Binding Sites of Monoclonal Antibodies and Dihydropyridine Receptor α_1 Subunit Cytoplasmic II−III Loop on Skeletal Muscle Triadin Fusion Peptides†

Hongran Fan,‡ Neil R. Brandt,‡ Mei Peng,§ Arnold Schwartz,§ and Anthony H. Caswell*,‡

Department of Molecular and Cellular Pharmacology, University of Miami School of Medicine, Miami, Florida 33136, and Institute of Molecular Pharmacology and Biophysics, University of Cincinnati, Cincinnati, Ohio 45267-0828

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ABSTRACT: Triadin binds to the dihydropyridine receptor (DHPr) and the junction foot protein (JFP) in Western blot protein overlay experiments. Fusion peptides were synthesized using an expression system, pGSTag, which includes a protein kinase A phosphorylation site. Expressed peptides are $DHPr_{664-799}$ encoding rabbit skeletal DHPr α_1 subunit amino acids 664−799, triadin 1 (1−49), triadin 2 (68−389), triadin 2′ (110−389), triadin 2a (68−278), triadin 2a1 (67−163), triadin 2a2 (165−240), triadin 2b (242−389), triadin 2b1 (242−299), triadin 3 (370−706), triadin 3a (370−562), triadin 3b (551−706), triadin 3b1 (551−672), and triadin 3b2 (673−706) (the numbers in parentheses correspond to the amino acid sequence of triadin). The triadin monoclonal antibodies, GE4.90 and AE8.91, bind to intact triadic vesicles as well as to vesicle fragments prepared after treatment with Triton X-100, indicating that they have cytoplasmic epitopes. MAb AE8.91 binds to triadin 2, 2′, 2a, and 2a1, while mAb GE4.90 binds to triadin 3, 3b, and 3b2 indicating that residues 110−163 and the C-terminal 34 amino acids contain cytoplasmic domains. Radiolabeled $DHPr_{664-799}$ binds to triadin in intact vesicles under nonreducing and reducing conditions. It binds to triadin fusion peptides, triadin 2, 2a, 3, 3b, and 3b1, but not to triadin 1 or triadin 3b2. The binding to triadin 2a is the most prominent. Direct binding between $DHPr_{664-799}$ and JFP was not seen. These experimental findings indicate that triadin contains an extensive cytoplasmic domain that binds to the domain of DHPr which is considered critical for signal transmission during skeletal muscle excitation−contraction coupling.

In vertebrate skeletal muscle, E−C[1] coupling requires the transmission of the signal of surface membrane depolarization into the interior of the muscle. The signal changes character from an electric potential at the surface to the specific release of Ca^{2+} from the SR. Transmission appears to be confined to the triad junction which is composed of two TCs on either side of the T-tubule. The DHPr in the T-tubule (Rios & Brum, 1987; Tanabe *et al.*, 1987) and JFP in SR (Cadwell & Caswell, 1982; Lai *et al.*, 1987; 1988; Campbell *et al.*, 1987; Inui *et al.*, 1987; Imagawa *et al.*, 1987; Hymel *et al.*, 1988) are two essential components in skeletal muscle E−C coupling.

The DHPr consists of five subunits of which the α_1 is the central component; it functions as a Ca^{2+} channel and as a transducer of E−C coupling (Vaghy *et al.*, 1987; Smith *et al.*, 1987; Talvenheimo *et al.*, 1987; Rios & Brum, 1987; Tanabe *et al.*, 1987; Sharp *et al.*, 1988). The critical role of the DHPr in E−C coupling was confirmed by experiments which showed that both the intramembrane charge movement and E−C coupling, which are absent in myotubes from dysgenic mice lacking the DHPr α_1 subunit, could be restored by introduction of cDNA for this subunit into the muscle (Numa *et al.*, 1990). The restoration can be effected by cDNA for either the skeletal or cardiac isoform of the protein, but with significant differences in the mode of transmission. The cardiac cDNA recreates contraction which requires external Ca^{2+}, while the skeletal isoform generates contraction similar to that of wild-type which does not require Ca^{2+} influx (Tanabe *et al.*, 1990). The α_1 subunit of the DHPr has four internal repeats of homologous sequences joined by putative cytoplasmic domains. The insertion of chimerae containing both skeletal and cardiac sequences of the DHPr into dysgenic fibers has allowed the designation of the cytoplasmic loop between the second and third homologous repeats as the likely controling site of muscle activation (Tanabe *et al.*, 1990; Beam *et al.*, 1992).

The JFP was originally described and isolated as a high molecular weight protein (Cadwell & Caswell, 1982), which was later identified as the ryanodine sensitive Ca^{2+} release channel in SR (Imagawa *et al.*, 1987; Lai *et al.*, 1988; Smith *et al.*, 1988). A targeted mutation in the skeletal muscle ryanodine receptor has shown that the skeletal muscle receptor is essential for both muscle maturation and E−C coupling (Takeshima *et al.*, 1994). The channel is not strongly voltage gated and therefore cannot be activated directly by membrane potential changes in the T-tubule or

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* Author to whom correspondence should be addressed.

‡ University of Miami School of Medicine.

§ University of Cincinnati.

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[1] Abbreviations: SR, sarcoplasmic reticulum; TC, terminal cisternae; T-tubule, transverse tubule; DHPr, dihydropyridine receptor; JFP, junctional foot protein; E−C coupling, excitation−contraction coupling; mAb, monoclonal antibody; GST, glutathione *S*-transferase; PCR, polymerase chain reaction; SDS−PAGE: sodium dodecyl sulfate−polyacrylamide gel electrophoresis; LMP, low melting point; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; IPTG, isopropyl-β-D-thiogalactopyranoside; MOPS, 3-(*N*-morpholino)propanesulfonic acid; PMSF, phenylmethanesulfonyl fluoride; Tris, tris(hydroxymethyl)-aminomethane; TMBS, 2, 2′-azinobis(3-ethylbenzthiazoline-6-sulfonic acid).

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SR membranes (Catterall, 1991). The action potential must therefore generate a chemical or physical signal that activates the Ca^{2+} release channel in the SR.

Various hypotheses have been proposed to describe the mechanism of E−C coupling in skeletal muscle. Several low molecular weight compounds, including Ca^{2+} (Ford & Podolsky, 1970; Endo *et al.*, 1970), inositol 1,4,5-trisphosphate (IP_3) (Volpe *et al.*, 1990; Vergara *et al.*, 1990), cADP-ribose (Morrissette *et al.*, 1993), H^+ (Shoshan *et al.*, 1981), and thiols (Tatsumi *et al.*, 1988; Zaidi *et al.*, 1989) have been considered as soluble transmitters in this process, but none has been found to have the correct physiological effects and kinetics of action. Recent studies have focused on a mechanism which invokes direct communication between the DHPr and the JFP (Schneider & Chandler, 1973; Rios & Brum, 1987; Tanabe *et al.*, 1987; Block *et al.*, 1988; Kim *et al.*, 1990). The molecular formulation of this hypothesis proposes that the DHPr is the T-tubule voltage sensor and the JFP is the SR Ca^{2+} release channel. Although no evidence has been presented to show the direct binding of these two proteins, the expressed cytoplasmic loop between the second and third homologous domain of the DHPr has been reported to activate opening of the JFP in lipid bilayers and to enhance the binding of ryanodine to SR vesicles (Lu *et al.*, 1994).

We have identified a protein, triadin, as an intrinsic membrane protein confined to the junctional region of the TC which binds both to the DHPr and the JFP (Brandt *et al.*, 1990; Kim *et al.*, 1990; Motoike *et al.*, 1994). It has an apparent molecular mass of 95 kDa and was observed in vesicles as a ladder of multimers formed by disulfide bonds from the 95 kDa monomers (Caswell *et al.*, 1991). A monoclonal antibody against triadin (GE4.90) inhibits T-tubule induced Ca^{2+} release from SR, while it has no effect on directly stimulated Ca^{2+} release by caffeine (Brandt *et al.*, 1992). Liu and Pessah (1994) have proposed that triadin and the JFP may be joined by disulfide bonds when the Ca^{2+} release channel is activated. These experimental results indicate that triadin not only may physically hold the T-tubule in contact with the SR through its binding to the DHPr but also may functionally mediate the signal transmission between the DHPr and JFP and be an essential link or modulator of muscle activation.

Recently, the cDNA sequence for triadin has been determined (Knudson *et al.*, 1993; Peng *et al.*, 1994). The membrane topology was predicted from the hydropathy plots to contain a single transmembrane α helix. Primarily on these grounds, the authors concluded that triadin contained a short cytoplasmic N-terminal sequence followed by a hydrophobic α helix which comprises the single transit across the SR membrane so that the rest of the protein was located in the lumen. The limited extension of the protein in the cytoplasm was considered to cast doubt on the reality of the role of triadin in binding to the DHPr and in maintaining the T-tubule−SR contact.

In this report we have studied the triadin membrane topology directly using two monoclonal antibodies with cytoplasmic epitopes to triadin and have resolved the binding sites of triadin to the $DHPr_{664-799}$ by the use of fusion peptides of both the rabbit skeletal muscle DHPr α_1 subunit and triadin. On the basis of our experimental evidence, we are proposing modified triadin membrane topologies, either of which give extensive cytoplasmic domains which may interact with other cytoplasmic triadic proteins.

MATERIALS AND METHODS

Antibody−Membrane interactions. The relative binding of antibodies to intact and Triton X-100-treated membranes was assessed either by observation of the depletion of free antibody in the presence of antigen or by assaying the retention of the antibody−membrane complex after centrifugal washing. In both procedures, TC/triads were isolated by the method of Caswell *et al.* (1976) and resuspended in 250 mM sucrose, 20 mM MOPS, pH 7.4, and 5 mM EDTA at 2 mg/mL. The preparation was divided in two, and to one portion Triton X-100 (0.5 mg/mg of protein) was added. The suspensions were centrifuged to equilibrium on a step gradient of 20% sucrose in 20 mM MOPS and 65% sucrose. The material at the 20%/65% interface was collected and diluted to the same volume as the starting material in 250 mM sucrose and 20 mM MOPS, pH 7.4 (sucrose/MOPS).

The mAb GE4.90 was purified from mouse ascites and diluted to a stock concentration of 5 μg/mL in sucrose/MOPS; mAb AE8.91 was used directly from tissue culture medium or in some cases purified from mouse ascites and diluted into sucrose/MOPS. Equal volumes of antibody and intact or Triton-treated membranes were mixed and incubated for approximately 2 h with continuous rocking at 22 °C. Aliquots (100 μL) were centrifuged on a 20%/65% sucrose step gradient in 250 μL tubes in a Beckman Airfuge.

The depletion of antibody from suspension was assayed as the fraction of antibody remaining in suspension after incubation with TC membranes and separation of bound antibody by centrifugation on sucrose step gradients. The total antibody was determined from a control antibody solution without membranes which was centrifuged identically to the membrane preparations. Aliquots (10 μL) from the uppermost layer of sample and control were added to 200 μL TBS (200 mM NaCl and 10 mM Tris-Cl, pH 7.4) and serially diluted in 2-fold steps in microtiter plates. The wells were blocked with 5% nonfat dry milk in TBS−Tween 20 (0.1%) and developed with biotinylated anti-mouse Ig and streptavidin peroxidase (Amersham) with ABTS (Sigma) and H_2O_2 as substrates. Depletion was calculated from the ratio of antibody retained in the control supernatants to that retained in the membrane reacted supernatants using the means of the ELISA absorbances at the different dilutions.

The retention of the antibody−membrane complexes was assayed in the material banding at the 20%/65% sucrose interface. The antibody−membrane incubation mixtures were centrifuged on the sucrose step gradients described above and the upper 150 μL was removed. The material banding at the 20%/65% interface was mixed with 50 μL of 20 mM MOPS, underlayed with 20% and 65% sucrose, and centrifuged again. The washed interface was diluted with 50 μL of 20 mM MOPS, and aliquots (10 μL) were added to 200 mL of TBS and serially diluted in 2-fold steps in microtiter plates in duplicate. One of the duplicate dilution series served for the assay of bound antibody. The second series was used to measure total antibody sites by incubation for 2 h with the appropriate mAb after removal of unabsorbed material and washing with TBS−Tween. The mAb/vesicle protein ratio is minimally 20-fold higher in the total site assay compared to the binding reaction in solution. All assays

(bound antibody and total sites) for the duplicate samples of intact and Triton X-100 vesicles were assayed on the same microtiter plate. Control samples of antibody in the absence of membrane were passed through the same centrifugation procedures and aliquots similarly laid on microtiter plates and developed. The absorbance was subtracted from those of the samples which contained membranes. The mean ratio of retained bound antibody to total sites was determined from the means of the ELISA absorbances at the different dilutions.

The calsequestrin content in the intact and Triton-treated vesicle suspensions was determined for the same preparations as those used in the antibody reactions. Samples were retained from the membrane preparation in 250 mM sucrose, 20 mM MOPS, pH 7.4, and 5 mM EDTA at 2 mg/mL before centrifugation as well as from the supernatants and resuspended interfaces after the sucrose step gradient fractionation. The interface material was diluted to the volume of the sample applied to the sucrose gradient. Equal volume aliquots from the total, supernatant, and membrane samples for both the intact and Triton-treated preparations were electrophoresed on 8% slab gels (Laemmli, 1970). The gels were stained with Coomassie Blue and the calsequestrin content in each lane determined by densitometric scanning.

Constructs for Expression of the Fusion Proteins. We have used the plasmid construct pGSTag (Gift of Dr. David Ron, Laboratory of Molecular Endocrinology, Massachusetts General Hospital, Harvard Medical School), which was derived from the bacterial expression plasmid pGEX-KG (Guan & Dixon, 1991), as the expression vector to synthesize the fusion peptides, since this contains an engineered protein kinase A phosphorylation site to allow labeling of the peptide outside the region of the insert (Ron & Dressler, 1992). Each of the parent inserts was synthesized from cDNA using the polymerase chain reaction (PCR) which included a 5′ restriction site for *Eco*RI and a 3′ site for *Xho*I on the primers to allow ligation into the multiple cloning sites of the construct.

PDHPr$_{664-799}$. Primers, 5′-ATCAGAATTCTGGCCGAGGCGGAGAG and 3′-TGATCTCGAGGTGGCGTTGACGATGCG (underlined nucleotides represent the recognition sites for *Eco*RI and *Xho*I, respectively) were synthesized corresponding to nucleotides 1990−2006 and 2379−2397 of the rabbit skeletal muscle DHPr α_1 subunit, respectively. These were used to synthesize the DNA fragment encoding amino acids 664−799 by the PCR using rabbit skeletal DHPr α_1 subunit as a DNA template. Vent polymerase (Promega) was employed to extend the DNA. The PCR products were purified by LMP agarose gel (Baxter) electrophoresis (Favre, 1992). After digestion with *Eco*RI and *Xho*I, the DNA fragment was purified by phenol/chloroform extraction and sodium acetate (pH 5.2)/ethanol precipitation and ligated into the *Eco*RI/*Xho*I sites of pGSTag using the DNA T4 ligase (New England Biolabs) to form pDHPr$_{664-799}$. The plasmids were then transformed into *Escherichia coli* strain JM 109 and selected on LB-agarose-ampicillin (100 mg/mL) plates (Promega technical manual). All other DNA restriction enzymes if not specifically stated were purchased from New England Biolabs.

PTriadins. The construction of triadin fusion peptides is shown in Figure 1. The amino acid numbers and restriction sites used to construct the plasmids are shown along the top.

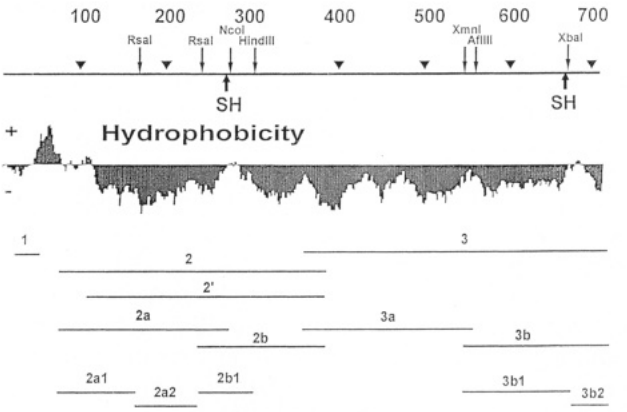

FIGURE 1: Construct of the triadin peptides. The hydrophobicity plot of triadin is shown with the corresponding amino acid sequence above. Also indicated are the two cysteines at residues 270 and 671, together with the restriction enzyme (*Nco*I, *Rsa*I, *Xmn*I, *Afl*III, and *Xba*I) cutting sites used for the preparation of the constructs. The position and nomenclature of the different triadin expression peptides are shown below the hydrophobicity plot.

The hydropathy plot indicates a single strongly hydrophobic domain covering residues 48−68 and three short nodes at residues 102−110, 267−278, and 665−674, while the rest of the protein is extremely hydrophilic. Triadin 1, 2, and 3 cover the full hydrophilic domains of the molecule. Triadin 1, 2, 2′, and 3 are the parent plasmids, encoding amino acids 1−49, 68−389, 110−389, and 370−706 of triadin, respectively, and were synthesized as described above using *Eco*RI and *Xho*I recognition sites at the 5′ and 3′ ends and rabbit skeletal muscle triadin cDNAs as the template (Peng *et al.*, 1994). PTriadin 2a, encoding amino acids 68−278, was synthesized by excising the pTriadin 2 insert with *Eco*RI and *Nco*I and purifying by LMP agarose gel electrophoresis. The fragment was ligated into the *Eco*RI/*Nco*I sites in pGSTag. PTriadin 2a1, 2a2, and 2b, encoding amino acids 68−163, 165−240, and 242−389, respectively, were synthesized by excising the pTriadin 2 insert (excised by *Eco*RI and *Xho*I) with *Rsa*I and ligated into the pGSTag polylinkers at sites *Eco*RI/*Xba*I blunt end (filled in with Klenow fragment, GIBCO-BRL), *Eco*RI blunt ends (filled in with Klenow fragments), and *Eco*RI blunt end (filled in with Klenow fragment)/*Xho*I separately. PTriadin 2b1 was derived from pTriadin 2b by treating the plasmid with *Hin*dIII and religating. PTriadin 3a, encoding amino acids 370−562, was derived from pTriadin 3 by excising the insert and digesting it with *Afl*III. The fragment was purified by LMP agarose electrophoresis and ligated into the *Eco*RI/*Nco*I sites in pGSTag. PTriadin 3b, encoding amino acids 551−706, was derived from pTriadin 3 by excising the insert and treating with *Xmn*I. The fragment was purified by electrophoresis and ligated into the *Sma*I/*Xho*I sites of the polylinker region of pGSTag. PTriadin 3b1 and 3b2 were derived from pTriadin 3b by excision of the insert using *Bam*HI and *Xho*I and cleavage by *Xba*I. Both DNA fragments, encoding amino acids 551−672 and amino acids 673−706, were purified by LMP agarose gel electrophoresis and ligated into the *Bam*HI/*Xba*I (pTriadin 3b1) and *Xba*I/*Xho*I sites (pTriadin 3b2), respectively.

Plasmid Purification and Protein Induction. The plasmids were purified following the Promega technical manual. The ligations of foreign DNA into the pGSTag vectors were tested by restriction enzyme digestion. The fidelity of the

parent PCR products was checked by direct sequencing. Cell cultures were grown overnight in 3 mL of LB/ampicillin (0.1 mM) medium, inoculated into 30 mL of fresh medium, and allowed to grow for additional 2 h. The protein induction was initiated by adding 0.1 mM IPTG (Ambion) into the cell culture medium. After 3 h of induction, cells were pelleted (5000 *g* for 5 min) and washed three times with 1 mL of phosphate-buffered saline (PBS: 150 mM NaCl, 50 mM KP_i, pH 7.4, GIBCO-BRL, 1 mM EDTA, 1 mM DTT, and 1 mM PMSF). The cells were then lysed on ice by sonicating three times for 20 s with the dial set at 3 (Sonifier Cell Disruptor, Model W185D from Heat Systems Inc., microtip). The crude bacterial lysates were then electrophoresed and blotted for the Western blot and protein overlay experiments.

Purification of the Expression Peptides. The purification of the expression peptides was carried out by the procedure described by Ron and Dressler (1992) with some modifications. Generally, the fusion peptide was first separated from the bacterial debris by centrifugation and the supernatant loaded onto a glutathione-Sepharose gel (Pharmacia) and incubated for 15 min at 22 °C. The column was washed with 1 mL of PBS three times (see above). The peptides were either eluted with 10 mM reduced glutathione in 1 M NaCl or radiolabeled (see below). The eluate was then electrophoresed and blotted.

Labeling and Purification of the $DHPr_{664-799}$. Affinity chromatography of the peptide with glutathione-Sepharose followed the protocol described above. Beads (80−100 μL) containing the $DHPr_{664-799}$ fusion peptide were transferred to a microcentrifuge tube, washed with 1 mL of the phosphorylation buffer DK (50 mM KP_i, pH 7.2, 10 mM $MgCl_2$, 5 mM NaF, and 4.5 mM DTT), and resuspended in 100 μL reaction volume containing 0.2 unit/mL of the catalytic subunit of protein kinase A (Sigma) and 0.25 mCi of [γ-^{32}P]ATP (6000 Ci/mM, DuPont) in buffer DK for 30 min at 22 °C. The unincorporated label and enzyme were removed by washing the beads three times in 1 mL of PBS supplemented with 5 mM NaF. The labeled beads were washed with 1 mL of TB (150 mM NaCl, 50 mM TrisCl, pH 8.0, 2.5 mM $CaCl_2$, and 1 mM DTT), resuspended in 150 μL of TB containing 1.5 μg of thrombin (Sigma) and rocked for 20 min at 22 °C in order to separate the fusion peptide from the glutathione transferase carrier and release it from the column. The released radiolabeled peptide was further purified by electrophoresis and excision of the peptide of correct M_r. The protein was electroeluted from the gel (Schleicher and Schuell Elutrap) in Tris−glycine buffer (25 mM Tris, pH 8.3, and 192 mM glycine) for 2 h at 200 V. Approximately 0.5 mL of solution containing radiolabeled purified fusion fragment was collected and employed as the probe.

Electrophoresis, Western Blot, and Protein Overlay. SDS−PAGE was performed as described by Laemmli (1970). Gels were electroblotted onto the nitrocellulose (Bio-Rad) sheets for 2 h at 12 V in 50% Laemmli running buffer containing 20% CH_3OH in Genie Blotter (Ideal Scientific). The immunoenzymic staining of Western blots was carried out as described by Kawamoto *et al.* (1986) using a one-step reaction kit (Pierce) for the peroxidase reaction. In the protein overlay experiments, the nonspecific sites were blocked by 5% nonfat dry milk in 20 mM gluconic acid (potassium salt) and 20 mM MOPS−Tris medium. The blots were incubated with the radiolabeled probe in 2 mL of the same medium for 1 h at 22 °C. The blot was then washed, air dried and developed by radioautography.

Table 1: Antibody Interactions with Intact and Triton X-100-treated TC/Triad Vesicles[a]

	Activity Assayed	Intact Vesicles	Triton X-100 Treated
A	calsequestrin retained	100%	13%
	mAb GE4.90 depleted	27+10%	28 ± 9%
	mAb AE8.91 depleted	32 ± 5%	35 ± 3%
B	calsequestrin retained (n = 3)	93%	21%
	mAb GE4.90 bound (n = 4)	54 ± 8%	73 ± 10%
	mAb AE8.91 bound (n = 2)	4.5%	6.2%

[a] Calsequestrin retained in the vesicles was calculated as Coomassie blue stained in the membranes recovered after treatment as the percent of stained material in the original preparation. (A) Depletion of antibody from suspension was assayed as percent antibody removed from the supernatant after centrifugation of antibody/membrane suspension on sucrose step gradients. The antibody remaining in supernatant after incubation of control samples without membranes was taken as 100%. (B) MAb bound was calculated as antibody found in the membrane fraction at the 20%/65% sucrose interface after centrifugation of the antibody/membrane reaction mixture and washing of the membrane fraction as described in Materials and Methods. Total antibody sites recovered were determined for each final membrane fraction. Data are expressed as % total mAb binding sites recovered ± Standard Error Mean.

RESULTS

Location of the MAb Binding Epitopes. Two monoclonal antibodies, GE4.90 and AE8.91, have been prepared against triadin and evidence has been presented that both these antibodies have cytoplasmic epitopes (Brandt *et al.*, 1992; Lewis-Carl *et al.*, 1995). MAb GE4.90 inhibited the release of Ca^{2+} from actively loaded triadic vesicles which had been induced by depolarization of the T-tubule (Brandt *et al.*, 1992; Lu *et al.*, 1994). MAb AE8.91 has been employed to detect triadin in intact vesicles using immunoelectron microscopy; colloidal gold particles were visualized adjacent to the protruding foot structures on the outside of the vesicles (Lewis Carl *et al.*, 1995).

As a more direct test of epitope orientation, the ability of these mAbs to bind to intact and detergent-disrupted triadic vesicles was assayed. Triton treatment essentially removes all nonjunctional membrane (Brunschwig *et al.*, 1982). To estimate the extent of accessibility of luminal surface to externally added proteins, the decrease in calsequestrin content in the vesicle pellet was determined. Calsequestrin forms an insoluble matrix inside the SR unless it is dissolved by either hypertonic salt or strong Ca^{2+} chelating agents. In order to detect leaky vesicles which might have been generated at any stage during isolation, the vesicles were prepared without treatment with hypertonic salt or chelating agents prior to this centrifugation step. Thus the EDTA treatment will release calsequestrin from all leaky vesicles. The complete retention of calsequestrin by those vesicles that were not treated with detergent (but suspended in EDTA-containing medium) in Table 1A indicates that the amount of leaky vesicles generated during the isolation of this particular preparation of TC/triads was below the level of detection. Over all experiments (Table 1B), the TC/triad preparations remained 93% intact. For the experiment in Table 1A, Triton released 87% of the calsequestrin while

for overall experiments (Table 1B), about 80% of the vesicles became leaky with the detergent treatment.

To test for depletion of antibody from suspension, each mAb was incubated with intact or Triton-treated membranes, the reaction mixture was fractionated by centrifugation on a sucrose step gradient, and free antibody remaining in the supernatant determined. Parallel suspensions of mAb without membranes were carried through the same procedures, and the antibody present in the supernatant was assayed as the control for undepleted mAb. Table 1A shows that the intact vesicles removed both mAb GE4.90 and AE8.91 to the same extent as the Triton-treated membranes, again indicating that these antibody epitopes are on the cytoplasmic surface of the SR.

As a second test for epitope orientation, the amount of antibody bound to membranes that were centrifuged through the 20% sucrose barrier was determined. Since these membranes were resuspended and centrifuged twice more through a sucrose barrier to remove trapped but not bound antibody, it was found necessary to assay the total number of binding sites in the final pellet for each membrane/antibody reaction sample to account for the variations in membrane recovery. Each sample was laid down in duplicate on the same microtiter plate. One lane was directly assayed for bound antibody, and the paired lane was incubated with excess antibody to determine total antibody binding sites. By this protocol, the amount of antibody bound by the intact vesicles and Triton-treated membranes was determined as a fraction of the total antibody sites. Table 1B shows that, at the antibody and membrane concentrations employed, the sites on both intact and Triton-treated membranes were nearly saturated with mAb GE4.90. In addition, we found that the Triton-treated membranes bound twice as much mAb GE4.90 as the intact vesicles. Those same Triton-treated membranes, however, also had twice the number of total mAb GE4.90 binding sites when assayed with excess antibody after deposition of the membranes on microtiter plates. These differences can be attributed to the fact that the Triton treatment removes nonjunctional membrane protein which would compete with triadin for the microtiter plate surface.

In contrast to the data obtained with mAb GE4.90, bound mAb AE8.91 was detected only at a low level (Table 1B) when the material appearing at the 20%/65% sucrose interface was extensively washed. The extent of retention was similar in both the intact and Triton-treated vesicles. This low amount of mAb AE8.91, however, appears to be specifically bound since when the TC/triads were incubated with other nonspecific mAbs such as against cardiac troponin I, no IgG could be detected in the final membrane pellet. Since both antibodies were equally depleted from suspension (Table 1A) but were retained to a considerably different degree in the extensively washed pellets (Table 1B), it is likely that the two antibodies have considerably different affinities for their epitopes. That is, mAb GE4.90 has a tight association with its epitope while mAb AE8.91 has a lower affinity and dissociates during the centrifugation and washing procedures.

Epitope Binding Site of Monoclonal Antibodies. In order to study the membrane topology of triadin and its binding domains to the other triadic junctional proteins, different hydrophilic domains of triadin were expressed as described in Materials and Methods. Figure 2 shows the Western blot

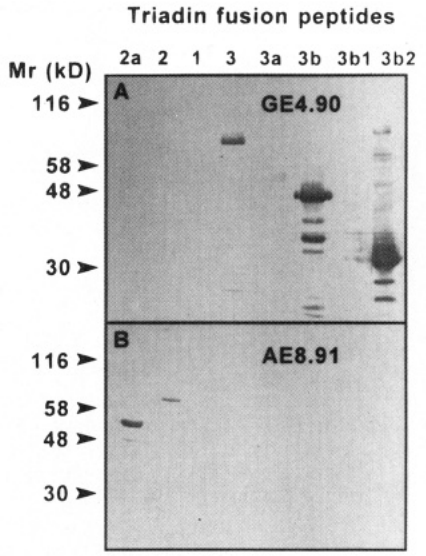

FIGURE 2: Binding of mAb GE4.90 and AE8.91 to triadin fusion peptides. A and B show the binding of GE4.90 (2.5 μg of protein/mL) and AE8.91 (cell culture supernatant) to the different triadin fusion peptides, respectively. The cell lysates (1.5 μg of protein/lane) were electrophoresed on the 10% SDS−PAGE and blotted onto a nitrocellulose sheet. Lanes labelled with 2a, 2, 1, 3, 3a, 3b, 3b1, and 3b2 refer to the different triadin fusion peptides as described in Figure 1.

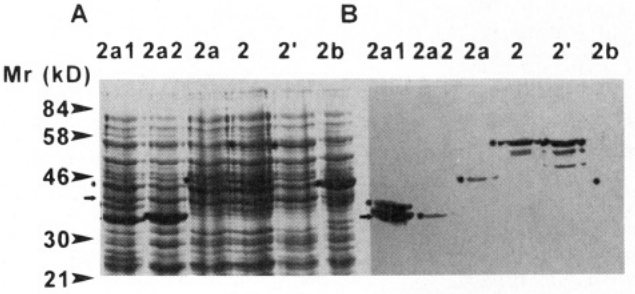

FIGURE 3: Binding of mAb AE8.91 to fusion peptides derived from triadin 2. Lanes labelled with 2a1, 2a2, 2a, 2, 2′, and 2b refer to the different triadin fusion peptides as described in the legend to Figure 1. The positions for the expression of the triadin fusion peptides are indicated by asterisks. (A) Coomassie Blue stain of the bacterial lysates (15 μg protein/lane) run on a gel as described in the legend to Figure 2. A proteolysis product of the expression peptide of triadin 2a1 is indicated by the arrow. (B) Same proteins (1.5 μg ofprotein/lane) that were electroblotted onto a nitrocellulose sheet and incubated with a cell culture supernatant containing mAb AE8.91.

of mAbs GE4.90 and AE8.91 onto the triadin peptides. MAb GE4.90 bound to triadin 3, 3b, and 3b2 (Figure 2A). Its strong binding to triadin 3b2 indicates that the epitope for mAb GE4.90 resides within the triadin C-terminal 34 amino acids which lies C terminal to both cysteines and to the modestly hydrophobic region at residues 665−674. MAb AE8.91 bound to triadin 2 and 2a specifically (Figure 2B). In order to locate the mAb AE8.91 epitope more precisely, triadin 2′, triadin 2a1, triadin 2a2, and triadin 2b were synthesized (see Figure 1). In Figure 3A,B, the position of the expressed fusion peptides 2a1, 2a2, 2a, 2, 2′, and 2b are indicated by asterisks, and the degradation product of triadin 2a1 is indicated by an arrow. Figure 3A shows the Coomassie Blue stain of the triadin fusion peptides and 3B shows the Western blot of mAb AE8.91 on the same peptides. Again the antibody binds to triadin 2a and 2 as was shown in Figure 2B. The relatively weaker binding to

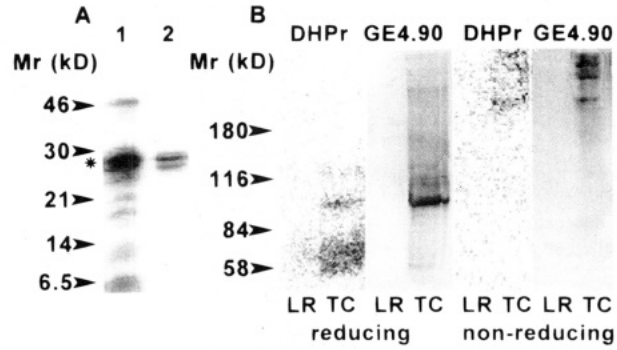

FIGURE 4: Binding of $DHPr_{664-799}$ to SR vesicle proteins. (A) Autoradiograph of expressed $DHPr_{664-799}$. Lane 1 shows the fusion product after partial purification and release by thrombin from a glutathione affinity matrix (14% SDS−PAGE). The parent product is indicated by asterisks. A slice of gel corresponding to the parent product was excised, and the peptide was collected by electroelution (lane 2) and employed as the probe to study the interactions of $DHPr_{664-799}$ with triadic proteins (Figure 4B) or triadin fusion peptides (Figure 5 and 6). (B) Skeletal muscle microsomes were centrifuged on a sucrose density gradient to separate the light SR preparation which is composed largely of longitudinal reticulum (LR) from the heavy fraction which contains predominantly TC/triads (TC). These were electrophoresed on the 6% SDS−PAGE either in the presence (reducing) or absence (nonreducing) of mercaptoethanol and transferred to a nitrocellulose sheet. Blots containing 30 μg of protein/lane were overlain with $DHPr_{664-799}$ in 2 mL of 20 mM potassium gluconate and 20 mM Tris MOPS (panel DHPr). Blots containing 3 μg of protein/lane were overlain with 2.5 mg/ml mAb GE4.90 in TBS (panel GE4.90).

2a in this experiment is a result of the poor expression of the peptide as shown by Coomassie Blue stain (compare Figures 3A and 6A, lane labeled with 2a) and is not indicative of a low affinity. MAb AE8.91 binds strongly to the triadin 2a1 and 2′ indicating that the binding epitope of mAb AE8.91 on triadin is between amino acids 110−162 (see Figure 1). A weak binding of mAb AE8.91 to triadin 2a2 was repetitively observed (Figure 3B, lane 2a2, asterisk), even though the peptide was strongly expressed (Figure 3A, lane 2a2, asterisk). Since there is no overlap between the sequences of 2a1 and 2a2 (see Figure 1 triadin fusion peptides construct), mAb AE8.91 must have two epitopes with considerably different affinities suggesting that similar but not identical sequences serve as its binding epitopes (see Figure 7 and Discussion).

Binding of the $DHPr_{664-799}$ with SR Fractions. Figure 4A shows the autoradiograph of the radio-labelled $DHPr_{664-799}$ released from the glutathione−Sepharose column by thrombin cleavage. The band corresponding to the intact $DHPr_{664-799}$ is indicated by asterisk (lane 1). Since some of its degradation products were also labeled (lane 1, lower bands), the undegraded $DHPr_{664-799}$ together with its nearby proteolytic product were further purified by electroelution (lane 2). The purified peptide was then employed as a probe to study the binding of the $DHPr_{664-799}$ to intact TC/triads (lanes TC) and longitudinal reticulum vesicles (lanes LR) under reducing and nonreducing conditions as shown in Figure 4B. Under reducing conditions, $DHPr_{664-799}$ binds to proteins of 95 and 60 kDa of the TC/triads (lane TC) but not to longitudinal reticulum (lane LR). The 95 kDa protein corresponds in position to the band of triadin, determined by mAb GE4.90 (lane TC). The 60 kDa protein is probably a breakdown product or homolog of triadin that is not recognized by mAb GE4.90 but is recognized by mAb

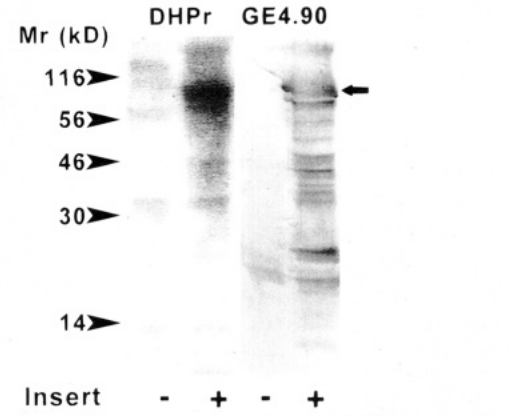

FIGURE 5: Binding of the $DHPr_{664-799}$ to the purified fusion peptides with or without insert of triadin 3. The bacterial extracts of the expression vector itself without (-) and with (+) triadin 3 insert were purified using the glutathione−Sepharose column, and the peptides were released from the affinity matrix by glutathione competition (see Materials and Methods for conditions). The eluates (15 μg of protein/lane 1 and 2 and 1.5 μg protein/lane 3 and 4) were then electrophoresed (8% SDS−PAGE) and transferred to a nitrocellulose sheet. Panel DHPr shows the autoradiography of the overlay of these peptides with the $DHPr_{664-799}$ probe. Panel GE4.90 shows the Western blot of the same peptides monitored by triadin mAb GE4.90. The position of the parent fusion peptide of triadin 3 is indicted by arrow. The conditions for the protein overlay and Western blot are those described in the legend to Figure 4.

AE8.91 (data not shown). Under nonreducing conditions, $DHPr_{664-799}$ binds to a series of proteins of high M_r (lane TC). The bands at 95 and 60 kDa are not present. This shift in electrophoretic mobility of the DHPr binding bands coincides with the disappearance of the monomeric triadin band and the appearance of a ladder of bands at high M_r detected with the antibody (GE4.90, lane TC). These data indicate that $DHPr_{664-799}$ binds to triadin specifically in the intact triad vesicles. No binding of $DHPr_{664-799}$ to the JFP or other junctional proteins (lanes TC) or SR proteins (lanes LR) was detected in this overlay blot under either reducing or nonreducing conditions.

Binding of the $DHPr_{664-799}$ with Triadin Expression Peptides. The purified radiolabeled $DHPr_{664-799}$ was then employed as a probe to study its binding with triadin fusion peptides. We first determined the specificity of the binding using the expression peptides of the pGSTag itself without insert (−) together with the triadin fusion peptide triadin 3 (+). These two expression peptides were purified by passing the bacterial lysates through the glutathione−Sepharose column and releasing from the column by glutathione competition (see Materials and Methods). The first two lanes of Figure 5 show the autoradiograph for the overlay of the $DHPr_{664-799}$ onto the GST expression peptides without insert (−) and with ptriadin 3 (+). The last two lanes show the Western blot of the same fusion peptides developed with mAb GE4.90. The $DHPr_{664-799}$ bound strongly to a peptide of 78 kDa found only in the preparation containing the ptriadin 3 insert. This molecular mass corresponds to that estimated for the insert together with the carrier peptide including GST and a band of that mobility was detected with mAb GE4.90 (indicated by arrow). The $DHPr_{664-799}$ probe also binds to peptides of lower molecular mass in the pTriadin preparation which correspond to the proteolytic products of the parent peptide detected in the immunoblot (panel GE4.90, + insert). Since triadin 3 proteolytic products were not detected when the crude bacterial lysate was

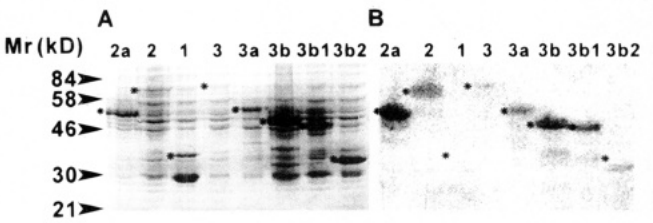

FIGURE 6: Binding of triadin fusion peptides with $DHPr_{664-799}$. Bacterial lysates containing 15 μg of triadin 2a, 2, 1, 3, 3a, 3b, 3b1, and 3b2 were electrophoresed on 10% SDS-PAGE. The positions of the expression peptides are indicated by asterisks. (A) Coomassie Blue stain; (B) same peptides which were electroblotted onto a nitrocellulose sheet and overlain with radiolabeled $DHPr_{664-799}$. Lanes labeled with 2a, 2, 1, 3, 3a, 3b, 3b1, and 3b2 refer to the different triadin fusion peptides described in the legend to Figure 1. The conditions for the protein overlay and Western blot are those described in the legend to Figure 4.

Western blotted (Figure 2A), the degradation must have occurred during the purification procedure. No specific labeling is observed at 31 kDa, the position corresponding to the carrier peptide GST, despite the fact that this peptide is expressed strongly in the induced bacteria. A few bands of bacterial origin were weakly labeled in both the GST alone and pTriadin insert preparations. The identities of the bacterial proteins were not determined, but it is known from previous work that glycolytic enzymes bind to the DHPr (Brandt *et al.*, 1990).

The binding of the $DHPr_{664-799}$ to a set of triadin fusion peptides is shown in Figure 6. The asterisks indicate the position of the expression peptides triadin 2a, 2, 1, 3, 3a, 3b, 3b1, and 3b2 in the Coomassie Blue stained gel (Figure 6A) and the $DHPr_{664-799}$ overlay (Figure 6B) of the bacterial sonicates. The expression level of each fusion peptide is quite variable (Figure 6A). $DHPr_{664-799}$ binds strongly to triadin 2a, 2, 3a, 3b, and 3b1 indicating the extensive interactions between these two molecules (Figure 6B). The intensity of labeling reflects in part the degree of expression of these peptides, but when this is taken into account, the data indicate a stronger binding to triadin 2a than to 3a or 3b. Poor expression rather than low affinity may account for the low binding of the probe to triadin 3. No overlay is observed between $DHPr_{664-799}$ and triadin 1 or 3b2 despite the considerable expression of the proteins (compare lanes 1 and 3b2 in panels A and B).

DISCUSSION

One of the most intractable problems in muscle activation has been to identify the proteins which form the stable contact between the TC and the T-tubule. The DHPr, the ryanodine receptor, and triadin are intrinsic membrane proteins which require both detergent and hypertonic salt to be dissolved from the membrane. In addition, triadin precipitates in isotonic salt. In an attempt to circumvent these problems, we employed fusion peptides which do not contain the transmembrane sequence. The use of the expression peptides has allowed us to investigate the submolecular binding domains of the proteins and to examine the transmembrane topology of triadin.

Triadin was first recognized as an intrinsic protein of the TC which bound to both the DHPr and to the JFP (Brandt *et al.*, 1990; Kim *et al.*, 1990). This suggested that it might serve the role of cementing the triad junction through its association with the DHPr in the T-tubule. For triadin to operate in this manner, it must be embedded in the TC membrane and be able to make contact across the junctional gap between the organelles, a distance in the range of 100 Å. The determination of the sequence of triadin led Knudson *et al.* (1993) to propose a topological model which was considered to be in conflict with this role. They proposed that triadin has a short 45 amino acid cytoplasmic N terminus followed by a single transit through the membrane of a hydrophobic α helix and that the rest of the molecule is luminal. With this topology the N terminus would be the only region capable of contacting the T tubule, but it was considered to be too short. The basis for this conclusion was not stated, but presumably implied that 45 amino acids do not have the requisite cytoplasmic extension. Since this conclusion is based on certain assumptions, it is necessary to evaluate these individually.

The assumption is that a short stretch of cytoplasmic amino acids will not allow contact between the protein and the T-tubule. We do not know how many amino acids are required to make contact across the junctional gap since the tertiary structure of the proteins is unknown, but certain factors can be evaluated. The proposed contact between triadin and the T-tubule is not from the protein to the membrane but from the protein to the DHPr. The latter contains extensive cytoplasmic domains including the 139 amino acid loop from residues 662 to 800 which is considered to determine the nature of muscle activation. Therefore, the DHPr may extend a considerable distance from the T-tubule membrane to make contact with triadin. If the peptide bond is fully extended, the distance of extension from the membrane is that of the peptide bond distance multiplied by the number of amino acids. With a peptide bond distance of 3.5 Å it is seen that the number of amino acids in the cytoplasmic domain required to traverse the distance of the junctional gap is not very large. For the more condensed structure of an α helix, the axial extension is 1.5 Å per amino acid, which is also adequate to allow for communication between triadin and the DHPr provided that both proteins contain short cytoplasmic residues. An example of a membrane protein with a cytoplasmic extension in the form of an α helix is phospholamban (Simmerman *et al.*, 1986).

The second assumption on the feasibility of triadin contacting the DHPr is that the topological model is correct. Since it is based extensively on the hydropathy analysis of the triadin sequence, it needs to be verified experimentally. Both the direct observations shown in Table 1 of this paper and the previous evidence that one of the antibodies inhibits release of Ca^{2+} from actively loaded vesicles (Brandt *et al.*, 1992; Liu & Pessah, 1994) and the other has an epitope which is defined by immunoelectron microscopy as residing close to the protruding junctional foot structures (Lewis-Carl *et al.*, in press) indicate that the epitopes are on the outside of the vesicle. The epitopes for mAb AE8.91 and GE4.90 lie between residues 110 and 163 and residues 673 and 706, respectively. Since neither of these domains includes the cytoplasmic N terminus (residues 1−47), our observations are at variance with the model of Knudson *et al.* (1993). With this new information the hydropathy plot can be reinterpreted. We agree with Knudson *et al.* that the N terminus is likely to be cytoplasmic since the cDNA sequence does not contain a hydrophobic N-terminal signal sequence. The absence of the signal sequence has been used to argue

the N-terminal cytoplasmic loci of many other membrane proteins (Catterall, 1988). This conclusion is further supported by the recent report that the N-terminal of triadin is located in the cytosol using a site-directed antibody (Marty *et al.*, 1995). The hydrophobic region between amino acids 45 and 66 is consistent with the proposal that it crosses the membrane in the form of an α helix. This places the amino acids immediately after this in the lumen including asparagine 75 which could be glycosylated. The hydropathy plot in Figure 1 indicates no further regions which are likely to transverse the membrane in the form of α helices, but three short segments (residues 102−110, 267−274 or 278, and 665−674) are relatively hydrophobic, and one or more of these might cross the membrane in the form of hydrogen-bonded β sheets or barrels. Thus we adduce two possible models for the transmembrane topology of triadin in which there are either two or four membrane transits. The first model predicts that triadin contains two transmembrane domains, residues 47−68 cross the membrane as an α helix and residues 102−114 cross the membrane in the reverse direction as a β sheet which leaves the rest of the sequence in the cytosol. The second model predicts that the triadin molecule contains four transmembrane domains. In addition to the two membrane-spanning regions proposed in model one, residue 267−278 and residues 665−674 each cross the membrane as a β sheet or similar extended structure. This model places the triadin N-terminal 47 amino acids, residues 115−266, and the C terminus 34 amino acids in the cytosol; residues 69−101 and the middle part of triadin, residues 279−664, are in the lumen of the SR. Both models provide the cytoplasmic loci for the two antibodies. The first model places the disulfides in the cytosol, while in the second model the two cysteines at residue 270 and 671 are in the membrane. In the second model the potential glycosylation site at asparagine 625 (Knudson *et al.*, 1993) is located in the lumen of the SR. Either of these two models will give triadin additional cytoplasmic domains which can interact with other cytoplasmic junctional proteins. The resolution between these models will require further experimental data [see Fan *et al.* (1995)], but both models place the C-terminal in the cytoplasm.

Marty *et al.* (1995) have recently concluded on the basis of C-terminal antibody depletion and protease experiments that the C-terminus of triadin is luminal. Our finding with antibody GE4.90 disagrees with their conclusions. We did not find that detergent treatment greatly increased the depletion of mAb GE4.90 from solution but did find that Triton treatment almost doubled the total number of sites available for binding on the membranes. A similar enhancement in the number of accessible antibody binding sites by detergent and alkali treatment has been reported for the C-terminal of the Ryr, which Grunwald and Meissner (1995) attributed to disruption of protein−protein interactions rather than an opening of the TC membrane. Furthermore, a luminal location for the C-terminal of triadin cannot account for the effect of mAb GE4.90 on T-tubular depolarization-induced release of Ca^{2+} from the SR (Brandt *et al.*, 1992).

We repeatedly observed, in addition to the strong binding of mAb AE 8.91 to triadin 2a1, a weak binding to triadin 2a2 (Figure 3B, lane 2a2). This suggests that regions of similarity between triadin 2a1 and 2a2 (Figure 7, framed) might be the domains where the binding occurs. Within the first framed segment (Figure 7) the top sequence is a part of

SEQUENCE OF TRIADIN

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MTEITAEGNA STTTTVIDSK NGSVPKSPGK VLKRTVTEDL VTTFSSPAAW    50
LLVIALIITW SAVAVVMFDL VDYKNFSASS IAKMGSDPLK LVHDAVEETT   100
DWIYGFFSLL SDIISSDGDE EDDEGDEDTA KGEIEEPPLK              140
 RKDIHKEKI EKQEKPERKI PTK         VVHKEKEKEK EKVKEKEKP    181
EKKATHKEKL EKKEKPETKT VTKE                               205
EKKARTKEKI EEKTKKEVKG VKQE       KVKQTVAKAK EVQKTPKPKE   249
KESKETAAVS KQEQKDQYAF CRYMIDIFVH GDLKPGQSPA IPPPSPTEQA   299
SRPTPALPTP EEKEGEKKKA EK         KVTTETK KK AEK EDAKK    338
KSEKETDIDM KKKEPGKSPD TKPGTV     KVTTQAATKK DEKKEDSKK    383
AKKPAEEQPK GKKQEKKEKH EEPAKSTKKE HAAPSEKQAK AKIERKEEVS   433
AASTKKAVPA KKEEKTTKTV EQETRKEKPG KISSVLKDKE LTKEKEVKVP   483
ASLKEKGSET KKDEKTSKPE PQIKKEEKPG KEV KP     KP           520
                  PQ PQIKKEEKPE QDIMKP      EKTALHGKPE     548
EKVLKQVKAV T          TEKHVKPKPA KKAEHQ  EK EPPSIKTDKP   587
KSTSKGMPEV TESGKKKIEK SEKEIKV PA RRESHQ     LQNVTKAEKP   632
ARGSKEGFED VPASKKAKEE AEEVSSTKKQ KSPISFFQCV YLDGYNGYGF   682
QFPVTPAQYP GESSGKPNSP GPKQ                               706
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FIGURE 7: Amino acid sequence of triadin designating putative membrane-spanning domains and regions of homology. The transmembrane α helix and β sheet or barrel structures are shaded. The two cysteines at residues 270 and 671 are shown in bold larger type. A repetitive sequence ***KKEEK*** (with one substitution allowed) is indicated in bold. Extensive homologous sequences are boxed. Identities of residues in these sequences are shown by dotted lines between the residues.

2a1 while the two lower sequences are present in 2a2. The resemblance between residues 141−162 and residues 182−205 is the greatest suggesting that the former contains the strong epitope and the latter the weaker one.

We synthesized a fusion peptide of DHPr α_1 subunit covering the residues of the putative cytoplasmic loop between homologous segments II−III, since this portion of protein is proposed to be the site where the communication between the voltage sensor and the Ca^{2+} release channel occurs (Beam *et al.*, 1992; Lu *et al.*, 1994, 1995). The $DHPr_{664-799}$ binds to the triadin fusion peptides extensively. The binding is specific since it does not bind to the carrier protein GST (Figure 5, panel DHPr with − insert) nor to the heavily expressed triadin fusion peptides, triadin 1, and triadin 3b2 (Figure 6, lanes labeled with 1 and 3b2). The binding data indicate a quite extensive domain or series of domains for the DHPr onto triadin. The peptides which bind to the DHPr peptide are basic in character, but it is unlikely that this is the determining factor in binding since the DHPr peptide contains a net charge of +2 with a fairly even distribution of positive and negative charges. In addition, we have shown previously that intact DHPr binds even in the presence of 0.5 M NaCl. Figure 7 highlights with bold a ***KKEEK*** sequence or a nearly identical one which is distributed throughout the triadin molecule except for triadin 1 and triadin 3b2. These regions may be sites for the binding of the $DHPr_{664-799}$ to triadin. In the first model of triadin topology, these sequences are located entirely in the cytoplasm and, therefore, may all potentially interact with the DHPr. On the other hand, in the second model, some of the sequences are cytoplasmic while others reside in the lumen. In this case the domains available to bind to the DHPr will be confined to those accessible in the cytoplasm.

A broad range of evidence supports the conclusion that triadin is a major triadic protein which exhibits a strong coassociation with the DHPr and JFP in mature and developing muscle and in normal and genetically altered tissue (Flucher *et al.*, 1993). This paper further characterizes its topological properties and gives more explicit submolecular detail of its binding to other junctional proteins. Although the data do not address the central question of the role of

triadin in the physiological coupling of muscle activation, they place triadin in a strategic site adjacent to the cytoplasmic loop II−III of the DHPr which could allow it to mediate signal transmission during E−C coupling.

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