The primary amino acid sequence for a highly abundant junctional sarcoplasmic reticulum glycoprotein (triadin) has been deduced from the cDNA sequence. Based on both biochemical analysis and the predicted amino acid sequence we suggest that this protein is an intrinsic membrane glycoprotein containing a single transmembrane domain that separates the protein into cytoplasmic and luminal domains. The cytoplasmic domain is proposed to contain the amino-terminal 47 amino acids. The remainder of the protein including the carboxyl terminus is proposed to be found within the lumen of the sarcoplasmic reticulum and contains an extremely high concentration of basic residues. Protease analysis of intact triads was consistent with the topological predictions. Western and Northern blots suggest that the protein is specifically expressed in skeletal muscle and not cardiac muscle or brain. The abundance and localization of this protein suggest that it plays an important regulatory or structural role in excitation-contraction coupling in skeletal muscle.

Considerable research has been focused on characterizing and identifying the molecular components that regulate the release of calcium from intracellular stores (Fill et al., 1989). Because of its abundance and importance, skeletal muscle has been a rich source of information in the biochemical characterization of many of these components. In the previous paper (Knudson et al., 1993), we have used junctional face membranes and triads derived from skeletal muscle in the production and characterization of monoclonal antibodies against junctional specific proteins (Campbell et al., 1987). Antibodies were produced against an approximately 94-kDa glycoprotein which is highly enriched in junctional face membranes and was localized to the junctional sarcoplasmic reticulum membrane. The protein was shown to have a very characteristic pattern of migration on SDS-PAGE when run in the absence of reducing agents. This pattern of staining has recently been described for a 95-kDa protein which is proposed to stabilize the triad junction by providing a link between the dihydropyridine receptor and the ryanodine receptor (Caswell et al., 1991). Based on these results, the authors have proposed that the 95-kDa protein be named triadin. Given the similar localization and the unique migration pattern on nonreducing SDS-PAGE, these proteins are almost certainly identical. In this study, the cDNA sequence for the 95-kDa glycoprotein of the junctional sarcoplasmic reticulum has been determined by molecular cloning using monoclonal antibodies. The sequence encodes a protein that is predicted to contain a single transmembrane domain near the amino terminus. Topological analysis predicts a small cytoplasmic domain of only 47 amino acids. This prediction is consistent with proteolytic analysis of intact and permeabilized membranes. The luminal portion of the protein is highly charged, containing 44.7% charged residues with an excess of basic residues resulting in an isoelectric point of 10.18. These results may provide an alternative explanation to the studies of Caswell et al. (1991). Interestingly, the protein contains only 2 cysteines, which minimizes the combinations of potential disulfide linkages. The tissue distribution of the 94-kDa glycoprotein was assessed using both Western and Northern blots, which showed that the protein is expressed in skeletal muscle but not cardiac muscle or brain. Combined, these results suggest that the 94-kDa glycoprotein performs an important function in calcium regulation at the triad junction. Although the specific function of the 94-kDa glycoprotein is not known, several possibilities are discussed.

**EXPERIMENTAL PROCEDURES**

Isolation and Characterization of Membranes—Adult rabbit triads were isolated by a modification of Mitchell et al. (1983) as described previously (Sharp et al., 1987). Fresh rabbit hearts were rapidly frozen using liquid nitrogen. Cardiac microsomes were prepared from frozen rabbit heart muscle as described (Campbell et al., 1984). Microsomal fractions were prepared as described (McPherson and Campbell, 1980). Protein was determined by the method of Lowry et al. (1951) as modified by Peterson (1977). Protein samples were analyzed by SDS-PAGE using the buffer system of Laemmli (1970) and either stained with Coomassie Blue or transferred to nitrocellulose according to Towbin et al. (1979). Monoclonal antibodies against the 94-kDa glycoprotein were prepared as described (Campbell et al., 1987). Polyclonal antibodies against the 94-kDa glycoprotein were prepared by injection of SDS gel slices according to the method of Tsang (1983). Indirect immunoperoxidase staining of nitrocellulose blots was
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performed using nonfat dry milk as a blocking agent as described previously (Leung et al., 1987).

α-Chymotrypsin Digestion of Rabbit Skeletal Triads—Rabbit skeletal muscle triads were treated with a 1:160 or 1:20 ratio of α-chymotrypsin to protein in the presence or absence of 0.25% CHAPS for 15 min at 37 °C. The samples were quenched with 2 mM phenylmethylsulfonyl fluoride and 3% SDS in Laemmli sample buffer and analyzed by 5–12% SDS-PAGE.

Molecular Biological Methods—Monoclonal antibody XIIH112 was used as a probe to isolate two clones (p94k1 and p94k3) from an oligo(dT)-primed cDNA expression library constructed in λgt11 from young rabbit back skeletal muscle poly(A)-enriched RNA (Ellis et al., 1988; Jay et al., 1990). The purified inserts were subcloned into the EcoRI site of Bluescript SK(+). The two overlapping clones contained a long open reading frame extending from the 5′ end of both clones to a region that contained multiple stop codons in all three reading frames (Fig. 1). The entire insert of p94k3 was used to rescreen the same oligo(dT)-primed λgt11 library. Five clones were purified, subcloned into Bluescript SK(+), and partially sequenced. Since none of these clones extended to the 3′ end of the cDNA, the 5′ HindIII fragment of p94k9 (Fig. 1) was used to rescreen a random primed λgt11 library (Jay et al., 1990). Three clones were plaque purified, subcloned into Bluescript SK(+), and sequenced including p94k15 (Fig. 1) which extended to the 5′-translated region of the gene based on nonsense or stop codons present in all three reading frames.

Northern Analysis—Poly(A)-enriched RNA from rabbit brain, heart, and skeletal muscle was prepared as described (Chomczynski and Sacchi, 1987). Four µg of each sample was electrophoresed on a 1.5% agarose gel containing 5% formaldehyde gel and transferred to GeneScreen nylon (Du Pont-New England Nuclear) membranes. The membranes were baked, prehybridized, and hybridized according to the manufacturer's specifications. The filter was washed with 2 X SSC (50 mM sodium citrate/ HCl, pH 7.0) for 2 X 5 min at room temperature, with 2 X SSC + 1% SDS for 2 X 30 min at 65 °C and with 0.1 X SSC for 2 X 30 min at room temperature. The filter was placed between two sheets of Saran Wrap and subjected to autoradiography.

Nucleotide Sequence Determination—Sequencing of both strands was carried out with plasmid-specific or gene-specific primers using the dideoxy method of Sanger et al. (1977). Sequencing reactions were resolved on 60-cm gels (IBI base runner) containing 6% acrylamide and 6.5 M urea. The gels were dried and exposed to film at room temperature.

DNA Sequence Analysis—The sequence was analyzed using software provided by the genetics computer group (GCG) and by PC/GENE from IntelliGenetics Inc. (Mountain View, CA). Homology searches were performed against the NBRF and the Swissprote protein data bases using the FASTA program provided in the GCG software. Automated Acid Sequence Analysis—Automated Edman degradation was performed with an Applied Biosystems (Foster City, CA) model 470A Sequencer equipped with an on-line model 120A phenylthiohydantoin derivative analyzer using the manufacturer's standard programming and chemicals. Tryptic peptides were prepared for acquisition of internal sequence information by SDS-electrophoresis of 200 pmol of intact protein through 7.5% acrylamide gels, electroblotting to nitrocellulose paper, and digesting the immobilized protein with trypsin (Aebbersold et al., 1987). Peptides were purified by reverse phase HPLC using an Applied Biosystems model 130A HPLC system equipped with a 2.1 x 100-mm RP-300 column. Chromatography was performed initially in 0.1% trifluoroacetic acid at 0.05 ml/min, and individual peaks were repurified on the same column in 0.1% ammonium acetate. In both cases, elution was performed with a gradient of 0–29% acetonitrile.

Materials —Ipropyl-1-thio-β-D-galactopyranoside) was from Sigma. Nylon GeneScreen membranes were from Du Pont-New England Nuclear.

RESULTS
cDNA Sequence Determination of the 94-kDa Glycoprotein—Monoclonal antibodies were previously shown to be specific for the 94-kDa glycoprotein based on recognition of the antigenic site for monoclonal antibodies XIIH112 and IIG12 to the region between the threonine at position 437 and the carboxyl terminus of the protein. Subsequent hybridization screening and sequencing have resulted in the identification of a number of overlapping clones (Fig. 1) which have been compiled to yield a 4,588-nucleotide sequence that contains a 2,118-nucleotide open reading frame. The cDNA sequence and predicted primary amino acid sequence of the 94-kDa glycoprotein are shown in Fig. 2. The identity of these clones with the 94-kDa glycoprotein has been confirmed by Edman degradation sequencing of both the intact protein and tryptic peptides from the protein. A total of six tryptic peptides have been found in the protein predicted from the cDNA sequence and are underlined in Fig. 2. All of the unambiguous peptide sequences obtained by Edman degradation sequencing were identified in the predicted amino acid sequence for the 94-kDa glycoprotein. The deduced amino acid sequence predicts a protein of 706 amino acids with a predicted molecular mass of 79,134 Da (Table I). The discrepancy between the molecular mass of the protein predicted from the cDNA (79,134) and the apparent molecular weight based on SDS-PAGE (94,000) led to a close examination of the predicted translation initiation and termination sites of the protein. The translation initiation site is the 1st methionine found in the long open reading frame and was chosen based on multiple criteria: (i) two independent and distinct clones showed identical sequence through this region; (ii) the nucleotide sequence around the initiator methionine conforms well to the consensus sequence for initiation of protein synthesis in eukaryotes (Kozak, 1987); and (iii) amino-terminal sequencing of the

Fig. 1. Restriction map and sequencing strategy of the 94-kDa glycoprotein. The figure shows restriction map of the cDNA for the 94-kDa glycoprotein and some of the DNA clones which were used to determine the sequence of the 94-kDa glycoprotein. Clones p94k1, p94k3, and p94k9 were isolated from the oligo(dT)-primed λgt11 cDNA expression library; clone p94k15 was isolated from the random primed λgt11 cDNA expression library. See "Experimental Procedures" for details. kb, kilobases.
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![Sequence of the 94-kDa Glycoprotein](image)

**Fig. 2.** cDNA sequence of the 94-kDa glycoprotein (traddin). The figure shows the cDNA sequence of the 94-kDa glycoprotein and the deduced amino acid sequence for the protein. The underlined amino acids were confirmed by amino-terminal sequencing of either the intact protein or of HPLC-purified tryptic fragments. The double underline denotes the putative membrane spanning domain. The potential N-linked glycosylation sites are denoted by an asterisk (*). The potential polyadenylation signal sequences in the 3' untranslated region are also underlined.
intact protein was matched with amino acids 2–13 of the predicted protein. The amino-terminal methionine was not identified in the first cycle of sequencing and is apparently removed in vivo by post-translational processing of the protein or by in vitro treatment of the tissue or membranes. The sequence surrounding the termination codon has been confirmed by sequence analysis of four independent clones and contains multiple stop/nonsense codons in all three reading frames. Although a small portion of the discrepancy in molecular weight may be accounted for by glycosylation (Knudson et al., 1998), the majority is likely caused by intrinsic properties of the protein which result in altered migration on SDS-PAGE (see the following discussion). Although six polyadenylation signal sequences (AATAAA) (Wickens, 1990) were found in the long 3' untranslated region, the poly(A) tail was not identified. It is unlikely that the untranslated regions

**Fig. 2—continued**

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extend much beyond that shown in Fig. 2 as the size of the clone matches closely with an approximately 4.6-kilobase mRNA that was identified on Northern analysis (see Fig. 5).

**Protein Sequence Analysis**—To identify possible membrane spanning domains, hydropathy analysis of the predicted amino acid sequence has been performed using an algorithm based on the properties of each individual amino acid (Kyte and Doolittle, 1982). Fig. 3 shows that the 94-kDa glycoprotein contains only one hydrophobic stretch which is predicted to traverse the membrane a single time between residues 48 and 68. Consistent with Edman degradation sequencing of the intact protein, the amino terminus was not hydrophobic and is unlikely to form a signal sequence. Thus, the amino terminus is predicted to be cytoplasmic. The remainder of the protein is very hydrophilic and therefore unlikely to contain

### Table I

| Residue | No. | Mol % |
|---------|-----|-------|
| A = Ala | 49  | 6.94  |
| C = Cys | 2   | 0.28  |
| D = Asp | 30  | 4.24  |
| E = Glu | 103 | 14.88 |
| F = Phe | 12  | 1.70  |
| G = Gly | 29  | 4.10  |
| H = His | 11  | 1.55  |
| I = Ile | 27  | 3.82  |
| K = Lys | 157 | 22.23 |
| L = Leu | 21  | 2.97  |
| M = Met | 7   | 0.99  |
| N = Asn | 6   | 0.85  |
| P = Pro | 58  | 8.21  |
| Q = Glu | 27  | 3.82  |
| R = Arg | 11  | 1.55  |
| S = Ser | 47  | 6.65  |
| T = Thr | 54  | 7.64  |
| V = Val | 44  | 6.23  |
| W = Trp | 3   | 0.42  |
| Y = Tyr | 8   | 1.13  |
| A + G   | 78  | 11.04 |
| S + T   | 101 | 14.30 |
| D + E   | 133 | 18.83 |
| D + E + N + Q | 166 | 23.51 |
| H + K + R | 179 | 25.35 |
| D + E + H + K + R | 312 | 44.19 |
| I + L + M + V | 99  | 14.02 |
| F + W + Y | 23  | 3.25  |

*Fig. 3. Hydrophobicity analysis of the 94-kDa glycoprotein.* The figure shows the hydropathy plot for the 94-kDa glycoprotein by the method of Kyte and Doolittle (1982) using a window size of 19 residues.
based on the previously discussed topological model. We have not explored either the in vitro or in vivo phosphorylation of this protein, and hence the physiological importance of these sites is not known.

Data base searches of the 94-kDa glycoprotein at both the DNA and the protein level failed to identify significant similarity with any known proteins in the data base. However, several proteins were identified which contained limited similarity to the 94-kDa glycoprotein. The 94-kDa glycoprotein had limited similarity with both neurofilament H and neurofilament M. Amino acids 298-622 of the 94-kDa glycoprotein were 25.5% identical to amino acids 497-820 of murine neurofilament M (Levy et al., 1987). Amino acids 121-386 of the 94-kDa glycoprotein were 28.7% identical to amino acids 834-1085 of murine neurofilament H (Julien et al., 1988). The protein also contained 17.9% identity over a 546-amino acid overlap with chicken smooth muscle caldesmon (Bryan et al., 1989). However, the similarity of the 94-kDa glycoprotein to these proteins may not represent genetic similarity because similar scores are obtained when the 94-kDa protein sequence is randomized and the amino acid content is kept constant. This suggests that the similarity between these proteins can be attributed to the amino acid composition alone and does not arise from a genetic link. Consistent with this explanation, the sequence similarity occurs over regions of these proteins which both contain highly charged, lysine-rich regions. Interestingly, these proteins, like the 94-kDa glycoprotein, have an apparent molecular weight based on SDS-PAGE which is much larger than the predicted molecular weight from the cDNA (Julien et al., 1988; Bryan et al., 1989). Therefore, we propose the highly charged, basic nature of these proteins may account for their altered migration on SDS-PAGE.

Membrane Topology Analysis—The proposed membrane topology of the 94-kDa glycoprotein was tested by proteolytic digestion of intact and permeabilized triads. Ideally, antibodies specific for the amino terminus would be used to test for loss of immunoreactivity when triads are treated with protease. However, all of the available antibodies were found to recognize the predicted luminal domain based on testing with the expression clones. Thus, these antibodies were used by examining for the appropriately sized proteolytic fragments. Fig. 4 shows triads treated in the absence (lane 1) or presence (lanes 2–5) of two concentrations of chymotrypsin either in the absence (lanes 2 and 4) or presence (lanes 3 and 5) of 0.25% CHAPS to permeabilize the vesicles. Chymotrypsin was the protease chosen for two reasons. First, the 94-kDa glycoprotein contains 23 aromatic residues which are potential cleavage sites of which only the phenylalanine at residue 44 is predicted to be cytoplasmic. Second, chymotrypsin, in contrast to trypsin, is rapidly inhibited by SDS, which prevents further digestion when the samples are solubilized for SDS-PAGE. Fig. 4A shows Coomassie Blue staining and illustrates that the ryanodine receptor is highly sensitive to chymotryptic digestion both in the absence or presence of detergent. This is consistent with the predicted membrane topology of this protein with the majority of the protein being cytoplasmic and thus accessible to digestion (Takeshima et al., 1989). In contrast, calmodulin, a strictly luminal protein (Fliegel et al., 1989), is only extensively digested in the presence of detergent (Fig. 4A). The 94-kDa glycoprotein is not clearly visualized on Coomassie Blue staining of the triads because of interference by the highly abundant (Ca^{2+} + Mg^{2+})-ATPase. Thus, the 94-kDa glycoprotein and some of its fragments were identified by immunoblot analysis with monoclonal (Fig. 4B) and polyclonal (Fig. 4C) antibodies. The proposed membrane topology for the 94-kDa glycoprotein predicts minimal digestion in the absence of detergent and extensive digestion in the presence of detergent. Fig. 4, B and C, shows that in the absence of detergent the 94-kDa glycoprotein is only partially digested, which results in a slight increase in mobility on SDS-PAGE. This can best be accounted for when the protein is incompletely digested, and a doublet composed of the intact protein and the proteolytic fragment is present (Fig. 4, B and C, lane 2). Increasing the concentration of protease leads to a more complete cleavage of the intact protein but does not result in the identification of additional proteolytic fragments (Fig. 4, B and C, lane 4). The polyclonal antibody staining (Fig. 4C) does recognize additional proteins, but these are also recognized in the undigested triads and may represent proteolytic fragments of the 94-kDa glycoprotein. Permeabilization with detergent results in the near complete digestion of the 94-kDa glycoprotein. This suggests that the resistance to digestion in the absence of detergent is not an intrinsic property of the protein. Similar results have been obtained using trypsin instead of chymotrypsin (data not shown). Thus, these results strongly support the proposed membrane topology for the 94-kDa glycoprotein. An alternative explanation to these results is that the 94-kDa glycoprotein has intrinsic properties that render it resistant to digestion and that detergent treatment exposes cryptomorphic sites by protein denaturation. However, the relative low concentration of detergent (0.25% CHAPS) and the relative insolubility of the protein in CHAPS (data not shown) make this explanation unlikely. Thus, we feel the hydrophobic analysis (Fig. 3), the absence of a signal sequence (Fig. 2), the chymotryptic digestion (Fig. 4), and the likely glycosylation of asparagine 625 provide overwhelming support for the proposed membrane topology of the 94-kDa glycoprotein.

Tissue-specific Expression—The tissue-specific expression of the 94-kDa glycoprotein was examined by both Northern blot analysis of mRNAs isolated from rabbit tissues and by Western blots of microsomes from the same tissues. Fig. 5A shows that the probe for the 94-kDa glycoprotein recognized a 4.6-kilobase band in rabbit skeletal muscle but did not hybridize with mRNA from brain or cardiac muscle (lanes 2 and 3). The size of this transcript is consistent with the 4,588-nucleotide transcript that was sequenced in this study and suggests that only a small portion of the 3'- and possibly 5' untranslated region(s) was not identified. Consistent with the Northern blot analysis, both monoclonal (Fig. 5B) and polyclonal antibodies (Fig. 5C) failed to recognize a 94-kDa protein in both cardiac muscle and brain microsomes. Together, these data suggest that this protein/gene is not expressed in brain and heart and is likely involved in a function that is specific to skeletal muscle. However, these results do not exclude the possibility that cardiac muscle or brain expresses low levels of this protein or that a similar protein/isoform is expressed which is not recognized by the antibodies and does not cross-hybridize with the 94-kDa glycoprotein probes under the conditions used in this experiment.

DISCUSSION

The primary amino acid sequence of a major 94-kDa glycoprotein (triadin) has been deduced from the cloned cDNA sequence. The properties and predicted membrane topology of the 94-kDa glycoprotein are summarized in the model shown in Fig. 6. Based on hydrophobic analysis, the 94-kDa glycoprotein was proposed to contain a single transmembrane domain from amino acid residue 48 to 68. This proposed membrane topology was consistent with the proteolytic pattern of the 94-kDa glycoprotein when triads were digested in

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FIG. 4. \(\alpha\)-Chymotrypsin digestion of rabbit skeletal triads. Rabbit skeletal triads were treated with either a 1:160 (lanes 2 and 3) or 1:20 (lanes 4 and 5) ratio of \(\alpha\)-chymotrypsin to protein in the presence (+) (lanes 3 and 5) or absence (−) (lanes 2 and 4) of 0.25% CHAPS, analyzed by 3–12% SDS-PAGE, and either stained by Coomassie Blue (panel A) or transferred to nitrocellulose (panels B and C) as described under "Experimental Procedures." Lane 1 contains 100 \(\mu\)g of triads which were incubated in the absence of protease. Arrowheads to the right of panel A identify the migration and digestion of the skeletal ryanodine receptor (RyR) and calsequestrin (Cs). Panels B and C are immunoblots stained with anti 94-kDa protein antibodies revealing the tryptic fragments of the 94-kDa glycoprotein. Monoclonal antibody IIG12 was used to stain panel B, whereas polyclonal guinea pig anti 94-kDa antibodies were used to stain panel C. The molecular weight standards (\(\times 10^3\)) are indicated on the left.

FIG. 5. Tissue distribution of the 94-kDa glycoprotein. Panel A shows mRNA isolated from skeletal muscle (lane 1), brain (lane 2), and cardiac muscle (lane 3) which was probed with the entire insert to the p94k3 clone as described under "Experimental Procedures." A 4.6-kilobase transcript was identified in skeletal muscle (lane 1) but not in the other tissues. Monoclonal antibodies XIIH112 and IIG12 (panel B) and polyclonal antibodies (panel C) against the skeletal 94-kDa were used to probe 100 \(\mu\)g of microsomes prepared from whole rabbit skeletal (lane 1), brain (lane 2), and cardiac (lane 3) microsomes as described under "Experimental Procedures." The molecular weight standards (\(\times 10^3\)) are indicated on the left.

the presence and absence of detergent. The absence of a signal sequence as determined by both protein sequencing and amino acid analysis also supports this topology. Thus, the transmembrane domain is predicted to separate the small amino-terminal/cytosolic domain from the much larger carboxyl-terminal/luminal domain. The most striking property of the sequence is the highly charged nature of the protein with the large surplus of basic residues in the luminal domain. The luminal domain contains an excess of 46 basic residues which are spread throughout this domain and are represented in Fig. 6 by the positive symbols. The luminal domain is predicted to contain two potential N-linked glycosylation sites consistent with the biochemical results. Interestingly, the amino terminus of one tryptic fragment was sequenced and found to overlap with the sequence surrounding the asparagine at residue 625. This residue was not identified from this peptide sequencing, which strongly suggests that it was post-transla-
tionaly modified by the addition of an oligosaccharide chain. Various methodologies have identified proteins of 71 (Chadwick et al., 1988), 106 (Zaidi et al., 1989a, 1989b), and 95 kDa (Kim et al., 1990) which are proposed to be localized to the triad junction and important in calcium homeostasis. The 71-kDa protein is thought to be involved in coupling the T-system to the terminal cisternae based on reactivity with photolabeled ryanodine receptor (Chadwick et al., 1988). This protein was subsequently identified as albumin and is thus unlikely to perform this function (Knudson and Campbell, 1989).

The 106-kDa protein is proposed to be a calcium channel with channel properties based on bilayer experiments which are very similar to the ryanodine receptor/calcium release channel (Zaidi et al., 1989a, 1989b). These studies showed that the 106-kDa protein migrates just below or just above the (Ca^{2+} + Mg^{2+})-ATPase depending on gel conditions (Zaidi et al., 1989a). Thus, since the 94-kDa glycoprotein also migrates just below the (Ca^{2+} + Mg^{2+})-ATPase it introduces the possibility that the proteins are identical. However, two experiments suggest that the 106- and 94-kDa proteins are distinct. First, the 106-kDa protein was originally identified by sulfhydryl modifying agents that were susceptible to disulfide reducing agents (Zaidi et al., 1989a). Thus the protein was labeled only in the absence of reducing agents on SDS-PAGE, and we have shown that the 94-kDa glycoprotein migrates at much higher molecular weights when run in the absence of reducing agents (Knudson et al., 1990). Second, since the 106-kDa protein is proposed to be a calcium channel with properties very similar to those of the ryanodine receptor one might expect sequence homology between these proteins. In fact, no homology was found between the 94-kDa glycoprotein and the ryanodine receptor, and the proteins are predicted to have vastly different membrane topology. Thus, it seems unlikely that the 94-kDa glycoprotein is related to the 106-kDa protein.

The 95-kDa protein was identified by protein blot overlay with purified dihydropyridine receptor (Brandt et al., 1990) and ryanodine receptor (Kim et al., 1990). This protein has been purified and used to make an affinity column which was shown to bind to the ryanodine receptor and the alpha-subunit of the dihydropyridine receptor (Kim et al., 1990). Based on these results, the authors propose that the protein provides a physical link between these receptors and hypothesize that the 95-kDa protein may be involved in the mechanical coupling of the receptors. Several lines of evidence suggest that the 94-kDa glycoprotein described in this study and the 95-kDa protein are identical. Both proteins are thought to be junctional specific proteins found in the terminal cisternae of the sarcoplasmic reticulum. Similarly, both proteins are enriched in junctional sarcoplasmic reticulum and absent or reduced in T-system and nonjunctional sarcoplasmic reticulum (Brandt et al., 1990). Aside from the similar mobility on SDS-PAGE and the analogous localization of the two proteins, Caswell et al. (1991) showed that the 95-kDa protein has a similar immunostaining pattern on SDS-PAGE when run in the absence of reducing agents (Caswell et al., 1991). This unique property makes it highly likely that the 94-kDa glycoprotein and the 95-kDa protein are identical. The glycoprotein nature of the 95-kDa protein has not been addressed. They have proposed that the 95-kDa protein be named triadin based on its localization and their proposal that it directly interacts with both the dihydropyridine receptor and the ryanodine receptor/calcium release channel (Caswell et al., 1991). The proposed membrane topology in this study suggests that only a very small region of the amino terminus is available in the cytoplasm for interaction with T-system proteins such as the dihydropyridine receptor. However, the evidence in this study provides an alternative explanation to these results. The highly charged and basic nature of the luminal domain of the protein may provide a nonspecific ionic interaction between this protein and the two receptors in question. In fact, the dihydropyridine receptor blot overlay revealed an ionic dependent binding to the 95-kDa protein (Brandt et al., 1990). This hypothesis could be tested by proteolytic cleavage of the amino terminus shown in Fig. 4 followed by protein blot overlay to determine if the specific binding is lost. Thus, in our view, the protein's proposed function as a mechanical link between the two receptors remains highly controversial.

The multimeric nature of the 94-kDa glycoprotein in the absence of reducing agents (Knudson et al., 1993) can be further addressed in light of the sequence information which showed only 2 cysteines at positions 270 and 671 in the proposed luminal domain of the protein. Since the complex is likely composed of homomultimers of the 94-kDa glycoprotein, it would not be possible for intramolecular disulfide bonds to exist. Thus, the alternative is intermolecular bonds between each cysteine and either the analogous cysteine or the opposite cysteine. It is not possible to determine which of these occurs with the available data. Since only 2 cysteines were found in the sequence it is not possible for the multimers to exist as a branched chain. Thus the proteins must be linked in either a linear or a circular fashion.

Since the protein was not identified in either cardiac muscle or brain (Fig. 5), it is possible that the protein performs a skeletal muscle-specific function. The mechanism of calcium regulation in cardiac and brain is clearly distinct from skeletal muscle. Specifically, cardiac excitation-contraction coupling requires external calcium (Nabauer et al., 1989), whereas skeletal muscle excitation-contraction coupling does not (Armstrong et al., 1972; Spiecker et al., 1979; Nabauer et al., 1989). This distinct difference between calcium release suggests a fundamental difference in the regulation of sarcoplasmic reticulum calcium release in these tissues. One possibility is that skeletal muscle contains distinct proteins which account for this fundamental difference. The results shown in Fig. 5 are consistent with the 94-kDa glycoprotein performing a skeletal muscle-specific function, which may account for these differences in excitation-contraction coupling.

Although the exact function of the protein remains unknown, the high abundance and localization of the protein suggest that it plays an important role in excitation-contraction coupling. One possibility is that the protein performs a permissive role in excitation-contraction coupling. An example of this would be functioning as an ion channel which would counter the charge across the sarcoplasmic reticulum membrane which would develop if calcium release were opposed. Although the protein sequence did not show any homology to known ion channels, recent results suggest that proteins that contain a single transmembrane domain may be voltage-sensitive K^+ channels (Takumi et al., 1988). A more likely function of the 94-kDa glycoprotein is that the highly basic luminal domain binds to the highly acidic, calcium-binding protein calsequestrin. Calsequestrin is proposed to sequester calcium near its point of release in the junctional sarcoplasmic reticulum (Fliege et al., 1987). Calsequestrin has been proposed to remain associated with the junctional sarcoplasmic reticulum by interactions with elongated protein strands which were identified from deep-etched rotary-replicated freeze fracture of skeletal muscle fibers (Franzini-Armstrong et al., 1987). The high abundance, predicted charge,
predicted membrane topology, and multimeric nature of the 94-kDa glycoprotein all make it an excellent candidate for the protein that forms these strands and binds calsequestrin. In this model the highly basic luminal domain is proposed to form the strands and extend out from the junctional sarcoplasmic reticulum where it binds to and anchors calsequestrin near the sarcoplasmic reticulum junction.

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