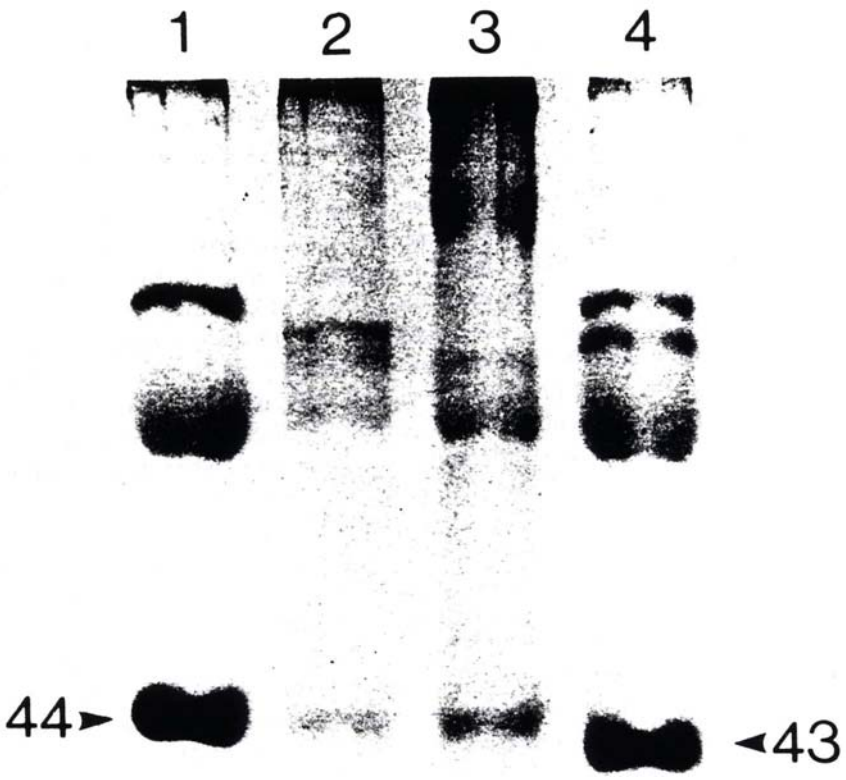


FIGURE 7. Analysis of sarcoplasmic reticulum vesicles from several different skeletal muscle sources. Sarcoplasmic reticulum vesicles were isolated from rabbit skeletal muscle (1), canine cardiac muscle (2), chicken skeletal muscle (3), and rat skeletal muscle (4), and proteins were separated by Weber and Osborn[71] SDS gel electrophoresis and stained with "Stains-all"[®67] The apparent molecular weights of calsequestrin was 44,000 (rabbit), 44,000 (canine cardiac). 44,000 (chicken), and 43,000 (rat skeletal). Numbers on the right represent $M_r \times 10^{-3}$.

protein, and its amino acid composition revealed that glutamic and aspartic acid constituted approximately 32% of the amino acid residues in the protein. Apparent molecular weight of canine cardiac calsequestrin was 55,000 when measured in alkaline SDS gels and 44,000 when measured in neutral SDS gels. Cardiac calsequestrin stains blue with "Stains-all"® and has been identified as a component of ryanodine-sensitive cardiac sarcoplasmic reticulum. Unlike skeletal muscle calsequestrin, cardiac calsequestrin was sensitive to Endo H. indicating that it contained a high mannose oligosaccharide.

Cala and Jones[115] have isolated cardiac skeletal calsequestrin using hydrophobic chromatography. Calsequestrin was extracted from the membrane with 0.1 *M* sodium carbonate at pH 11.4, then adsorbed to phenyl-Sepharose in the presence of EGTA and 0.5 *M* NaCl.[115] Calsequestrin is then eluted from the phenyl-Sepharose column with 1 m*M* Ca^{2+}. Thus, it appears that calsequestrin will interact with the hydrophobic column in the absence of Ca^{2+} ions and will not interact with the column in the presence of Ca^{2+} ions. Ultraviolet absorption and tryptophan fluorescence changes have indicated that aromatic residues in calsequestrin move from a polar surface to a hydrophobic interior when Ca^{2+} is bound to calsequestrin.[110-113] These aromatic residues could be the residues in calsequestrin that are interacting with the phenyl-Sepharose in the absence of Ca^{2+} ions.

Recently, Campbell et al.[116] have developed a direct purification procedure for skeletal or cardiac calsequestrin which does not require the isolation of sarcoplasmic reticulum

membrane vesicles. The procedure involves the extraction of calsequestrin directly from the muscle using an acidic extraction buffer, followed by precipitation of calsequestrin and DEAE chromatography. At this stage, calsequestrin is greater than 95% pure. The main advantage of this new procedure is the high yield (150 to 200 mg of skeletal calsequestrin per kilogram skeletal muscle or 20 to 40 mg of cardiac calsequestrin per kilogram of cardiac muscle), which is about 10 times the values previously obtained.[61,73,115]

Localization of calsequestrin in skeletal muscle[104,119-121] and in isolated sarcoplasmic reticulum vesicles[38,51,61] has been extensively studied. It is now generally agreed that calsequestrin is localized in the lumen of the terminal cisternae of the sarcoplasmic reticulum. Localization of calsequestrin to the lumen of the sarcoplasmic reticulum is supported by the following observations:

1. Calsequestrin is only released after disruption of the sarcoplasmic reticulum membrane by detergents,[61] by divalent cation chelators,[117] and by treatment with sodium carbonate at high pH[115]
2. Antibodies to calsequestrin do not aggregate sarcoplasmic reticulum vesicles[94]
3. Limited digestion of sarcoplasmic reticulum vesicles does not cleave calsequestrin[26,72]
4. Calsequestrin is not labeled with impenetrable complex of cycloheptaamylose-fluorescamine[72,118]

Jorgensen et al.[119] were the first to prove the luminal localization of calsequestrin by indirect immunoferritin labeling of ultrathin frozen sections of rat skeletal muscle.

Localization of calsequestrin to the terminal cisternae of the sarcoplasmic reticulum was first proposed by Meissner[51] from his studies of light and heavy sarcoplasmic reticulum vesicles. He suggested that the electron-dense material within the heavy vesicles was calsequestrin and/or M_{55}, (53,000-dalton glycoprotein), and that these proteins were localized in the terminal cisternae where the electron-dense matrix was localized. Campbell et al.[38] and Caswell and co-workers[17] have suggested that the electron-dense content in the heavy sarcoplasmic reticulum vesicles was calsequestrin, since heavy vesicles were enriched in calsequestrin, while several proteins of approximately 55,000 dalton were found in both the light and heavy vesicles.

Localization of calsequestrin to the terminal cisternae of the sarcoplasmic reticulum has been proven by Jorgensen et al.[104,119-121] Using immunofluorescent staining of cryostat-sectioned rat skeletal muscle, Jorgensen et al. [104] showed that calsequestrin was confined to the region of the A-I junction. The ultrastructural localization of calsequestrin in rat skeletal muscle was determined by indirect immunoferritin labeling of ultrathin frozen sections. [119] Calsequestrin was found in the lumen of the transversely and longitudinally oriented terminal cisternae, but was absent from most of the longitudinal sarcoplasmic reticulum. A fraction of calsequestrin was also located in vesicular structures in the central region of the I band. [119]

The localization of calsequestrin to the terminal cisternae of the sarcoplasmic reticulum suggests that in addition to lowering the luminal-free Ca^{2+} concentrations, calsequestrin also concentrates Ca^{2+} in the terminal cisternae. Somlyo et al.[12,13] have shown that Ca^{2+} is localized in the terminal cisternae in resting skeletal muscle and is released from the terminal cisternae during tetanus. Therefore, a major function of calsequestrin may be to concentrate Ca^{2+} in the region of the sarcoplasmic reticulum which is involved in Ca^{2+} release. The Ca^{2+}-calsequestrin complex in heavy sarcoplasmic reticulum vesicles is localized to the region of the junctional sarcoplasmic reticulum membrane[38] (Figure 1C). Calsequestrin may actually be attached to the junctional sarcoplasmic reticulum membrane since there appear to be fibrous strands of material which run from internal membrane structures in the area of the junctional feet to the matrix formed by calsequestrin.[24]

The recent localization of cardiac calsequestrin in chicken heart muscle or sheep Purkinje fibers is quite interesting since these heart cells contain no transverse tubular system and therefore no internal triads.[120,121] Calsequestrin was localized in the lumen of peripheral junctional sarcoplasmic reticulum, as well as in the lumen of membrane-bound structures present in the central region of the I band. It is likely that the calsequestrin containing structures present throughout the I band region of these muscle cells correspond to specialized regions of the free sarcoplasmic reticulum in the I band[120,121] called corbular sarcoplasmic reticulum. These results imply that two structurally different regions of the sarcoplasmic reticulum contain calsequestrin and could function as Ca^{2+} storage sites in cardiac muscle and raise the possibility that Ca^{2+} storage and/or release from these two sites might be regulated differently.

3. Intrinsic 53,000-Dalton Glycoprotein

The third major protein constituent of the sarcoplasmic reticulum is the intrinsic 53,000-dalton glycoprotein.[60,72] Early studies on the protein composition of sarcoplasmic reticulum vesicles using neutral gel electrophoresis claimed the high affinity Ca^{2+} binding protein was the third major protein constituent of the sarcoplasmic reticulum.[66] Recent analysis of sarcoplasmic reticulum vesicles on high resolution SDS gel clearly identifies the high affinity Ca^{2+} binding protein as a very minor protein component of the sarcoplasmic reticulum.[72] The major protein with a molecular weight in the range of 55,000 is the intrinsic 53,000-dalton glycoprotein.[60] Meissner and Fleischer[42,51] have previously referred to the major sarcoplasmic reticulum protein of approximately 55,000 daltons as M_{55} in their publications.

The glycoprotein nature of the 53,000-dalton protein was first identified using ^{125}I-Con. A binding to sarcoplasmic reticulum proteins separated by SDS gel electrophoresis.[72] The largest amount of ^{125}I-Con-A was bound to a 53,000-dalton protein and binding to this protein could be blocked by alpha-methyl-D-mannoside. Endo H digestion of sarcoplasmic reticulum proteins has provided the strong evidence for the identification of the glycoprotein nature of the 53,000-dalton protein. The apparent molecular weight of the glycoprotein was reduced from 53,000 to 49,000 after digestion with Endo H, and its reactivity with ^{125}I-Con-A (Figures 6 and 9). Endo H digestion of sarcoplasmic reticulum proteins has also showed that the oligosaccharide moiety of the 53,000-dalton glycoprotein was located in the lumen of the sarcoplasmic reticulum.[60] Cycloheptaamylose-fluorescamine labeling has shown that the 53,000-dalton glycoprotein is at least partially exposed on the cytoplasmic surface of the sarcoplasmic reticulum.72 Campbell et al.[122,123] have also shown that several monoclonal antibodies to the 53,000-dalton glycoprotein react with intact sarcoplasmic reticulum vesicles. Therefore, the 53,000-dalton glycoprotein must have several sites that are exposed on the cytoplasmic face of the sarcoplasmic reticulum. Gutweniger and Montecucco,[124] using photoreactive lipids, have recently provided the first direct evidence that the 53,000-dalton glycoprotein interacts with the hydrophobic domain of the sarcoplasmic reticulum membrane. Therefore, the 53,000-darton glycoprotein has been shown to be exposed to the cytoplasm, the lipid bilayer-and the lumen of the sarcoplasmic reticulum.

The intrinsic 53,000-dalton glycoprotein has been purified and characterized by Campbell and MacLennan.[60] The 53,000-dalton glycoprotein was first separated from the Ca^{2+} + Mg^{2+} ATPase by solubilization with low concentrations of deoxycholate in the presence of 1*M* KCl. The 53,000-dalton glycoprotein was then purified by two successive gel filtration steps. In the presence of deoxycholate, the glycoprotein was soluble, but in an aggregated or oligomeric form which elutes in the void volume of a gel filtration column When subsequently refiltered in the presence of sodium dodecyl sulfate, the glycoprotein eluted in pure form as a monomer. The SDS purified glycoprotein contains 48% nonpolar amino acids, 22% aspartic and glutamic amino acids, and about 12% lysine and arginine. It also contains 4 mol of glucosamine and 18 mol of mannose per mole of protein suggesting that it contains two chains of $(GlcNAc)_2$:$(Man)_9$.

The 53,000-dalton glycoprotein has also been partially purified without SDS using deoxycholate extracts and Con-A Sepharose.[60] The 53,000-dalton glycoprotein interacted strongly with Con-A Sepharose and was completely removed from deoxycholate extracts of sarcoplasmic reticulum by Con-A Sepharose 4B.[60] Elution with alpha-methyl-D-mannoside resulted in the copurification of 53,000- and 160,000-dalton glycoproteins[60] (Figure 9). Endo H digestion of the copurified 53,000- and 160,000-dalton glycoproteins reduced the molecular weight of each protein by 4,000 to 5,000 daltons, suggesting each has two chains of $(GlcNAc)_2$:$(Man)_9$. The 53,000-dalton glycoprotein was also purified by Chiesi and Carafoli[63] using CAPP-Sepharose 4B (trifluoperazine affinity column). The 53,000-dalton glycoprotein adsorbed to the affinity column in the presence of Ca^{2+} and was eluted in almost pure form with EGTA.

A 53,000-dalton intrinsic glycoprotein has also been identified in cardiac sarcoplasmic reticulum using ^{125}I-Con-A binding, Endo H digestion, and indirect antibody staining with skeletal muscle glycoprotein antiserum.[73] It has a 53,000 mol wt and its size is reduced to 49,000 daltons by Endo H digestion. In addition to identifying the 53,000-dalton intrinsic glycoprotein in canine cardiac sarcoplasmic reticulum, it has also been identified, using Endo H and ^{125}I-Con-A probes, in chicken skeletal muscle, rat cardiac, and porcine skeletal muscle sarcoplasmic reticulum, suggesting that it is an invariant component of sarcoplasmic reticulum from all muscle sources.[73]

Alteration in the content of 55,000-dalton protein in sarcoplasmic reticulum vesicles after denervation or following chronic nerve stimulation has been observed.[125,126] The quantity of 55,000-dalton protein (probably intrinsic 53,000-dalton glycoprotein) is increased relative to the Ca^{2+} + Mg^{2+} ATPase after denervation.[125] Chronic nerve stimulation also produces an increase in the 55,000 daltons.[126] It has been suggested that this change in protein composition accompanies a change from fast to slow twitch muscle fibers in both cases.

Meissner[18] originally found the M_{55} protein, which has been identified as intrinsic 53,000-dalton glycoprotein, to fractionate with heavy sarcoplasmic reticulum vesicles. Other investigators[17,38,127] have found the 55,000-dalton protein to fractionate with both light and heavy sarcoplasmic reticulum vesicles. Michalak et al.[72] using ^{125}I-Con-A binding, showed that the 53,000-dalton glycoprotein was found in both light and heavy sarcoplasmic reticulum. Recently, Campbell et al.[123] have used monoclonal antibodies to the 53,000-dalton glycoprotein to demonstrate that it is found in both light and heavy sarcoplasmic reticulum vesicles. Since the light sarcoplasmic reticulum vesicles are believed to be derived from the longitudinal reticulum, these results suggest that the 53,000-dalton glycoprotein is located throughout the sarcoplasmic reticulum and not confined to the terminal cisternae.

Localization of the intrinsic 53,000-dalton glycoprotein in skeletal muscle has been examined using indirect immunofluorescence.[128] Antiserum to the 53,000-dalton glycoprotein was produced in sheep and antibodies were then affinity purified. The affinity-purified antibodies bound to the 53,000- and 160,000-dalton glycoproteins and 49,000- and 155,000-dalton carbohydrate-free form of the glycoproteins. Indirect immunofluorescence suggested that the 53,000- and 160,000-dalton glycoproteins were confined to the region of the terminal cisternae. Thus, the biochemical localization data and the immunofluorescent localization data disagree. Hopefully, the ultrastructural localization of the 53,000-dalton glycoprotein using monoclonal antibodies and colloidal gold labeling will provide us with a defined localization.

The role of the 53,000-dalton glycoprotein in sarcoplasmic reticulum function is not completely understood. It was initially proposed that the 53,000-dalton glycoprotein might function in the regulation of Ca^{2+} transport, since it was found to be present in a constant ratio with the Ca^{2+} + Mg^{2+} ATPase in both light and heavy sarcoplasmic reticulum vesicles,[72] and since it was found to reconstitute with the Ca^{2+} + Mg^{2+} ATPase following detergent solubilization of sarcoplasmic reticulum vesicles.[72] It was also suggested the 53,000-

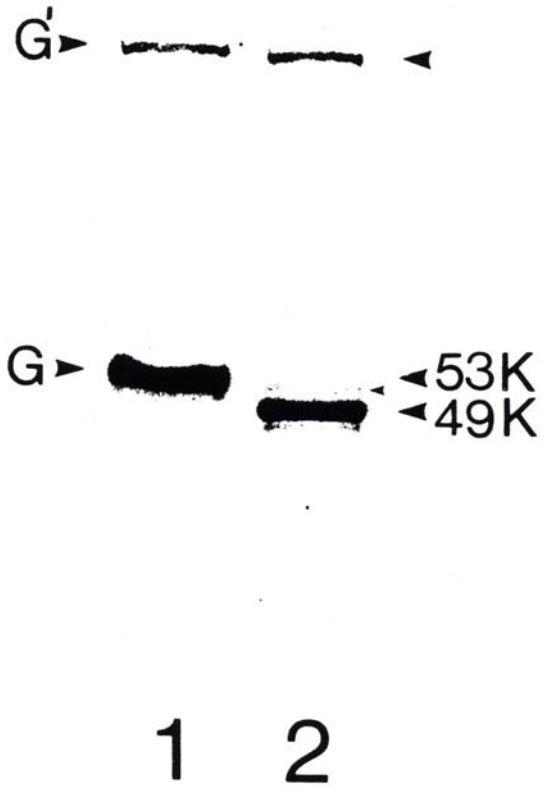


FIGURE 9. Endo H digestion of the Con-A Sepharose-purified 53,000- and 160,000-dalton glycoprotems. The 53,000-dalton glycoprotein was purified using Con-A Sepharose and then treated with 0.1 unit/mg of Endo H for 4 hr at 37°C in the presence of 0.1 % SDS Lane 1 the 53,000 (G) and 160,000-dalton (G′) glycoproteins; lane 2, following Endo H treatment; 49,000 and 155,000 daltons. respectively. Small arrow indicates intermediate in Endo H digestion of the 53,000-dalton glycoprotein. (Reproduced from Campbell, K. P. and MacLennan, D. H., *J. Biol. Chem*., 256, 4626, 1981. With permission of the American Society of Biological Chemists.)

dalton glycoprotein might function in the cotransport of anions during Ca^{2+} transport since the reconstituted vesicles with the glycoprotein did not require an internal Ca^{2+} precipitating anion for Ca^{2+} loading. Covalent labeling of sarcoplasmic reticulum vesicles with an inhibitor of anion transport[130] did not support this proposal. Finally it was also initially proposed that the 53,000-dalton glycoprotein of the sarcoplasmic reticulum might be comparable in structure and function to the 53,000-dalton glycoprotein subunit of the $Na^+ + K^+$ ATPase.[132] The major difference between the glycoproteins is that in contrast to the tight association found between the $Na^+ + K^+$ ATPase glycoprotein and the alpha subunit of the $Na^+ + K^+$ ATPase, the 53,000-dalton glycoprotein can be easily removed from the $Ca^{2+} + Mg^{2+}$ ATPase.

The role of the 53,000-dalton glycoprotein in the regulation of Ca^{2+} transport has also been suggested by the work of Chiesi and Carafoli[63] and Leonards and Kutchai.[131] Chiesi and Carafoli[63] have found that the Ca^{2+}-dependent inhibition of ATPase activity bv trifluoperazme was largely lost after removal of the glycoprotein from the ATPase. They proposed that mhibuion of Ca^{2+} uptake and Ca^{2+} ATPase activity by tritluoperazine was the result of the interaction of trifluoperazine with the 53,000-dalton givcoprotein. Leonards and Kutchai have reconstituted the $Ca^{2+} + Mg^{2+}$ ATPase with various amounts of glyco-

protein and then determined the degree of coupling between ATP hydrolysis and Ca^{2+} transport in the reconstituted vesicles. Their results suggest that the 53,000-dalton glycoprotein may be intimately involved in the regulation of coupling of ATP hydrolysis to Ca^{2+} transport. The possibility that the 53,000-dalton glycoprotein regulates Ca^{2+} transport is very attractive, but no direct evidence has been obtained which demonstrates regulation of the Ca^{2+} + Mg^{2+} ATPase by the 53,000-dalton glycoprotein.

One possible mechanism of regulation of sarcoplasmic reticulum function is through the action of membrane-bound protein kinases. 8-AZIDO-(^{32}P)-ATP (8-N_3-ATP) has been used by Campbell and MacLennan[129] to identify possible membrane-bound protein kinases in the sarcoplasmic reticulum. The radioactive 8-N_3-ATP was specifically incorporated into proteins of molecular weights 53,000, 105,000, and 160,000 daltons when intact sarcoplasmic reticulum vesicles were incubated with 0.14 to 1.6 μ*M* 8-N_3-ATP. Analysis of various membrane fractions during purification of the sarcoplasmic reticulum from muscle homogenates showed concomitant purifications of the 53,000-, 105,000-, and 160,000-dalton proteins that bound 8-N_3-ATP. The 8-N_3-ATP-labeled proteins had identical mobilities to the 53,000-dalton glycoprotein, the 105,000-dalton Ca^{2+} + Mg^{2+} ATPase, and the 160,000-dalton glycoprotein, respectively. 8-N_3-ATP labeling of deoxycholate extracts of sarcoplasmic reticulum resulted in the specific labeling of two proteins of 40,000 and 53,000 daltons. while calsequestrin was not labeled. The 53,000-dalton-labeled protein bound to Con-A Sepharose columns was eluted by alpha-methyl-D-mannoside. Endo H digestion of 8-N_3-ATP-labeled vesicles reduced the molecular weight of the 53,000- and 160,000-dalton 8-N_3-ATP-labeled proteins. Antibodies against the 53,000-dalton glycoprotein which cross-reacted with both glycoproteins also immunoprecipitated both the 53,000- and 160,000-dalton 8-N_3-ATP-labeled proteins. The binding of ATP with high affinity suggests that the 53,000- and 160,000-dalton glycoproteins might be membrane-bound protein kinases, but no experimental evidence has been obtained showing there is protein kinase activity in the purified glycoproteins.

B. Minor Protein Components

1. Low Molecular Weight Proteins

a. Proteolipid

The lowest molecular weight protein component of the sarcoplasmic reticulum is a proteolipid of approximately 6,000 to 12,000 daltons.[62] A proteolipid by definition is soluble in chloroform-methanol, but insoluble in the mixture upon addition of ethyl ether. MacLennan et al.[62] were the first to purify and characterize the sarcoplasmic reticulum proteolipid. They isolated the proteolipid in acidified chloroform methanol, precipitated it with ether, and purified it using silica gel chromatography followed by Sepharose LH-20 chromatography. The proteolipid was enriched in arginine and glutamic acid, and it had two molecules of fatty acid covalently bonded per molecule of 12,000 daltons. The proteolipid is an intrinsic membrane protein that fractionates with the ATPase,[74,133] but it is not essential for Ca^{2+} ATPase activity.[134,135] The role of the proteolipid in sarcoplasmic reticulum function is not known. It has been shown not to act as an ionophore in bilayer membranes.[100,136] It is possible that the proteolipid might play a structural role in the sarcoplasmic reticulum by providing hydrophobic and hydrophilic nuclei around which proteins and lipids could organize in the formation of the sarcoplasmic reticulum membrane. Rabbit skeletal sarcoplasmic reticulum membranes contain at least two types of proteolipid[134] which seem to have different distribution within light and heavy vesicles.[38]

b. Calmodulin

Calmodulin was originally identified as a component of skeletal sarcoplasmic reticulum by Campbell and MacLennan[64] using boiled EGTA extracts of sarcoplasmic reticulum ves-

icles. These extracts were shown to stimulate the phosphorylation of EGTA-washed sar coplasmic reticulum vesicles in the same manner as calmodulin. Chiesi and Carafoli[63] have been able to purify calmodulin from skeletal muscle sarcoplasmic reticulum using a CAPP Sepharose 4B affinity column. They estimated that the sarcoplasmic reticulum contained approximately 1 μg of calmodulin per milligram of sarcoplasmic reticulum protein Cal modulm can also be identified as a component of isolated sarcoplasmic reticulum vesicles using "Stains-all"® staining of sarcoplasmic reticulum proteins[67] (Figure 3) "Stains-all"® has been shown to stain several known Ca^{2+} binding proteins (calsequestrin, calmodulin troponin C, and S-100) either dark blue or purple.[67]

The function of calmodulin in skeletal sarcoplasmic reticulum is not known It is likely that it is involved in the regulation of the calmodulin-dependent protein kinase system in the sarcoplasmic reticulum.[63,64,137] Calmodulin is known to exist largely as an intermyofibrillar protein m skeletal muscle. Harper et al.[138] have localized calmodulin in skeletal muscle at the level of the I band which contains glycogen panicles and the terminal cisternae The recent work of Seiler et al.[59] has shown that the high molecular weight 300,000- to 350,000-dalton proteins are the major site for calmodulin binding in both skeletal and cardiac sarcoplasmic reticulum vesicles. Since these proteins are localized in the junctional sarcoplasmic reticulum membrane, it is possible that calmodulin plays a role in Ca^{2+} release or the regulation of Ca^{2+} release.

c. Intrinsic 30,000-Dalton Protein

Intrinsic 30,000-dalton protein was originally identified as a component of isolated sarcoplasmic reticulum by Sarzala et al.[139] Initially, several groups[118,139,140] reported that the 30,000-dalton protein was a glycoprotein, but recently it has been shown that this protein does not bind ^{125}I-Con-A and is not sensitive to Endo H digestion.[60,72] Therefore it seems unlikely that this protein is a glycoprotein. Hidalgo and Ikemoto[118] have shown that approximately 40% of the free amino groups in the intrinsic 30,000-dalton protein is exposed to the cytoplasm. They suggested that the 30,000-dalton intrinsic protein was a component of the sarcoplasmic reticulum feet since such a large percentage of the molecule was exposed to the cytoplasm.[118] Campbell and Shamoo[65] originally identified the 30,000-dalton intrinsic protein as a component of heavy sarcoplasmic reticulum vesicles. In subsequent studies Campbell et al.[38] have shown that the 30,000-dalton band seen by previous investigators actually consisted of several proteins of molecular weights 30,000, 33,000, 34,000 and 38,000. Campbell et al.[38] have provided data that the extrinsic 34,000- and 38,000-dalton proteins are most likely components of the sarcoplasmic reticulum feet. Gutweniger and Montecucco[124] have recently provided evidence that the intrinsic 30,000-dalton protein is an integral membrane protein using photoreactive lipids.

Alteration of the quantity of intrinsic 30,000-dalton protein in isolated sarcoplasmic reticulum vesicles has been observed after denervation by Tate et al.[125] There is an approximate four-fold increase in the intrinsic 30,000-dalton protein in the sarcoplasmic reticulum of denervated muscle. The ratio of Ca^{2+} + Mg^{2+} ATPase to 30,000-dalton protein went from 16:1 in control muscles to 3:1 in denervated muscle sarcoplasmic reticulum. Similar changes have also been observed following chronic nerve stimulation.[126]

d. Extrinsic 34,000- and 38,000-Dalton Proteins

The extrinsic 34,000- and 38,000-dalton proteins were first identified as sarcoplasmic reticulum proteins by Campbell et al.,[38] and were found to be enriched in heavy sarcoplasmic reticulum vesicles which are decorated with junctional sarcoplasmic reticulum feet Campbell et al. have proposed that the extrinsic 34,000- and 38,000-dalton proteins are components of the Junctional sarcoplasmic reticulum feet because treatment of the heavy sarcoplasmic reticulum vesicles with KCl results in the concurrent loss of the 34,000- and 38,000-dalton

proteins and a visible portion of the junctional feet. Triton® X-100 extraction of heavy sarcoplasmic reticulum vesicles leaves an insoluble fraction which consists of electron-dense material.[38] This insoluble fraction is enriched in calsequestrin along with the intrinsic 30,000-dalton protein and the extrinsic 34,000- and 38,000-dalton proteins, suggesting that the 30,000-, 34,000-, and 38,000-dalton proteins are associated with calsequestrin in the heavy vesicles.[38]

Corbett and Caswell[141] have recently reported that the extrinsic 34,000-dalton protein promotes the formation of triadic junctions from isolated transverse tubular vesicles and terminal cisternae vesicles. Therefore, it is possible that the extrinsic 34,000-dalton protein is a peripheral component of the "junctional feet" that promotes the formation of the triad junction.

e. High Affinity Ca^{2+} Binding Protein

High affinity Ca^{2+} binding protein was first identified and purified from skeletal sarcoplasmic reticulum by Ostwald and MacLennan.[66] It binds about 1 mol of Ca^{2+} per mole of protein with a dissociation constant of about 4 μ*M*.[66] The function of high affinity Ca^{2+} binding protein is not known. High resolution gels have indicated that it is a very minor protein component of the sarcoplasmic reticulum membrane. Isolated transverse tubular vesicles appear to be enriched in the high affinity Ca^{2+} binding protein, but it is also present in light and heavy sarcoplasmic reticulum vesicles.

f. 20,000- and 60,000-Dalton Phosphoproteins

Phosphorylation of skeletal muscle sarcoplasmic reticulum was first observed by Campbell and Shamoo.[142] Calmodulin plus Ca^{2+}-dependent phosphorylation was detected in skeletal muscle and in sarcoplasmic reticulum vesicles by Campbell and MacLennan.[64] Phosphorylation of two proteins with molecular weights 20,000 and 60,000 mol wt was enriched in sarcoplasmic reticulum vesicles and was enhanced when purified sarcoplasmic reticulum vesicles were extracted with EGTA to remove endogenous calmodulin. Phosphorylation of the 20,000- and 60,000-dalton proteins was stimulated by NaF and not affected by cAMP.[64] Maximal levels of phosphorylation were observed in the presence of 0.6 μ*M* calmodulin and 0.3 μ*M* free Ca^{2+}. The pH optimum of phosphorylation of the 60,000-dalton protein was below 6.0, and phosphorylation was inhibited over 90% at pH 8.0. Chiesi and Carafoli[63] also observed calmodulin-dependent phosphorylation of skeletal sarcoplasmic reticulum proteins with apparent molecular weights of 57,000, 35,000, and 20,000.

Characterization of the 60,000-dalton phosphoprotein has also been performed by Campbell and MacLennan.[64] The 60,000-dalton phosphoprotein does not appear to be a glycoprotein since digestion with Endo H or Endo D failed to alter its molecular weight.[64] Two-dimensional gel electrophoresis showed that the 60,000-dalton phosphoprotein was not related to calsequestrin.[64] In addition, the 60,000-dalton phosphoprotein was not immunoprecipitated with antibodies against calsequestrin. The SDS-purified 60,000-dalton phosphoprotein contained phosphoserine and phosphothreonine. Therefore, it is not likely that the 60,000-dalton protein is related to $pp60^{SRC}$, which is phosphorylated on a tyrosine residue. Chiesi and Carafoli[143] have shown that the 20,000-dalton phosphoprotein is an acidic proteolipid distinct from phospholamban.

The physiological function of the calmodulin-dependent phosphorylation of the 20,000- and 60,000-dalton proteins is not known. Since the phosphorylation system is integral to the sarcoplasmic reticulum, it is likely that it plays a regulatory role in sarcoplasmic reticulum function. Since the stoichiometry between the ATPase and the 60,000-dalton phosphoprotein is about 60:1, it is unlikely that the 60,000-dalton phosphoprotein is a regulator of Ca^{2+} transport by skeletal sarcoplasmic reticulum vesicles. A functional role for the phosphorylation of the 60,000-dalton protein in the regulation of Ca^{2+} release has been suggested.[64]

Trifluoperazine, a potent inhibitor of calmodulin-dependent reactions, has been shown to inhibit the calmodulin-dependent phosphorylation of sarcoplasmic reticulum and to uncouple Ca^{2+} accumulation from Ca^{2+} ATPase activity.64 Compound 48/80 has been shown to inhibit calmodulin-dependent phosphorylation and Ca^{2+} uptake under conditions in which Ca^{2+} ATPase activity is fully functional.[137] EGTA extinction of sarcoplasmic reticulum vesicles, which removes endogenous calmodulin and lowers phosphorylation levels, and high pH, which inhibits phosphorylation, also lead to an uncoupling of Ca^{2+} accumulation from Ca^{2+} ATPase activity. The uncoupling of Ca^{2+} transport by these procedures could be due to the opening of a Ca^{2+} release channel. This would suggest that the calmodulin-dependent phosphorylation system was involved in the closing of Ca^{2+} release channels following the elevation of cytoplasmic Ca^{2+} initiated by Ca^{2+} release.

2. High Molecular Weight Proteins

a. Intrinsic 160,000-Dalton Glycoprotein

The intrinsic 160,000-dalton glycoprotein was originally identified by ^{125}I-Con-A staining of sarcoplasmic reticulum proteins separated by SDS gel electrophoresis.[72] It was present in both light and heavy sarcoplasmic reticulum vesicles, and reduced in a fraction that contained transverse tubular membrane vesicles. The 160,000-dalton glycoprotein, like the 53,000-dalton glycoprotein, reconstituted into vesicular structures when detergents were removed from detergent-solubilized sarcoplasmic reticulum.[72] Copurification of the 53,000- and 160,000-dalton glycoproteins on Con-A Sepharose was obtained by Campbell and MacLennan[60] (Figures 6 and 9). The apparent molecular weight of the partially purified 160,000-dalton glycoprotein was reduced from 160,000 to 155,000 after digestion with Endo H (Figure 9). There was no change in the molecular weight of the 160,000-dalton glycoprotein when sealed vesicles were treated with Endo H, but the molecular weight was reduced when sarcoplasmic reticulum vesicles were solubilized with deoxycholate (Figure 6). Gutweniger and Montecucco[124] have provided direct evidence that the 160,000-dalton glycoprotein is an integral component of sarcoplasmic reticulum membranes using photoreactive lipids. Therefore, the 160,000-dalton glycoprotein is also transmembrane glycoprotein like the 53,000-dalton glycoprotein.

The 53,000- and the 160,000-dalton glycoproteins share several biochemical characteristics: ^{125}I Con-A binding, Con-A-Sepharose binding, DEAE binding, Endo H sensitivity, 8-N_3-ATP binding, and antiserum cross-reactivity. Even though they share these character istics, the 160,000-dalton glycoprotein is not just an oligomer of the 53,000-dalton glycoprotein.[60] One chemical property that reveals a difference between the 53,000- and 160,000-dalton glycoproteins is "Stains-all"® staining. "Stains-all"® stains the 160,000-dalton glycoprotein blue, while the 53,000-dalton glycoprotein stains pink. (Figures 3 and 6) This suggests that the 160,000-dalton glycoprotein may contain a region which is not related to the 53,000-dalton glycoprotein. "Stains-all"® blue staining (Figures 3, 4, and 6) and the effect of Ca^{2+} on the mobility of the 160,000-dalton glycoprotein (Figure 8) suggest that the 160,000-dalton glycoprotein is a Ca^{2+} binding protein. Cardiac sarcoplasmic reticulum also contains a "Stains-all"® blue-staining 130,000-dalton glycoprotein that is similar to the 160,000-dalton glycoprotein of skeletal muscle sarcoplasmic reticulum. We have invariably observed two high molecular weight proteins which stain blue with "Stains-all"® in sarcoplasmic reticulum isolated from rabbit, chicken, pig and rat skeletal muscle and canine cardiac muscle. These proteins had a range of molecular weights, (i.e., canine cardiac muscle 130,000 and 140,000 daltons), but one was sensitive to Endo H, and the other changed apparent molecular weight upon two-dimensional gel electrophoresis.

Recently, Campbell et al.[122,123] have shown that the monoclonal antibodies to the 53,000-dalton glycoprotein cross-react with the 160,000-dalton glycoprotein, while monoclonal antibodies to the 160,000-dalton glycoprotein do not cross-react with the 53,000-dalton

glycoprotein. Therefore, it is probable that the 160,000-dalton glycoprotein consists of a polypeptide region that is almost identical to the 53,000-dalton glycoprotein and a polypeptide region that is different from the 53,000-dalton glycoprotein. A marker for this latter region is Stains-all blue staining. Monoclonals to the 160,000-dalton glycoprotein have also shown that it is contained in both light and heavy sarcoplasmic reticulum vesicles.[123] The function of the 160,000-dalton glycoprotein is not known, but it is likely to function in a similar manner as the 53,000-dalton glycoprotein.

b. 170,000-Dalton Protein

The 170,000-dalton protein was initially identified as a component of isolated sarcoplasmic reticulum by Campbell et al.[67] It is one of the four proteins in skeletal sarcoplasmic reticulum vesicles which stains blue with "Stains-all"® (Figures 3 and 4). It is not related to glycogen-debranching enzyme, since this enzyme stains red or pink with "Stains-all"®. Unlike the 160,000-dalton glycoprotein. the 170,000-dalton protein is not sensitive to Endo H (Figure 6). It is my present contention that the 170,000-dalton protein is related to calsequestrin in both structure and function. The 170,000-dalton protein is related to calsequestrin in both structure and function. The 170,000-dalton protein and calsequestrin stain blue with "Stains-all"®, precipitate with Ca^{2+}, bind to Ca^{2+} phosphate, and are enriched in heavy sarcoplasmic reticulum vesicles. We have also found that the addition of Ca^{2+} to sarcoplasmic reticulum samples prior to SDS gel electrophoresis will enhance the mobility of the 170,000-dalton protein and calsequestrin (Figure 8). Finally, the 170,000-dalton protein has a shift in apparent molecular weight on two-dimensional gel electrophoresis (Figure 10). This is the same type of pH-dependent mobility change which is observed for calsequestrin (Figure 5). Therefore, it is likely that the 170,000-dalton protein shares some primary sequence and structural properties with calsequestrin.

It is not known whether the 170,000-dalton protein is a transmembrane protein or where it is exactly located in the sarcoplasmic reticulum. If it is transmembrane and located in the junctional sarcoplasmic reticulum, it could function in the formation of a junctional calsequestrin complex that is seen in heavy sarcoplasmic reticulum vesicles.[38] (Figure 1C).

c. 300,000 to 350,000-Dalton Proteins

A pair of very large molecular weight proteins were first identified as very minor components of isolated triad junctions by Cadwell and Caswell.[54] The apparent molecular weights of these proteins was 300,000 and 325,000 daltons. Proteolytic digestion of the isolated triad junction caused the separation of the transverse tubule vesicles from the terminal cisternae vesicles and the degradation of the two high molecular weight proteins. Experiments using ^{125}I-labeled transverse tubular vesicles and unlabeled terminal cisternae vesicles indicated that the ^{125}I-labeled high molecular weight proteins were able to transfer from the transverse tubule vesicles to terminal cisternae vesicles during reformation and breakage of the isolated triad junction. Cadwell and Caswell[54] have suggested that this is a property of the protein which spans the gap between the triad junction and that the 300,000- and 325,000-daltons proteins are possible components of the sarcoplasmic reticulum feet.

Recently, Seller et al.[59] have identified these high molecular weight proteins in junctional sarcoplasmic reticulum isolated from both cardiac and skeletal muscle. Seiler et al.[59] have shown that these high molecular weight proteins are not found in free sarcoplasmic reticulum or purified sarcolemma membranes. The high molecular weight proteins were shown to be the major calmodulin-binding proteins in the isolated skeletal or cardiac sarcoplasmic reticulum. The high molecular weight proteins were also substrates for the endogenous Ca^{2+} calmodulin protein kinase and exogenously added catalytic subunit of cAMP-dependent protein kinase. Heavy sarcoplasmic reticulum vesicles are also enriched in the high molecular weight proteins when compared to light sarcoplasmic reticulum vesicles. In addition, a

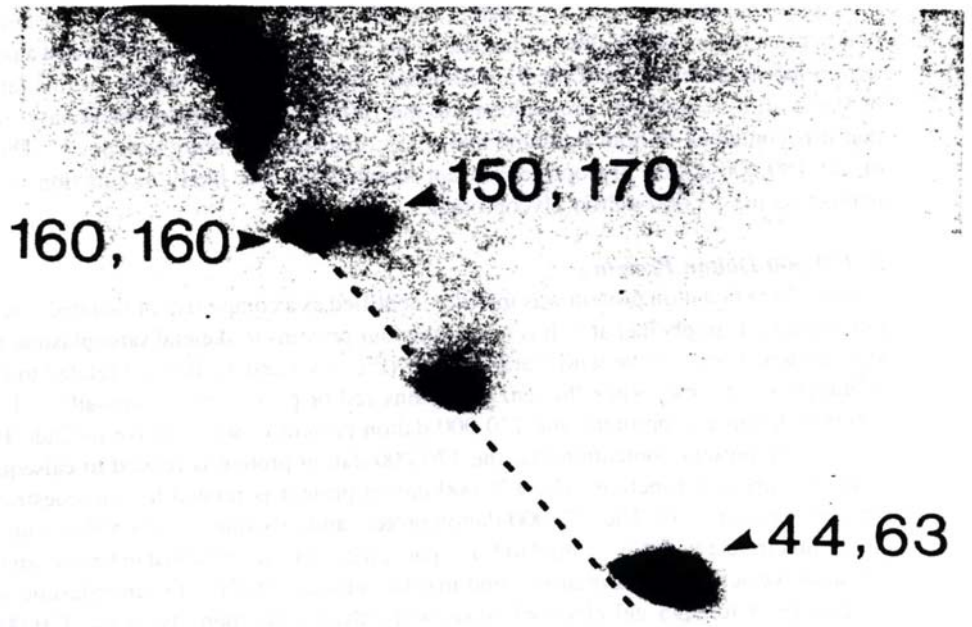


FIGURE 10. Two-dimensional gel electrophoresis of rabbit skeletal muscle sarcoplasmic reticulum. Rabbit sarcoplasmic reticulum proteins were separated by two-dimensional gel electrophoresis[72] (5% acrylamide in the first (horizontal) dimension and 7.5% acrylamide in the second (vertical) dimension) and stained with "Stains-all".® Calsequestrin (indicated by 44,63) ran with a mobility of 44,000 daltons in the horizontal dimension and 63,000 daltons in the vertical dimension. The 160,000-dalton glycoprotein (indicated by 160,160) ran with the identical mobility in both dimensions, while the 170,000-dalton protein (indicated by 150,170) ran with a mobility of 150,000 daltons in the horizontal dimension and 170,000 daltons in the vertical dimension.

dantrolene-sensitive fraction of heavy sarcoplasmic reticulum vesicles is enriched in the high molecular weight proteins (Figure 11).

The functions of the high molecular weight proteins are not known, but it is likely that they are important components of the junctional sarcoplasmic reticulum membrane. The high molecular weight proteins could be related to microtubular-associated proteins.[144,145] Both sets of proteins have similar molecular weights, are substrates of various protein kinases, are able to bind calmodulin, and are easily degraded with proteases. If the high molecular weight proteins are related to the microtubule-associated proteins, then it is possible that they are involved in the binding of microtubules to the junctional sarcoplasmic reticulum membrane.

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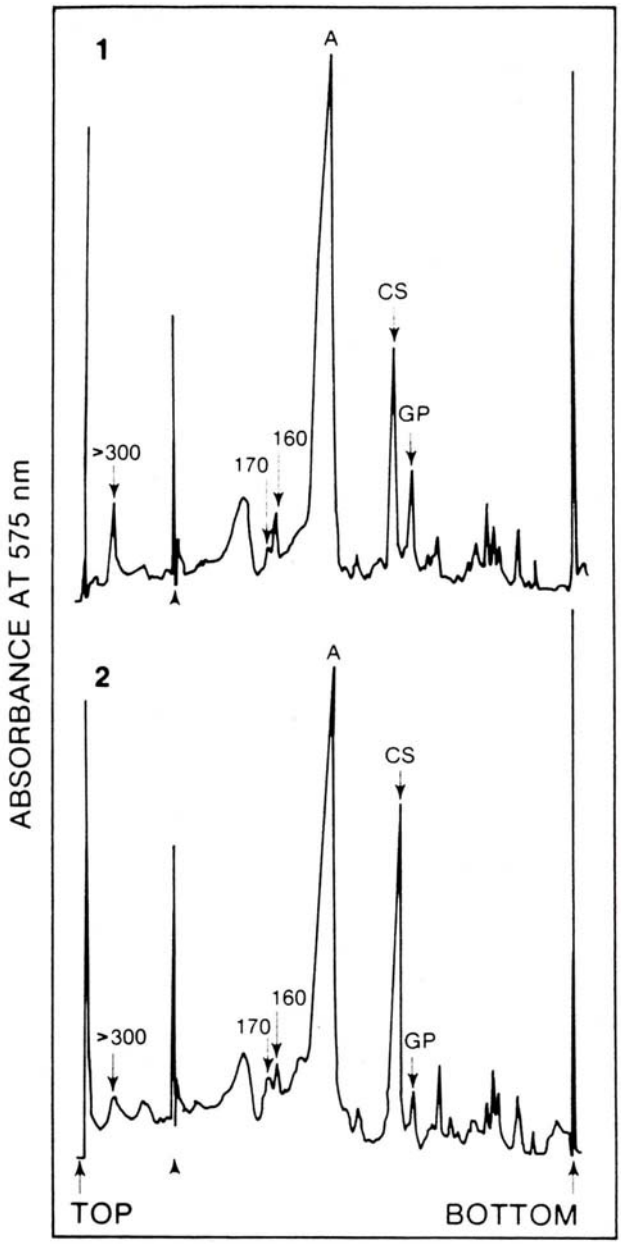

FIGURE 11. Densitometric scans of dantrolene-sensilive sarcoplasmic reticulum vesicles (1) and dantrolene-insensitive sarcoplasmic reticulum vesicles (2) separated by SDS gel electrophoresis (5 to 15% gradient slab gels) and stained with Coomassie blue. A, Ca^{2+} + Mg^{2+} ATPase (105,000 daltons); CS, calsequestrin (63,000 daltons); GP, glycoprotein (53,000 daltons); 160, 160,000-dalton glycoprotein; 170, 170,000-dalton protein; >300, 300,000- to 350,000-dalton protein. The top of the stacking gel is indicated by an arrow and the top of the running gel is indicated with an arrowhead. Gels were scanned at 615 nm.

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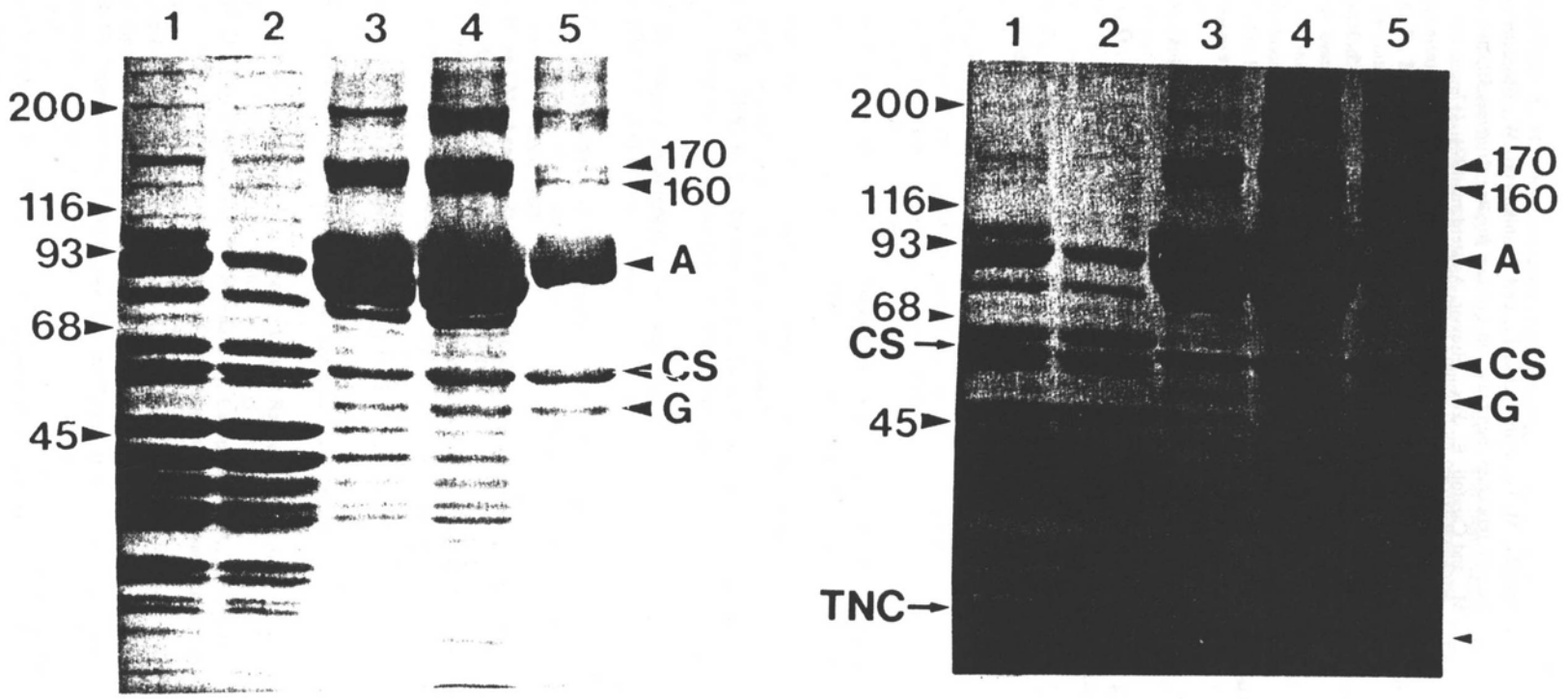

FIGURE 3. Coomassie blue and Stains-all® staining of skeletal muscle proteins. Fractions in the purification of sarcoplasmic reticulum vesicles from rabbit skeletal muscle according to Campbell and MacLennan[60] were analyzed by SDS-polyacrylamide gel electrophoresis (5 to 15% gradient gels) and stained with Coomassie blue (A) or Stains-all® (B) as described.[67] Lane 1, supernatant from rabbit skeletal muscle homogenate following centrifugation at 10,000 × *g* for 20 min; lane 2, supernatant from rabbit skeletal muscle homogenate following centrifugation at 50,000 × *g* for 1 hr; lane 3, pellet obtained from 50,000 × *g* centrifugation; lane 4, supernatant following 7000 × *g* centrifugation; lane 5, KCl-washed sarcoplasmic reticulum vesicles. Coomassie blue-stained gel contained 100 μg of protein in each lane, while the Stains-all®-stained gel contained 200 μg of protein in each lane. A, $(Ca^{2+} + Mg^{2+})$ATPase (105,000 daltons); CS, calsequestrin (63,000 daltons); G, glycoprotein (53,000 daltons). 160, 160,000 dalton glycoprotein; 170, 170,000 dalton protein. TNC, troponin C. The numbers on the left side represent molecular weight × 10^{-3} of molecular weight standards. The small arrowhead indicates a lower molecular weight blue-staining protein, possibly calmodulin, in the purified sarcoplasmic reticulum vesicles. (Reproduced from Campbell, K. P., MacLennan, D. H., and Jorgensen, A. O., *J. Biol. Chem.*, 258, 11267, 1983. With permission of the American Society of Biological Chemists.)

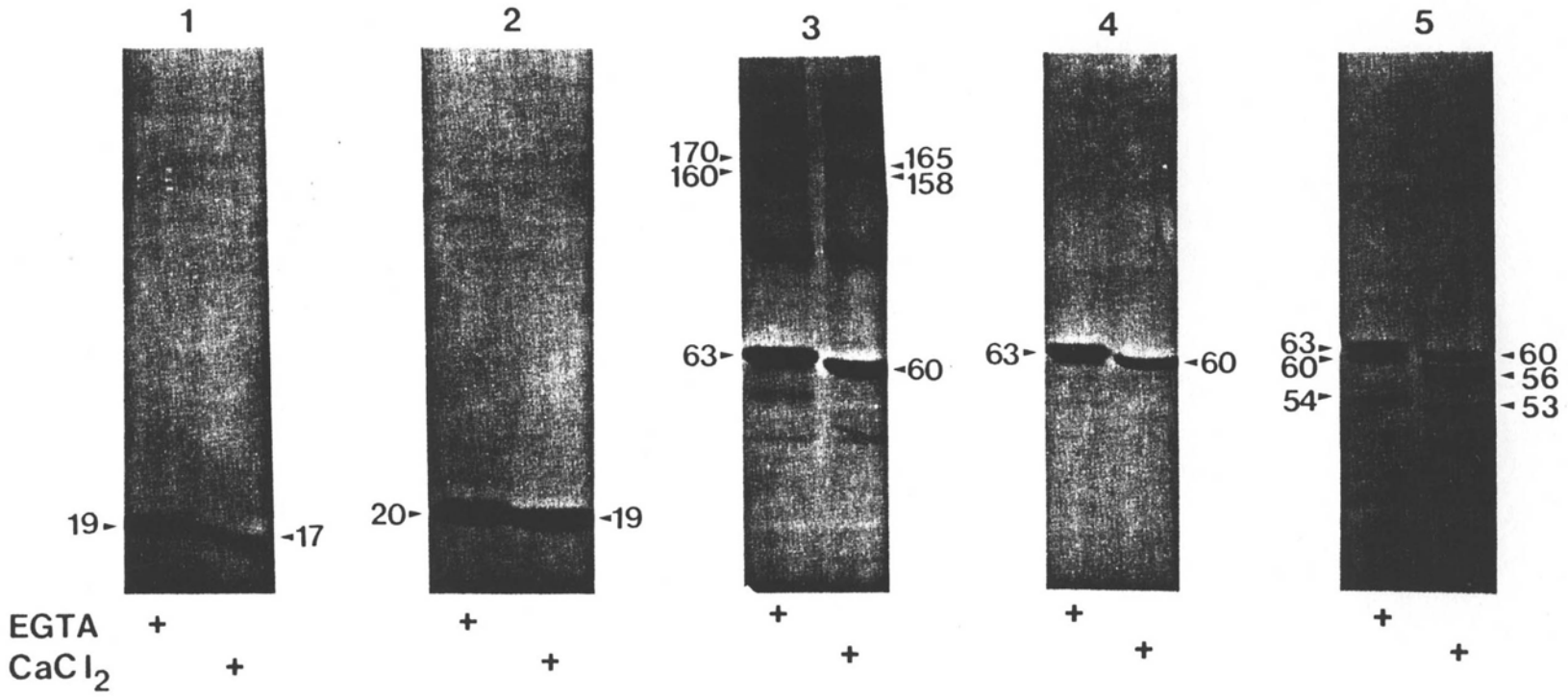

FIGURE 8. Mobility of calsequestrin in SDS gel electrophoresis in the presence of EGTA or Ca^{2+}. EGTA (1 m*M*) or Ca^{2+} (1 m*M*) was added to each sample prior to electrophoresis. Gel electrophoresis was performed according to Laemmli[70] and the gel lanes were stained with "Stains-all"®.[67] Sample 1, calmodulin; sample 2, troponin C; sample 3, skeletal sarcoplasmic reticulum vesicles; sample 4, purified calsequestrin; sample 5, degraded calsequestrin.