Disulfide Bonds, N-Glycosylation and Transmembrane Topology of Skeletal Muscle Triadin†

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ABSTRACT: Native triadin is a disulfide linked homopolymer of variable subunit number. Two monoclonal antibodies (mAbs), AE8.91 and GE4.90, recognize cytoplasmic regions of triadin between amino acids 110 and 163 and at the C-terminal 34 amino acids, respectively. Triadin in intact triads is largely unaffected by trypsin, while triads whose membrane has been disrupted by hypotonicity or by treatment with the detergent Triton X-100 yield both soluble and membrane bound fragments. Soluble fragments monitored by mAb GE4.90 appear to be formed sequentially during the course of proteolysis at 28, 16, 10 and 7 kDa in the presence of mercaptoethanol. Higher molecular weight bands are observed under nonreducing conditions. A two-dimensional electrophoresis immunoblot (first nonreducing; second reducing) of the soluble fragments developed with mAb GE4.90 shows the presence of several bands which can be interpreted as containing a dimer formed by a combination of any two of the fragments of 16, 10, or 7 kDa present in the digest. MAb AE8.91 does not detect these fragments. This observation indicates that one of the intermolecular disulfide bonds is formed between the identical domains of two triadin molecules at cysteine 671. Immunoblots performed with and without mercaptoethanol of the insoluble fragments using mAb AE8.91 indicate the presence of a dimer formed between identical domains of two triadin molecules with a subunit of 60 kDa. This fragment which was not detected with GE4.90 indicates an intermolecular disulfide linkage at cysteine 270. The glycosidase endo F/N-glycosidase F changed the mobility of intact triadin in TC/triads and its proteolytic fragments detected by mAb GE4.90. It decreased the mass of the 46, 28, and 16 kDa soluble fragments, detected by mAb GE4.90, but not the 10, 7, and 5 kDa species confirming that the asparagine at residue 625 is glycosidated. The cytoplasmic location of the mAbs together with the conditions of tryptic digestion and the glycosylation site at N_{625} provides the framework of a model of the transmembrane topology of triadin in which each disulfide resides in a separate segment of a membrane-spanning β sheet or similar extended structure. An additional β sheet segment and an α helix comprise two more membrane-spanning domains of triadin giving rise to a model with four membrane transits and extensive cytoplasmic and luminal regions. The α helix segment shares some identity with the M2 segment of the ryanodine receptor while the β sheet segments resemble the sequences of the dihydropyridine receptor which are considered to line the pore of the Ca^{2+} channel. This latter comparison suggests the possibility that triadin may be a channel.

The triad junction of skeletal muscle contains the components which transduce the signal of depolarization of the T-tubule[1] to evoke Ca^{2+} release from the SR. Despite years of exhaustive research by many investigators, it is still unclear how the rapid Ca^{2+} release from SR is coupled to the T-tubule membrane depolarization. It is now generally thought that the primary mechanism of signal transmission in skeletal muscle is through direct communication between the DHPr as the voltage sensor in T-tubule and the JFP as the Ca^{2+} release channel in SR (Schneider & Chandler,1973; Rios & Brum, 1987; Tanabe *et al.*, 1987; Block *et al.*, 1988; Kim *et al.*, 1990). There is to date no evidence indicating the direct interactions between these two proteins. A third intrinsic protein of the triad junction, triadin, is in immediate juxtaposition to the JFP in SR (Lewis Carl *et al.*, 1995) and interacts with both the DHPr and JFP (Brandt *et al.*, 1990; Kim *et al.*, 1990). Triadin is therefore a candidate for holding the T-tubule in contact with the SR through its binding to the DHPr. On the basis of these experimental findings, we proposed that triadin may be important for maintaining a tight association of triad junction structure and possibly also to mediate the signal transmission between the DHPr and JFP as an essential link of skeletal muscle activation (Kim *et al.*, 1990).

Recently, the cDNA sequence for triadin was determined (Knudson *et al.*, 1993b). The authors proposed, largely on the basis of the hydropathy plot, that triadin has only one transmembrane domain between residues 48 and 68 that separates the protein into a short cytoplasmic and a long luminal domain. In the accompanying paper (Fan et al., 1995), we have employed triadin fusion peptides to localize the binding epitopes for the two triadin mAbs AE 8.91 and GE4.90to residues 110−163 and 670−706, respectively. Since both antibodies have cytoplasmic epitopes, there is at

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[1] Abbreviations: SR, sarcoplasmic reticulum; TC, terminal cisternae; T-tubule, transverse tubule; DHPr, dihydropyridine receptor; JFP, junctional foot protein; mAb, monoclonal antibody; SDS−PAGE: sodium dodecyl sulfate−polyacrylamide gel electrophoresis; EDTA, ethylenediaminetetraacetic acid; PMSF, phenylmethanesulfonyl fluoride; Tris, tris(hydroxymethyl)aminomethane; Tween 20, polyoxyethylene-sorbitan monolaurate.

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least one and possibly two other cytosolic domains of triadin in addition to the N-terminal 47 amino acids.

The previous data were not sufficient to distinguish a definitive membrane topology for this protein. In this paper we have added further information on the structure of triadin through observation of its disulfide bond and glycosylation and proteolytic degradation patterns which allows us to present a more refined picture of the organization of the protein in the membrane. A comparison of the membrane-spanning regions of triadin with those of related and associated Ca^{2+} channel proteins suggests a new interpretation of the role of triadin in muscle activation.

MATERIALS AND METHODS

Trypsin Digestion. TC/triad vesicles were prepared from rabbit back muscle as described by Caswell *et al.* (1978). The pellet was resuspended at a protein concentration of 8 mg/mL either in 1 M NaCl, 20 mM Tris-HCl, pH 7.4, 0.1 mM $CaCl_2$, and the protease inhibitors leupeptin and pepstatin (NaCl buffer) or when vesicles were treated with 1% Triton X-100 in 250 mM sucrose, 2 mM histidine, pH 7.4, 0.1 mM $CaCl_2$, and the protease inhibitors (sucrose buffer). Trypsin digestion was carried out at 22 °C with 6.25 μg of trypsin/mg of protein. The medium used is specified in the figure legends. Reactions were terminated by addition of 25 μg of soy bean trypsin inhibitor/mg of protein. All the chemicals were purchased from Sigma unless specially stated.

For the two-dimensional immunoelectrophoresis, water-soluble trypsin fragments were partially purified and enriched by DEAE chromatography. TC/triads were suspended in NaCl buffer and trypsinized for 15 min. The reaction mixture was then diluted 2-fold with NaCl buffer and centrifuged in a Beckman rotor Ti 70 at 40 000 rpm for 45 min. The supernatant was dialyzed against 4 L of 20 mM Tris-HCl, pH 7.4, at 0 °C overnight and loaded onto a 2 mL DEAE Sepharose column pre-equilibrated in 20 mM Tris-HCl, pH 7.4. Peptides were eluted by 100 mM NaCl, and 20 mM Tris-HCl, pH 7.4 and assayed for triadin by ELISA with mAb GE4.90. The fractions which gave significant antibody response were pooled and dialyzed against 4 L of 10 mM NH_4HCO_3 for 4 h and then lyophilized.

Electrophoresis and Western Blotting. SDS−PAGE was performed as described by Laemmli (1970) or by Schagger and Von Jagow (1987) as specified in the figure legends. For the two-dimensional electrophoresis, the proteolytic fragments were first electrophoresed under nonreducing conditions on 0.75 mm Laemmli slab gels. A lane was excised, incubated in 50 mM Tris-HCl, pH 7.4, 1% SDS, and 2.5% mercaptoethanol for 15 min at 22 °C and loaded into a preformed well on a 1 mm Laemmli slab gel. Thioglycolic acid (1 mM) was added into the upper chamber buffer. Gels were electroblotted onto nitrocellulose (Bio-Rad) for 2 h at 12 V in 50% Laemmli running buffer containing 20% CH_3OH. The immunoenzymic staining of Western blots was carried out as described by Kawamoto *et al.* (1986) with the modifications described in the companion paper (Fan et al., 1995).

Deglycosidation. Triadic fragments produced by trypsinolysis as specified in the figure legends were incubated in 50 mM KP_i, pH 7.2, 50 mM EDTA, 2% Triton X-100, 0.2% SDS, and 1% 2-mercaptoethanol (omitted when the experiment was carried out under nonreducing conditions) containing 0.5 unit of endo F/N-glycosidase F (Boehringer Mannheim) for 18 h at 37 °C. Reactions were quenched by the addition of 2% SDS, and the products were analyzed by Western blotting. Control samples were incubated and processed in parallel without the addition of endo F/N-glycosidase F.

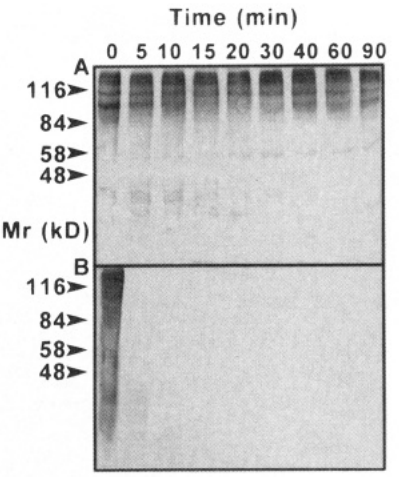

FIGURE 1: Time course for the loss of the mAb GE4.90 epitope of triadin from TC/Triads membranes by trypsinolysis. TC/triads were suspended in sucrose buffer in the absence (A) or presence (B) of Triton X-100. At the times indicated, 200 μL aliquots were quenched by addition of trypsin inhibiter and held on ice. All samples were then centrifuged in a Beckman Airfuge (20 psi for 15 min), and the pellets were resuspended in the original volume. Aliquots (10 μg of protein based on the original TC/triad suspension) were electrophoresed on 6% Laemmli Slab gels under nonreducing conditions and transferred to nitrocellullose. Western Blots were developed with mAb GE4.90. Relative moecular mass was determined with Pre-Stained Molecular Weight Marker kit (Sigma).

RESULTS

Disulfide Bonds. In order to delineate different domains of triadin, the protein was cut by proteolysis, and the fragments were analyzed by Western blotting. Of the several proteases evaluated, trypsin proved to be the most successful at producing soluble fragments detectable by our antibodies. Figure 1 shows the immunoblot for triadin remaining in the pelletable fraction after treatment of TC/triads with trypsin in the presence of isotonic sucrose in the absence (A) and presence (B) of Triton X-100. The gels were run under nonreducing conditions and triadin fragments stained with mAb GE4.90. Figure 1A shows that little breakdown or loss of triadin occurs in the intact TC/triad vesicles for up to 90 min of trypsinization. In constrast, when detergent was added into the digestion buffer (Figure 1B), the detectable triadin fragments disappeared immediately from the pelletable junctional complex. This difference indicates that the major trypsin cleavage sites are occluded in the intact vesicles and may be in the luminal portion of the protein.

The formation of water-soluble proteolytic fragments of triadin from TC/triad vesicles incubated in the absence of an impermeable osmotic buffer such as sucrose is shown in Figure 2. The reaction medium contained 1 M NaCl to enhance the solubility of the triadin fragments. The Western blots were developed with mAb GE4.90 whose epitope is at the C-terminus of triadin. Figure 2A shows the immunoblot of the soluble triadin fragments electrophoresed under

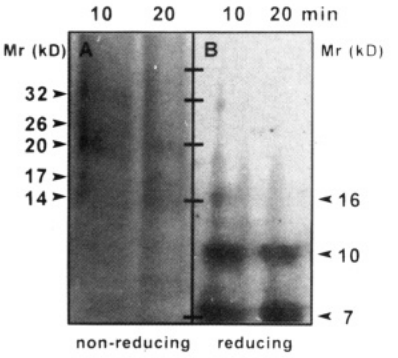

FIGURE 2: Generation of water-soluble disulfide-linked fragments of triadin by trypsinolysis. TC/triads were suspended in NaCl buffer and incubated with trypsin for the times indicated above the gel lanes. Reactions were quenched with trypsin inhibiter and centrifuged. Aliquots from the supernatant were electrophoresed on 5−15% gradient Laemmli slab gels under (A) nonreducing and (B) reducing conditions. Proteins were transferred to nitrocellulose and the Western blots developed by mAb GE4.90. Relative molecular mass was estimated with Rainbow Standards (Amersham).

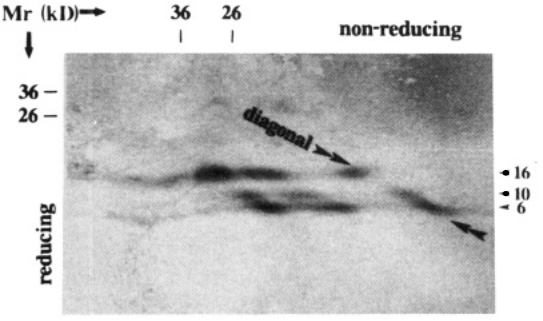

FIGURE 3: Analysis of trypsin generated water-soluble disulfide-linked fragments of triadin by two-dimensional electrophoresis. Water-soluble triadin fragments were partially purified by DEAE chromatography as described in Materials and Methods. The lyophylized peptides were resuspended in 20 mM Tris-HCl, pH 7.4, and 2% SDS and electrophoresed under nonreducing conditions (horizontal dimension). One lane was excised, equilibrated with 2% 2-mercaptoethanol, and re-electrophoresed under reducing conditions (vertical dimension) (see Materials and Methods for details). Peptides were transferred to nitrocellulose and the Western blot was developed with mAb GE4.90. The arrows indicate the position of the diagonal in which the protein migrates at the same R_f in each dimension.

nonreducing conditions. The major fragments have molecular masses of 32, 26, 23, 20, 17, and 14 kDa. Under reducing conditions (Figure 2B), the main bands were observed at 16, 10, and 7 kDa. Smaller peptides may also be generated which are not detected in this particular gel. The fragments seen under nonreducing conditions roughly matched the sums of the different combinations of dimers formed from fragments observed under reducing conditions, suggesting the presence of disulfide bridges between the proteolytic fragments. The higher molecular mass moieties seen under both electrophoretic conditions disappeared when the vesicles were incubated with trypsin for a longer time. This suggests that there is more than one trypsin cutting site and that proteolysis can occur sequentially.

Figure 3 shows a two-dimensional electrophoresis immunoblot of partially purified peptides obtained from the solubilized fraction of TC/triads after trypsin treatment. The first dimension was run in the absence of mercaptoethanol, and the second dimension was run at right angles after reduction. The major fragments identified by immunoelectrophoresis under reducing conditions have molecular masses of 16 and 7 kDa while a minor component appeared at 10 kDa (indicated by arrowheads). Spots corresponding to these monomers are observed lying along the diagonal of the gel (indicated by double arrowhead). In addition, spots are seen lying off the diagonal to the left of the monomer spots indicating the presence of disulfide bridged peptides in the original sample. Each of the spots are interpretable as originating from dimers formed from the combination of the three fragments. These are as follows: 16 kDa + 16 kDa = 32 kDa, 16 kDa + 10 kDa = 26 kDa, 16 kDa + 7 kDa = 23 kDa, 10 kDa + 10 kDa = 20 kDa, 10 kDa + 7 kDa = 17 kDa, and 7 kDa + 7 kDa = 14 kDa. There was no indication of the existence of higher polymers. This electrophoretic pattern demonstrates that one of the intermolecular disulfide bonds of triadin is present in these fragments. The previous identification of the binding epitope of mAb GE4.90 as the C-terminal end of triadin limits the region of these peptides to the C terminus. Only the one cysteine at amino acid 671 occupies a position such that it can be present in a peptide of 16 kDa and contain the antibody epitope.

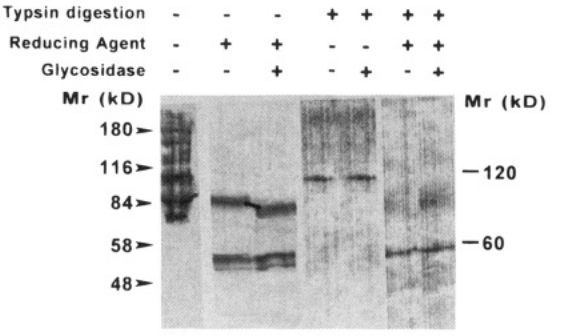

FIGURE 4: Deglycosylation of intact and water-insoluble trypsin fragments of triadin which retain the mAb AE8.91 epitope. TC/triads were trypsinized in sucrose medium containing 1% Triton X-100. Reaction mixtures were then centrifuged and the pellets were incubated in the absence or presence of endo F/N-glycosidase F as described in Materials and Methods. Each sample was electrophoresed on a 7.5% Laemmli slab gel, the proteins were transferred to nitrocellulose, and the Western blots were developed with mAb AE8.91. Lanes 1−3 show the intact triadin under nonreducing without endo F/N-glycosidase F (lane 1) and reducing conditions without (lane 2) or with endo F/N-glycosidase F (lane 3). Lanes 4−7 show triadin proteolytic fragments under nonreducing (lanes 4 and 5) and reducing conditions (lanes 6 and 7) without (lanes 4 and 6) or with endo F/N-glycosidase F treatment (lanes 5 and 7).

Consistent with our previous observation using mAb GE4.90 (Caswell *et al.*, 1991), triadin, monitored by mAb AE8.91, forms disulfide bridged multimers in the absence of the reducing agent, mecaptoethanol (Figure 4, lane 1). Upon the addition of 1% (v/v) mecaptoethanol (Figure 4, lane 2), this ladder of bands was replaced by monomeric triadin with a molecular mass of 95 kDa. A lower band of 60 kDa is detected by mAb AE8.91 which may be a breakdown product or a homolog of triadin. When trypsin was incubated with the TC/triad vesicles in the presence of Triton X-100, the ladder of triadin multimers (lane 1) was replaced by a single band of 120 kDa (lane 4) under nonreducing conditions and 60 kDa (lane 6) under reducing conditions. The change in molecular mass upon addition of reducing agent indicates the presence of a disulfide-linked dimer, composed of identical sized triadin fragments. Since mAb AE8.91 has an epitope in the region between amino acids 110−163 which is close to that of cysteine at amino

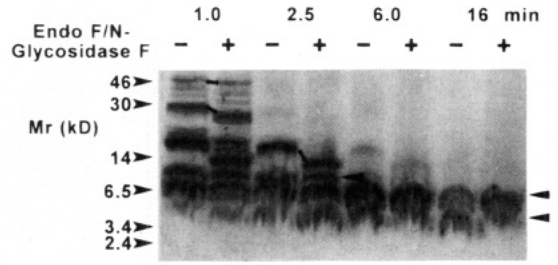

FIGURE 5: Deglycosylation of trypsin fragments of triadin which retain the mAb GE4.90 epitope. TC/triads were suspended in sucrose medium containing 1% Triton X-100 and centrifuged. The pellet was resuspended in sucrose medium and incubated with trypsin. At the times indicated above the paired gel lanes, samples were quenched with trypsin inhibiter and incubated in the absence or presence of EndoF/N-Glycosidase F as described in Materials and Methods. Equal volume aliquots from each sample were electrophoresed in adjacent lanes on a Schagger−Von Jagow gel. Proteins were transferred to nitrocellulose and the Western blot developed with mAb GE4.90. Proteolytic fragments whose molecular masses stayed constant after endo F/N-glycosidase F treatment are indicated by arrowheads. Those bands shifted in molecular by deglycosidation are indicated by double arrows.

acid 270, trypsin treatment generates a membrane-bound fragment formed by an intermolecular bond between two cysteines at amino acid 270. Neither band was detected with mAb GE4.90.

Glycosylation sites. Figure 4 shows the effect of endo F/N-glycosidase F on the mobility of intact triadin in TC/triads and the insoluble triadin proteolytic fragments detected by the antibody AE8.91. The shift of intact triadin from 95 kDa to 92 (lane 2 and 3, indicated by double-arrow) upon addition of the glycosidase indicates the presence of N-glycosylation in triadin. On the other hand the lower molecular mass band is unaffected by the glycosidases. The comparison of the proteolytic fragment of trypsin treatment under nonreducing (lane 4 and 5) and reducing conditions (lanes 6 and 7) indicates no discernible shift in molecular mass associated with glycosidase treatment indicating that the peptide does not contain an N-glycosylation site.

Figure 5 shows the immunoblot of triadin proteolytic fragments of Triton X-100 treated vesicles obtained by trypsin treatment for varying times with or without subsequent incubation with endo F/N-glycosidase F monitored by mAb GE 4.90. The main bands which were observed after short trypsin treatment without endo F/N-glycosidase F are of 44, 28, 16, 10, 7, and 5 kDa. When incubated with endo F/N-glycosidase F, the higher molecular mass bands were replaced by lower bands of 42, 25.5, and 13.5 kDa, respectively. The shift of the mobilities are indicated by double arrows in Figure 5. The mobility change of these fragments after the glycosidase treatment clearly indicates the presence of an N-glycosidated peptide. The three lower bands of 10, 7, and 5 kDa (indicated by arrowheads) remain unchanged indicating the absence of a glycosidated residue. The only asparagine residue which is contained within a consensus site for glycosylation (NxS/T) and which could be detected by mAb GE4.90 is that at position 625.

DISCUSSION

In the previous paper, we determined that the binding site for mAb GE4.90 on triadin is at the C-terminal 34 amino acids while that for mAb AE8.91 is between residues 110 and 163, which gave rise to two possible triadin membrane topologies. In this report we employed both of these antibodies to track the disposition of the disulfide bonds and *in vivo* glycosylation sites after trypsin proteolysis.

Triadin in SR vesicles exists in the form of a homopolymer composed of a variable number of disulfide linked monomers (Caswell *et al.*, 1991). Triadin only contains two cysteines throughout its sequence, and therefore both of these must participate in the intermolecular disulfide linkage. Three possible modes of linkage are represented by (1) cysteine 270 forming a bond with cysteine 671 on the neighboring molecule, (2) cysteine 270 binding to cysteine 270 on its neighbor, while cysteine 671 binds to cysteine 671 on a second neighbor, and (3) an intermediate model in which some bonds are to the identical cysteine of a neighbor while others are to the complementary cysteine. Our observations are consistent with the second proposition in which the disulfide bonds occur between identical cysteines of neighbors. The observations on the water-soluble fragments using GE4.90 indicate that several peptides are formed sequentially which are detected by the antibody. The molecular mass of the peptides is too small to include cysteine 270. These peptides, nevertheless, are present as dimers formed with each other. We cannot totally preclude the possibility that a second peptide which is not detected by the antibody or by antibody, AE8.91, is linked by disulfide bonds to the first. However, the data would then require that this peptide have the same identical three masses as those detected by the antibody under reducing conditions and are present in approximately the same ratio. Since this is unlikely, we have made the minimalist assumption that the peptides forming the disulfide link are identical. The primary insoluble fragment detected with AE8.91 also is present as a dimer of two units of 60 kDa. This fragment includes the epitope for the antibody at amino acids 110−163 but does not include the glycosylation site at amino acid 625. The fragment therefore contains cysteine 270, but not 671. Thus tryptic digestion separates triadin into distinct peptides each of which contains a single cysteine intermolecularly linked by a disulfide bridge. These observations support proposition 2.

Triadin is known to be a glycoprotein which contains four consensus sites for N glycosylation at amino acids 9, 21, 75, and 625. Knudson *et al.* (1993b) have excluded amino acid 9 as a site since their direct sequencing revealed an unmodified asparagine. They suggested that amino acid 625 could be glycosidated since their sequencing failed to detect the asparagine residue in this region. Our observations in this paper directly confirm the glycosylation of residue 625, since this is the only site which could be present in the soluble peptides which are modified in size by endo F/N-glycosidase F. The larger peptides detected by mAb GE4.90 down to 16 kDa are all cut by the glycosidase. This estimate of molecular mass is only approximate since triadin does not migrate in SDS gels according to its true molecular mass determined by sequencing (Knudson *et al.*, 1993b). A peptide which just includes residue 625 as well as the C terminus should have an molecular mass of approximately 9 kDa. If allowance is made for the slow migration of the peptide in the gel relative to its calculated size, the minimum peptide size which will contain both N_{625} and the C terminus will be 10 kDa, consistent with the response to the glycosidase.

It has proved more difficult to establish whether the other asparagines are glycosidated. Residue 21 is an unlikely

candidate, since it is most likely to be cytoplasmic. Residue 75 is considered to be luminal in the model of Knudson *et al.* (1993b) or in either model which we presented in the previous paper. Two lines of evidence, however, generally support the view that N_{75} is unmodified. First, the extent of alteration of molecular mass of intact triadin caused by endo F/N-glycosidase F is 3 kDa according to our estimates (Figure 4, lanes 2 and 3) and 2 kDa according to Knudson *et al.* (1993a) using endoglycosidase H. We find that the tryptic peptides of 44, 28, and 16 kDa which are modified by endo F/N-glycosidase F are reduced by 2, 2.5, and 2.5 kDa in order of decreasing molecular mass. The data are most accurate for the smallest peptide. Thus, within experimental error, a single site of N glycosylation can account for the effect of endo F/N-glycosidase F on intact triadin. Secondly, the 60 kDa peptide detected by AE8.91 is unaffected by endo F/N-glycosidase F. This peptide does not include N_{625} but does include the epitope for the antibody and C_{270}. Unfortunately, this leaves some margin for doubt as to whether it includes N_{75}. However, a further line of reasoning suggests that it is predominantly an N-terminal peptide. The first major C-terminal peptide detected with GE4.90 has a molecular mass of 28 kDa which is rapidly cut further. If this peptide represents the first major cutting site, then it should split triadin into fragments of 67 and 28 kDa. The 67 kDa fragment is close to the size of the 60 kDa fragment detected with AE8.91, which is therefore likely to include the N-terminal region and certainly will include N_{75}. Our evidence is, therefore, generally in favor of a single glycosylation site at residue 625.

The observations of this paper are sufficient to delineate the two transmembrane topologies considered in the previous paper. These models are based on certain assumptions: (1) We presume that the N terminus is cytoplasmic, since triadin contains no signal sequence which would allow for the N terminus to cross the endoplasmic reticulum membrane and posttranslational cleavage is confined to the single N terminal methionine (Knudsen *et al.*, 1993a). The absence of the signal sequence has been used to argue the N-terminal cytoplasmic loci of many other membrane proteins (Catterall, 1988). (2) We assume that amino acid residues 48−68 cross the membrane as an α helix, since this region is uniformly hydrophobic and of sufficient length to cross the membrane. In addition, the hydropathy plot detects three other regions at residues 102−111, 267−278, and 665−674 which are sufficiently hydrophobic and of sufficient length that they could cross the membrane as β sheets, β barrels, or similar structures. The rest of the sequence is highly hydrophilic. (3) The antibody binding data from the previous paper indicate that residues 110−163 and 675−706 are cytoplasmic.

Two lines of evidence favor a luminal location of residues from 278 to 665. First, trypsin is ineffective in cleaving triadin unless the membrane is rendered permeable by either hypotonic breakage or by detergent treatment. When the vesicles are permeable, the principal sites of cleavage are in the region of amino acids from 470 to 650 indicating a luminal locus. Second, N-glycosylation occurs inside the endoplasmic reticulum during protein processing so that sites of glycosylation are luminal. This indicates that residue 625 is luminal.

The combined observations and analyses of these two papers suggest the model of triadin shown in Figure 6. This

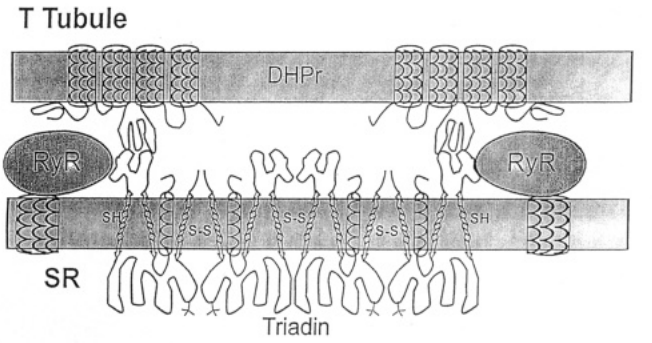

FIGURE 6: Model for the membrane topology of triadin and its interactions with the DHPr and the JFP in the triad junction. This model depicts triadin transiting the TC membrane by one α-helix (residues 48−67) and three β sheets or similar extended structures (residues 102−111, 267−278, and 665−674 respectively). Both of the cysteines, C_{270} and C_{671}, are located in the transmembrane domains, and the intermolecular disulfide linkages between the identical domains of triadin are shown by S−S. The experimentally identified glycosylation site at N_{625} is located in the lumen of the SR as indicated by the forks. A cytoplasmic site of interaction between the II−III loop of the DHPr and triadin is indicated. The JFP, located in the SR, could interact with triadin in the cytosol, in the membrane, or in the lumen of SR since both proteins have extensive cytoplasmic and luminal domains.

figure places the N terminus in the cytoplasm. Residues 48−68 form a membrane-spanning α helix, and residues 102−111 form a β sheet segment to transmit the peptide back into the cytoplasm so that the binding epitope for mAb AE8.91 (residues 110−163) is cytoplasmic. A second membrane-spanning β sheet domain at residues 267−278 carries the structure into the lumen and forms a disulfide linkage with a second triadin molecule within the membrane. This luminal re-entry allows a luminal locus for both the trypsin cleavage sites and asparagine 625. A final membrane-spanning β sheet domain gives rise to a second disulfide bridge in the membrane and stipulates a cytoplasmic C terminus which is the epitope site for mAb GE4.90. The data from the previous paper indicate that the domain from residues 110−267 contains a binding site for the DHPr. The binding region for the JFP has not been designated from these papers but may include cytoplasmic, membrane, or luminal sites.

Although in the discussion above we have concluded that the membrane spanning domains include three β sheet domains, it remains possible that the membrane transit could be accomplished by some other structure. The β structure format is likely to be favored, since it satisfies the hydrogen bonds of the peptide backbone within the membrane. Similar β structures have been proposed for the voltage gated channels and the nicotinic receptor (Yellen *et al.*, 1991; Hartmann *et al.*, 1991; Morrissette *et al.*, 1993; Tang *et al.*, 1993; Unwin, 1993). In the former case the concept that the structure serves as the lining of the channel pore has given rise to the proposal that the hydrogen bonds between the segments are folded round to give a β barrel. This is also a reasonable structure for triadin, since it avoids the need to consider unbonded edges to the β sheet. We cannot predict how many β segments would be required to close the ring. Triadin forms a polymer of varying size which might suggest a variability of the size of the ring, but it is also possible that the polymer contains more than one barrel.

The proposed existence of both α helices and β barrels in the membrane has prompted us to search for possible

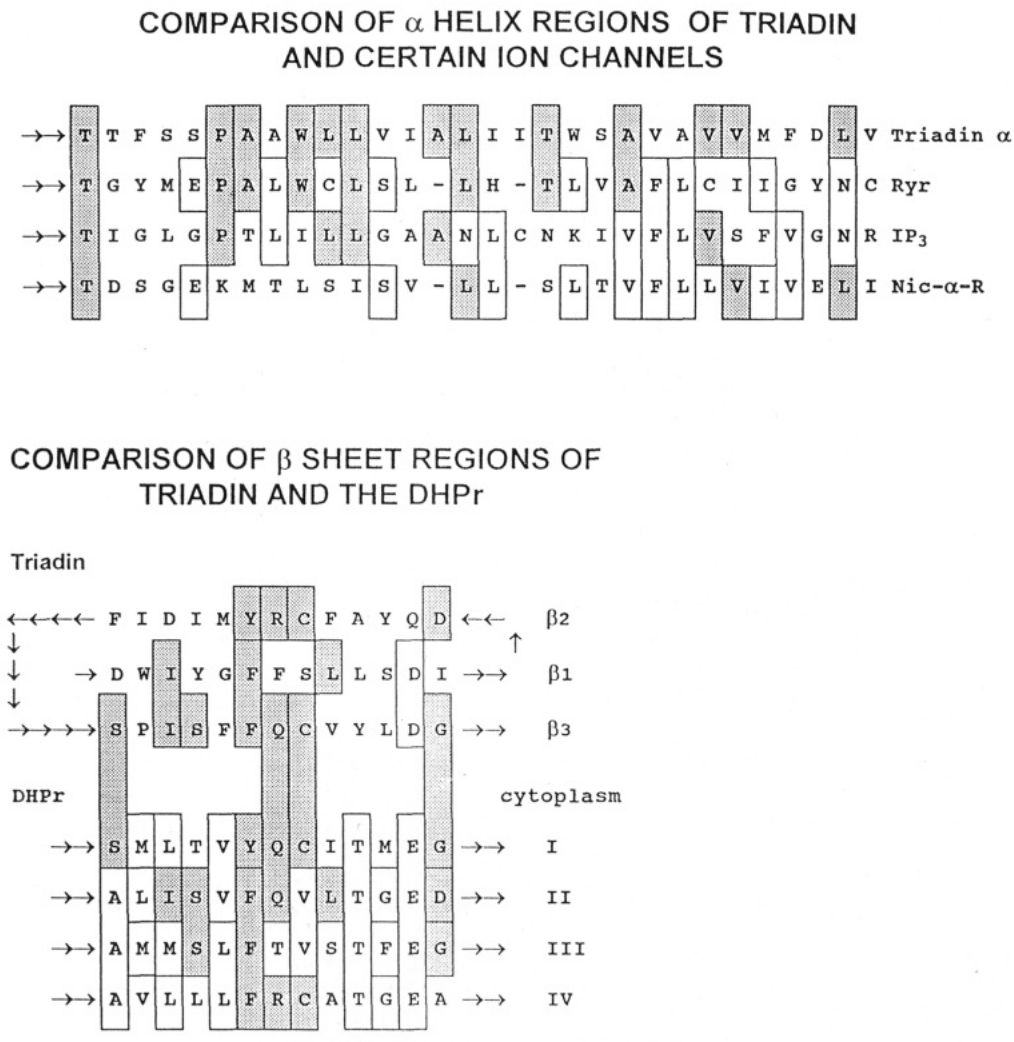

FIGURE 7: Comparision of α-helix and β sheet regions of triadin with transmembrane domains of other proteins. The upper part compares the putative α helix domain of triadin (residues 47−68) with the M2 segment (residues 4637−4664) of the skeletal muscle JFP, inositol 1,4,5-trisphosphate receptor (residues 2301−2330) and m2 segment (residues 237−264) of the nicotinic receptor α subunit. The shaded boxes indicate the alignment of triadin to any of the other three proteins. The open boxes show the alignment between the other three proteins but not with triadin. In the lower part, the putative β structures of triadin are compared to the putative pore-lining sequences of the DHPr α_1 subunit. The top panel shows the putative β2 (residues 267−278), β1 (residues 102−111), and β3 (residues 665−674) sequences of triadin. I (residues 281−293), II (residues 603−615), III (residues 1003−1015), and IV (residues 1312−1324) are the partial sequences of the putative β sheets between the transmembrane segment S5 and S6 in the first, second, third, or fourth homologous repeats of the DHPr α_1 subunit transmembrane domains. The arrows indicate the orientation of the transmembrane domains. The shaded boxes indicate the identity of residues between the proteins, while the open boxes represent identity within either protein.

resemblances of these domains with those of other proteins. Figure 7 is a comparison of membrane-spanning domains of triadin with select regions of other muscle proteins which reveals a surprising range of sequence resemblance of triadin to segments of these proteins. The top portion shows that the putative α helix of triadin aligns with the M2 segment (Takeshima *et al.*, 1989) of the JFP and to some extent with the inositol 1,4,5-trisphosphate receptor (IP_3r, Südhof *et al.*, 1991) and the m2 segment of the nicotinic receptor α subunit (Noda *et al.*, 1983). In order to enhance the alignment, it is necessary to assume that two deletions or insertions have occurred. This is also true in aligning the IP_3r with the JFP. Since the deletions or insertions are associated with a rotation of the residues around the axis of the helix, it is unlikely that a specific functional feature is contained in these alignments. On the other hand, the sequence resemblance implies a distant evolutionary kinship of each of these four proteins.

More tantalizing is the sequence resemblance of the putative β structures of triadin with the putative pore lining sequences of the DHPr α_1 subunit (Tanabe *et al.*, 1987; Tang *et al.*, 1993) shown in the lower portion of Figure 7. This alignment has been made taking account of the direction of transit across the membrane such that the first and third β segments (DWIG−−, SPIS−−) run from left to right, while the second (DQYA−−) runs from right to left. For both proteins, the right side is cytoplasmic. The shaded boxes represent identity of residues between the proteins, while the open boxes indicate residue identity within either protein. The alignment of residues is at least as good as that among different classes of voltage gated channels. Certain features of this alignment should be emphasized. The third segment of triadin (β3) is so similar to the first (I) and second (II) SS1 segments of the DHPr that every residue is represented by an identical one or a broadly conservative substitution. The alignment does not require the inclusion of insertions or deletions. The penultimate residue (E) in the DHPr, which is considered to determine the ion selectivity, is retained in triadin as a D. Negatively charged residues are also represented at the other end of the putative membrane-

spanning region. A good alignment is seen with triadin segment 2 even though this requires comparing residues running in reverse along the sequence. All of these features suggest the conjecture that triadin may itself be an ion channel which contains possible negatively charged energy wells at either end to facilitate cation transport and to give the ion selectivity. Although there is no direct evidence for a channel function for this protein, we are unaware of any experimental evidence which rules this out.

It is interesting to speculate how such a channel might operate to facilitate signal transmission in muscle. The II–III cytoplasmic loop of the DHPr may attach to the cytoplasmic segment of triadin to form a rigid rod connecting these two proteins. The II–III segment may itself be connected through the membrane-spanning domains of the DHPr to the positively charged s4 segments which are physically translated outward (~5–10 Å) during membrane depolarization. Thus it is possible that the connection between the two proteins may serve as a plunger which allows the DHPr to activate the triadin Ca^{2+} channel (cf. Chandler *et al.*, 1976). The efflux of Ca^{2+} from the SR may in its turn open the JFP through its Ca^{2+} activation function to allow amplification of the signal. Although we recognize that this mechanism is speculative, it might serve to revive the concept of a Ca^{2+}-triggered Ca^{2+} release which is broadly analogous to the mechanism which applies in the heart, but in which the source of the trigger Ca^{2+} is the SR rather than the T-tubule. A similar mechanism has been proposed by Jaquemond *et al.* (1991), but in their model the JFP fulfills both the roles of primary triggering and of secondary release.

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