Localization and Characterization of the Calsequestrin-binding Domain of Triadin 1

EVIDENCE FOR A CHARGED β-STRAND IN MEDIATING THE PROTEIN-PROTEIN INTERACTION

Received for publication, March 13, 2000, and in revised form, March 29, 2000
Published, JBC Papers in Press, March 30, 2000, DOI 10.1047/jbc.M002091200

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Triadin is an integral membrane protein of the junctional sarcoplasmic reticulum that binds to the high capacity Ca\(^{2+}\)-binding protein calsequestrin and anchors it to the ryanodine receptor. The luminal domain of triadin contains multiple repeats of alternating lysine and glutamic acid residues, which have been defined as KEKE motifs and have been proposed to promote protein associations. Here we identified the specific residues of triadin responsible for binding to calsequestrin by mutational analysis of triadin 1, the major cardiac isoform. A series of deletional fusion proteins of triadin 1 was generated, and by using metabolically labeled calsequestrin in filter-overlay assays, the calsequestrin-binding domain of triadin 1 was localized to a single KEKE motif comprised of 25 amino acids. Alanine mutagenesis within this motif demonstrated that the critical amino acids of triadin binding to calsequestrin are the even-numbered residues Lys\(^{210}\), Lys\(^{212}\), Glu\(^{214}\), Lys\(^{216}\), Gly\(^{218}\), Gln\(^{220}\), Lys\(^{222}\), and Lys\(^{224}\). Replacement of the odd-numbered residues within this motif by alanine had no effect on calsequestrin binding to triadin. The results suggest a model in which residues 210–224 of triadin form a β-strand, with the even-numbered residues in the strand interacting with charged residues of calsequestrin, stabilizing a “polar zipper” that links the two proteins together. This small, highly charged β-strand of triadin may tether calsequestrin to the junctional face membrane, allowing calsequestrin to sequester Ca\(^{2+}\) in the vicinity of the ryanodine receptor during Ca\(^{2+}\) uptake and Ca\(^{2+}\) release.

Calsequestrin is a high capacity Ca\(^{2+}\)-binding protein located in the lumen of the junctional SR\(^{1}\) in cardiac and skeletal muscle. Calsequestrin sequesters large amounts of Ca\(^{2+}\) in the vicinity of the RyR/Ca\(^{2+}\) release channel, where the protein acts as a storage depot for the Ca\(^{2+}\) that is released during muscle contraction \(3\). In both cardiac and skeletal muscle, RyRs are visualized by electron microscopy as “feet” lined up on the cytoplasmic face of the junctional SR membrane; calsequestrin appears as an electron-dense matrix in the SR lumen, closely opposed to the RyRs from the luminal side of the membrane \(1–3\). Electron microscopy and deep-etch studies also reveal the presence of thin strands or “anchoring filaments” in the junctional SR lumen, which appear to form a reticulated network that connects the electron-dense matrix (calsequestrin) to the Ca\(^{2+}\) release channels \(4\). Recently, triadin \(5\) and junctin \(6\), single-span membrane proteins localized to junctional SR in cardiac \(6, 7\) and skeletal muscle \(6, 8\), were suggested to be components of these anchoring strands, based on the abilities of the two proteins to bind to each other, to calsequestrin, and to the RyR \(7, 9, 10\). Triadin and junctin are homologous proteins, each projecting a highly charged, carboxyl terminus into the junctional SR lumen \(6, 8\). A quaternary complex between calsequestrin, triadin, junctin, and the RyR, stabilized from the inner surface of the junctional SR membrane, was proposed \(10\), which could serve both to concentrate Ca\(^{2+}\) at the inner face of the junctional SR membrane as well as to provide a molecular conduit for the rapid flow of Ca\(^{2+}\) ions from calsequestrin to the RyR during activation of Ca\(^{2+}\) release in cardiac and skeletal muscle.

Triadin was first identified and purified from rabbit skeletal muscle SR as an integral membrane protein localized to skeletal muscle triads \(5, 12\). Several triadin isoforms have since been found in cardiac and skeletal muscle, all of which appear to arise from alternative splicing of the same triadin gene \(7, 8, 11\). In cardiac junctional SR, the predominant isoform of triadin expressed is triadin 1, the smallest of the known triadins \(11\). Triadin 1 accounts for more than 95% of the total triadin in mammalian myocardium \(11\). The protein is partially glycosylated at asparagine residue 75 in heart, accounting for its appearance as a doublet of 35- and 40-kDa molecular mass proteins on SDS-PAGE \(11\). All triadin isoforms in a given species have virtually identical amino acid sequences over the first 250–260 residues \(7, 8, 11\). This common region encompasses the short amino-terminal cytoplasmic domains, the membrane-spanning segments, and the highly charged luminal domains (residues 69–257 for canine triadin). The charged luminal domains are basic and are responsible for binding to calsequestrin and to the RyR \(7, 9, 10\). Dispersed throughout these common luminal domains are multiple clusters of charged amino acids, of alternating positive and negative charge, that are particularly enriched in lysine and glutamic acid residues. These mixed charged clusters have been referred to as “KEKE motifs” \(13\) and have been proposed to facilitate protein-protein associations by acting as
polar zippers (14–16). Guo and co-workers (9, 10) first localized the calsequestrin-binding domain of triadin to the common luminal region (residues 69–264 for the rabbit triadins), where short runs of alternating lysine and glutamic acid residues motifs are present in very high content. The luminal region of junctin also contains a high density of KEKE motifs and also binds calsequestrin avidly (6, 10). Thus, it was proposed that KEKE motifs may be responsible for triadin (and junctin) binding to calsequestrin (10). This idea is reinforced by the fact that calsequestrin is a highly charged, acidic protein (17), suggesting that KEKE association motifs of triadin (and junctin) could bind to KEKE-binding sites (13) of calsequestrin. To date, however, the region of triadin (or junctin) binding to calsequestrin has not been definitively localized, and the role of the KEKE motifs, if any, in the relatively specific interaction between these proteins has not been investigated.

Here we have utilized deletional and site-specific mutagenesis of triadin 1 in combination with the radiolabeled calsequestrin overlay technique (6, 18) to localize the calsequestrin-binding domain of cardiac triadin. Our results demonstrate that a charged cluster of KEKE residues is indeed required for triadin binding to calsequestrin but that only one of several KEKE motifs of triadin is essential for binding to occur. Alanine mutagenesis suggests that the charged residues of triadin interacting with calsequestrin form a β-strand, in which only one face of the putative β-strand contributes amino acid side chains that bind to calsequestrin.

**EXPERIMENTAL PROCEDURES**

**Materials**—Restriction enzymes were purchased from New England Biolabs. Growth media constituents for plasmid propagation in JM109 *Escherichia coli* and protein induction in BL21 (DE3) *E. coli* were purchased from New England BioLabs and Sigma. Plasmids were obtained from Molecular Biochemicals. All other reagents were from Sigma unless otherwise stated. All PCR products were generated using AmpliTaq® DNA polymerase (Perkin-Elmer), and the fidelity of all constructs was confirmed by nucleic acid sequencing using Sequenase™ (Amersham Pharmacia Biotech). All oligonucleotides were purchased from Life Technologies, Inc. Radioactive reagents were obtained from NEN Life Science Products.

**Generation and Purification of Radiolabeled Calsequestrin—** cDNA encoding canine cardiac calsequestrin without its signal sequence was PCR-amplified from clone IC3A (17), using a 5′ primer with an NdeI restriction enzyme site, and a 3′ primer with a BamHI restriction enzyme site, then subcloned into the pET5α vector (Novagen). The calsequestrin/pET5α construct produced encodes mature cardiac calsequestrin (amino acid residues 1–391) (17) with an amino-terminal methionine contributed by the NdeI restriction enzyme site. (Amino acid residues 1–42 of the purified, recombinant protein were verified by Edman sequencing.) The calsequestrin/pET5α construct was then transformed into BL21 (DE3) *E. coli* cells (Novagen), and calsequestrin was metabolically labeled in *E. coli* and purified as follows.

A fresh colony of BL21 (DE3) cells carrying the calsequestrin/pET5α construct was inoculated into 10 ml of Luria broth supplemented with 125 μM ampicillin and grown at 37 °C to an *A* 600nm of 0.6. Isopropyl-β-D-thiogalactopyranoside was added at 0.5 mM to induce expression along with 10 μCi of 35S-EasyTag™ (specific activity 11750 Ci/mmol, 11 μCi/ml) Protein induction proceeded for 3 h at 37 °C with constant shaking. Cells were harvested and washed in cold phosphate-buffered saline and then resuspended in 5.0 ml of lysis buffer containing 50 mM Tris-Cl (pH 7.5), 5 mM DTT, 1 mM EDTA, and 0.1 mg/ml lysozyme, along with the protease inhibitors aprotinin (1 μg/ml), leupeptin (2 μg/ml), Pefabloc™ (100 μM), and benzamidine (1 mM). Cells were lysed on ice for 30 min and tip-sonicated with three 10-s bursts. The soluble fraction was separated from insoluble material by centrifugation at 50,000 × g for 30 min at 4 °C. 20 mM CaCl2 was added to the cleared bacterial lysate to precipitate the expressed calsequestrin (19), and the sample was incubated on ice for 20 min followed by centrifugation at 10,000 × g for 10 min at 4 °C. The Ca2+-precipitated pellet of calsequestrin was clarified by resuspension in 5 ml of buffer containing 20 mM MOPS (pH 7.2), 5 mM DTT, 10 mM NaCl, and 0.5 mM Na2S and allowed to sit on ice for 10 min. The solubilized recombinant calsequestrin was then loaded over a 1-ml phenyl-Sepharose column pre-equilibrated with 20 mM MOPS (pH 7.2), 5 mM DTT, 1 mM EDTA, and 0.5 mM NaCl, and the column was washed with 8 column volumes of the same buffer. Bound, metabolically labeled calsequestrin was subsequently eluted with 5 column volumes of elution buffer containing 20 mM MOPS (pH 7.2), 1 mM DTT, 10 mM CaCl2, and 0.5 mM NaCl (20). Elution fractions containing the concentrated, 35S-labeled calsequestrin was dialyzed against 20 mM MOPS (pH 7.2), 150 mM NaCl and stored frozen in small aliquots.

**Generation of Triadin Deletion Mutants—** To localize the calsequestrin-binding site of canine triadin 1, different triadin domains and subdomains with progressive amino- and carboxyl-terminal deletions encoding canine cardiac triadin 1 were generated as GST fusion proteins. GST fusion proteins were amplified by PCR using a series of oligonucleotides complementary to targeted regions of canine triadin 1 cDNA (11). Sense and antisense oligonucleotides were engineered with 5′ EcoRI and XhoI sites, respectively, to facilitate cloning into pGex4T-1. Constructs were transformed into BL21 (DE3) *E. coli* cells and grown overnight in Luria broth supplemented with 125 μg/ml ampicillin. Overnight cultures were washed two times, diluted 10-fold, and grown to an *A* 600nm of 0.6 at 37 °C. Optimal induction conditions to maximize expression with the least amount of protein degradation were determined empirically for each fusion protein. Protein expression proceeded for either 1–3 h at 37 °C or overnight at 22 °C with 0.1–0.5 mM isopropyl-β-D-thiogalactopyranoside for induction of expression. Cultures were harvested by centrifugation at 5,000 × g for 10 min, and cells were resuspended in 30 mM MOPS, pH 7.5, and cell pellets were then sonicated on ice with three 10-s bursts and then centrifuged at 10,000 × g for 10 min. Solubilized fusion proteins were purified using glutathione-Sepharose 4B chromatography (Amersham Pharmacia Biotech) (10). Protein concentrations were determined by the method of Schaffner and Weissman (21).

**Site-directed Mutagenesis of Amino Acids within the Calsequestrin-Binding Domain—** The GST fusion protein construct encoding residues 178–224 of canine triadin 1 was produced as described above. Using this fusion protein construct as template, mutations encoding alanine substitutions between residues 200 and 224 of triadin were generated by PCR following the methodology described in Kobayashi and Jones (11). Residues 178–224 of triadin with the targeted alanine mutations were then expressed and purified as a series of GST fusion proteins. Amino acid residues of triadin 1 that would appear on either side of the calsequestrin-binding domain when plotted on a β-sheet were mutated to alanine.

**Generation of the ΔCBD/Triadin 1 Construct—** Production of cDNA encoding canine cardiac triadin 1 with amino acid residues 200–224 deleted was done by a three-step PCR procedure. The first PCR reaction produced a product that encoded amino acid residues 1–230 of triadin 1 but with the coding sequence for residues 200–224 deleted. This PCR reaction utilized sense-Primer 1 (11), which encoded the first 9 amino acids of triadin 1 and had an engineered 5′ EcoRI restriction enzyme site just upstream of the Kozak initiation sequence and ATG start codon, and antisense-Primer 2 (5′ GACATTGGTTGGTGCATTTGCTTTC 3′), which comprised the antisense codons for amino acids 194–199 and 225–230 of triadin 1 but had the antisense codons for residues 200–224 deleted. The second PCR reaction produced a product that encoded amino acids 194–278 of triadin 1 with the coding sequence for residues 200–224 deleted. This PCR reaction utilized sense-Primer 1 (11), which encoded the first 9 amino acids of triadin 1 and had an engineered 5′ EcoRI restriction enzyme site just upstream of the Kozak initiation sequence and ATG start codon, and then an engineered 5′ XhoI restriction enzyme site just downstream of the TGA stop codon. The third PCR reaction used the products from the first and second PCR reactions and Primer 1 and Primer 3′ T1 to amplify the cDNA encoding the complete amino acid sequence of triadin 1 (amino acid residues 1–278) but with the calsequestrin-binding domain (residues 200–224) deleted (ΔCBD/triadin 1). The final PCR product was digested with EcoRI and XhoI and then ligated into the baculovirus transfer vector pBlueBac4.5 to produce ΔCBD/pBlueBac4.5 construct. ΔCBD/pBlueBac4.5 construct was co-transfected with Bac-N-Blue linearized baculovirus DNA (Invitrogen) into Sf21 insect cells (Invitrogen) according to manufacturer’s specifications. Isolation and amplification of the recombinant baculovirus encoding ΔCBD/triadin 1 was performed as described in baculovirus protocols (22). Recombinant wild-type triadin 1 and ΔCBD/triadin 1 were expressed in Sf21 cells and...
puriﬁed from Triton X-100-solubilized microsomes by phosphocellulose chromatography as recently described (11).

\[ ^{35}S \]-Calsequestrin Overlay Assay—A ﬁlter overlay assay (6, 10, 18) was used to localize the calsequestrin-binding domain of triadin 1. 2 μg of different puriﬁed GST-triadin fusion proteins or of full-length recombinant triadin 1 were separated by SDS-PAGE and transferred to nitrocellulose sheets. Blots were blocked for 1 h with 1% horse hemoglobin dissolved in 0.5 M LiCl, 150 mM KCl (pH 7.2), 1 μM EGTA. Blots were allowed to air dry and then pure to homogeneity in one additional step, employing 150 mM KCl, and 1 μM EGTA. Blots were allowed to air dry and were directly exposed to autoradiographic ﬁlm at room temperature.

SDS-PAGE, Immunoblotting, and Antibodies—SDS-PAGE was conducted according to Laemmli (23) or Porzio and Pearson (24) using 10 or 8% polyacrylamide, respectively. Immunoblotting with the T1-speciﬁc antibody was performed as recently described using 125I-protein A for antibody detection (11).

Miscellaneous Techniques—Canine cardiac junctional SR vesicles were isolated by sucrose density gradient centrifugation (26). Endoglycosidase H digestions were performed as described previously (11).

RESULTS
Expression and Puriﬁcation of Metabolically Labeled Calsequestrin—Previously, iodinated calsequestrin was used to identify calsequestrin-binding proteins in junctional SR vesicles isolated from heart (6, 18). However, iodination may oxidize amino acids and produce a heterogeneous pool of labeled product. Since the goal here was to localize and characterize the calsequestrin-binding domain of triadin, it was important to ensure that a high quality radiolabeled calsequestrin preparation was utilized for the studies. To this end, canine cardiac calsequestrin was expressed and metabolically labeled at cysteine and methionine residues in E. coli and then puriﬁed to homogeneity in two easy steps. Five criteria were used to verify that the metabolically labeled, recombinant calsequestrin was structurally and functionally equivalent to its native counterpart as follows: 1) the inherent ability of calsequestrin to aggregate in its Ca\(^{2+}\)-bound state (18, 19, 25); 2) the ability of calsequestrin to undergo a conformational change, internalizing a hydrophobic site and exposing hydrophilic sites in the presence of Ca\(^{2+}\) (17, 18, 20); 3) the solubility of recombinant calsequestrin; 4) the anomalous electrophoretic mobility of cardiac calsequestrin on SDS-PAGE (17); and 5) the characteristic “blue staining” of calsequestrin by the dye Stains-all (26).

Recombinant calsequestrin was readily expressed and puriﬁed. The protein was extracted from E. coli without use of detergents (Fig. IA, Sup), indicating that it was properly folded and soluble. Ca\(^{2+}\) in millimolar concentrations selectively precipitated the recombinant protein, purifying it to near homogeneity in one step (19) (Fig. IA, Ca\(^{2+}\) pel). The precipitated \(^{35}S\)-calsequestrin was resolubilized in Ca\(^{2+}\)-free buffer and then puriﬁed to homogeneity in additional step, employing Ca\(^{2+}\)-dependent elution from phenyl-Sepharose (20) (Fig. IA, lanes 5–9). Successful puriﬁcation with phenyl-Sepharose shows that the hydrophobic site of the protein is exposed at low Ca\(^{2+}\) concentration and internalized at high Ca\(^{2+}\) concentration, one of the hallmarks of native calsequestrin (17, 18, 20). Recombinant calsequestrin migrated with identical mobility on SDS-PAGE as native calsequestrin in cardiac SR vesicles, and the intense blue staining by Stains-all was preserved (Fig. IB). The apparent molecular mass of the protein on SDS-PAGE, 55 kDa, is substantially greater than the calculated molecular mass of 45 kDa (17), demonstrating that the anomalous electrophoretic mobility of calsequestrin was also retained by the recombinant protein. 10 μl (48.6 pmol) of the puriﬁed \(^{35}S\)-calsequestrin was run on an SDS-PAGE gel and exposed to ﬁlm (Fig. 1C), demonstrating the purity of the probe used in the overlay assays described below.

\(^{35}S\)-Calsequestrin Binding to Native and Recombinant Triadin 1—Binding of \(^{35}S\)-calsequestrin to native triadin 1 in SR vesicles and to puriﬁed recombinant triadin 1 was demonstrated by use of the calsequestrin overlay method (6, 18). Canine cardiac junctional SR proteins and puriﬁed triadin 1 were separated by SDS-PAGE, transferred to nitrocellulose, and blots were overlaid with \(^{35}S\)-calsequestrin. Three major \(^{35}S\)-calsequestrin-binding proteins were detected in cardiac SR vesicles (Fig. 2, 1st lane) as follows: a 26-kDa protein or junctin (6 asterisk), and a doublet of 35- and 40-kDa molecular mass proteins (arrows), corresponding to the deglycosylated and glycosylated (ϕ) forms of triadin 1 (T1), respectively (11). These same major calsequestrin-binding proteins in junctional SR vesicles were previously identiﬁed by probing overlays with native cardiac calsequestrin that had been iodinated (6, 18). The identities of the 35- and 40-kDa molecular mass proteins as triadin 1 were conﬁrmed with use of the T1-speciﬁc antibody (lane 2), which was raised to a peptide with the same sequence as the carboxyl-terminal 17 residues of triadin 1 (11). Recombinant canine triadin 1 precipitated from insect cells also bound \(^{35}S\)-calsequestrin strongly (3rd lane, arrows) and was recognized by the T1-speciﬁc antibody (4th lane). The two glycosylated forms of triadin 1 (ϕ) expressed by S/21 cells were noted previously (11) and also bound \(^{35}S\)-calsequestrin.
Localization of the Calsequestrin-binding Region of Triadin 1—Three major regions of canine triadin 1, excluding the transmembrane domain, were expressed and purified as GST fusion proteins to determine which domain binds 35S-calsequestrin (Fig. 3A). Fig. 3B shows a Coomassie Blue-stained gel of the expressed and purified fusion proteins along with purified recombinant triadin 1, and Fig. 3C shows the results of the 35S-calsequestrin overlay, following SDS-PAGE and transfer of identical samples to nitrocellulose. Neither the amino-terminal cytoplasmic domain common to all triadins (Fig. 3A, residues 2–47) nor the unique carboxyl terminus of triadin 1 (Fig. 3A, residues 258–278) bound 35S-calsequestrin (lane 2). The lumenal domain common to all triadins (Fig. 3A, residues 69–257), in contrast, bound 35S-calsequestrin strongly (Fig. 3C, lane 4), as did the purified recombinant protein (Fig. 3C, lane 1). These results concur with previous findings using a fusion protein pull-down assay, which showed that the calsequestrin-binding domain of rabbit triadin is located between residues 69 and 264 (7, 9).

The common lumenal domain of canine triadin 1 (residues 69–257) has several KEKE motifs (Fig. 4), defined as regions of sequence greater than 12 residues in length, with more than 60% Lys and Glu/Asp residues, and lacking five positively or negatively charged residues in a row (13). However, the proximal half of this region of triadin is more negatively charged than the distal half and contains only one KEKE motif (Fig. 4). To see which subdomain of this region binds 35S-calsequestrin,
residues 69–164 and 165–257 of triadin 1 were expressed separately (Fig. 5A) and purified as GST fusion proteins (Fig. 5B). 35S-Calsequestrin overlay assay (Fig. 5C) showed that the calsequestrin-binding domain resided on the more positively charged distal half of the common luminal domain (residues 165–257) (lane 3). The proximal half of the common luminal domain (residues 69–164) did not bind 35S-calsequestrin (lane 2), excluding the upstream KEKE motif from participating in the binding interaction. This assay narrowed the calsequestrin-binding domain of triadin 1 to 93 amino acids, located between amino acid residues 165 and 257. These amino acids (lane 3) bound 35S-calsequestrin as well as intact triadin 1 (lane 1).

To resolve the calsequestrin-binding domain with greater precision, residues 188–257 of triadin were progressively deleted from the carboxy-terminal end, holding residues 165–187 constant and anchored to GST (Fig. 6A). This approach should delimit the amino terminus of the calsequestrin-binding domain. The fusion proteins were expressed and purified (Fig. 6B) and then used for overlay assay (Fig. 6C). Analysis of the results showed that calsequestrin binding remained approximately constant when triadin residues 165–224 were retained by the fusion proteins (Fig. 6C, lanes 2–5) but dropped off precipitously with the fusion protein containing only residues 165–211 (lane 6), with no binding at all observed with the fusion protein containing residues 165–199 (lane 7). These results eliminated residues 165–199 of triadin from binding to calsequestrin and also excluded the two KEKE motifs in this region (Fig. 4) from interacting with calsequestrin.

A second series of fusion protein deletions was made to confirm the carboxy-terminal end of the calsequestrin-binding domain of triadin. This time residues 165–238 of triadin were progressively deleted from the amino-terminal side, holding the carboxy terminus constant (Fig. 7A). Overlay assay (Fig. 7C) of the purified fusion proteins (Fig. 7B) showed that only the two shortest fusion peptides tested (lanes 7 and 8) did not bind 35S-calsequestrin. This excluded residues 225–257 of triadin from binding to calsequestrin. Combining the data in Figs. 3 and Figs. 5–7, the calsequestrin-binding region of canine triadin is localized to amino acid residues 200–224, a select run of only 25 amino acids. This region also contains a KEKE motif. The high concentration of lysine and glutamic acid here is conserved across several mammalian species, especially upon aligning residues 208–224 of canine triadin with the homologous triadin regions from the other species (Fig. 8).

**Lack of Binding to 35S-Calsequestrin to δCBD/T1**—If the fusion protein analyses accurately localized the calsequestrin-binding domain of triadin to a single site at residues 200–224, then triadin 1 with these amino acids deleted (δCBD/T1) should not bind calsequestrin. This was tested by expressing and purifying 35S-calsequestrin as well as intact triadin 1 (Rec. T1) (Fig. 9B). However, the ability to bind 35S-calsequestrin (Fig. 10C). Considering the even-numbered residue mutations, the first fusion protein tested (carrying the E209A, I211A, K213A, and V215A mutations) (lane 2) exhibited an increased level of 35S-calsequestrin binding, relative to the binding obtained with the fusion protein with no mutations in this region (lane 1). However, the next two fusion proteins tested, which had downstream alanine substitutions on this side of the putative β-strand of triadin were mutated to alanine. Fusion proteins carrying the mutations were isolated (Fig. 10B) and tested for the ability to bind 35S-calsequestrin (Fig. 10C). The fusion proteins encompassing this region (K210A, K212A, E214A, and K216A) (lane 3) and (G218A, Q220A, K222A, and K224A) (lane 4), were incapable of binding 35S-calsequestrin. The next series of fusion proteins investigated contained the odd-numbered residue mutations that would appear on the opposite side of the putative β-strand (Fig. 10A). All of the fusion proteins encompassing this region (E201A, R203A, and T207A; lane 5) (E209A, I211A, K213A, and V215A; lane 6); and (G217A, K219A, E221A, and V223A; lane 7) bound 35S-calsequestrin as well as the fusion protein with no mutations (lane 1) or as well as intact triadin 1 (Rec. T1) (Fig. 10C).

These results provide strong evidence that the calsequestrin-binding domain of triadin is a β-strand. The amino acids of...
triadin 1 most critical to formation of the calsequestrin-binding site are the even-numbered, mostly charged residues located between residues 210 and 224 (Fig. 4).

**DISCUSSION**

In this study we have used deletional and site-specific mutagenesis to localize the calsequestrin-binding domain of triadin 1. Only a single binding site was found, located at residues 210–224 of triadin. Here, replacement of Lys210, Lys212, Glu214, and Lys216 by alanine or replacement of Gly218, Gln220, Lys222, and Lys224 by alanine abolished the ability of triadin to bind calsequestrin. The results are consistent with a model in which residues 210–224 of triadin form a β-strand. The even-numbered, mostly charged residues above may line up along one side of the β-strand to interact directly with charged residues of calsequestrin (17), forming a “polar zipper” (15) that links the two proteins together. Remarkably, replacement of charged

**FIG. 6.** Carboxyl-terminal deletional analysis of the triadin subdomain binding calsequestrin. A, schematic of the fusion proteins analyzed, containing triadin residues 165–257 (protein 2), 165–248 (protein 3), 165–224 (protein 5), 165–211 (protein 6), 165–199 (protein 7), and 165–187 (protein 8). Residues of triadin retained by the fusion proteins are indicated in the right-hand margin in parentheses. Purified recombinant triadin 1 was also analyzed (protein 1). B, Coomassie Blue-stained gel of proteins 1–8 in A. C, 35S-calsequestrin overlay of proteins 1–8.

**FIG. 7.** Amino-terminal deletional analysis of the triadin subdomain binding calsequestrin. A, schematic of the fusion proteins analyzed, containing triadin residues 165–257 (protein 2), 178–257 (protein 3), 188–257 (protein 4), 200–257 (protein 5), 212–257 (protein 6), 225–257 (protein 7), and 238–257 (protein 8). Residues of triadin retained by the fusion proteins are listed in the right-hand margin in parentheses. Protein 1 is purified triadin 1. B, Coomassie Blue-stained gel of proteins 1–8. C, 35S-calsequestrin overlay of proteins 1–8.

**FIG. 8.** Sequence comparison of the calsequestrin-binding domains from different species. The amino acid sequence of residues 200–224 of canine triadin is compared with similar sequences in triadins from different species including rabbits (8), humans (27), and mice. Vertical lines denote amino acid residues that are identical for all species. The hyphens denote a gap in the canine sequence. Triadin residue numbers are indicated.

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Residues on the opposite side of the proposed β-strand by alanine did not affect calsequestrin binding to triadin at all, demonstrating that only one face of the strand contributes amino acid side chains that are essential for binding to calsequestrin. Notably, all of the even-numbered, charged residues above are completely conserved across several mammalian species (Fig. 8), suggesting that they comprise a critical interaction site for calsequestrin binding to all triadins.

KEKE motifs have been defined as short runs of amino acid sequence greater than 12 residues in length, which contain at least 60% alternating lysine and glutamic/aspartic acid residues, lack five positively or negatively charged residues in a row, and are devoid of tryptophan, tyrosine, phenylalanine, and proline (13). Such KEKE motifs have been implicated in a variety of different types of protein associations (13–16). Since triadin (and junctin) are replete with KEKE motifs throughout their luminal domains (Fig. 4), we strongly suspected in initiating these studies that several charged sites of triadin, corresponding to KEKE motifs, would be required for tight binding to calsequestrin. Forming to KEKE motifs, would be required for tight binding to calsequestrin binding region of triadin is a β-strand.

The ΔCBD/triadin 1 mutant, which is full-length recombinant triadin 1 with residues 200–224 deleted, failed to bind to calsequestrin in the overlay assay (Fig. 9). Moreover, several small fusion peptides, which shared only residues 200–224 of triadin 1, bound calsequestrin as well as full-length triadin 1. However, these fusion peptides 6 and 7, which had charged residues on the opposite side of the putative β-strand changed to alanine, suggests that the charged side chains of these residues form hydrogen bonds with charged side chains of calsequestrin, stabilizing a β-sheet, which may zip the two proteins together (15). It should be noted, however, that the results of Fig. 10 called into question the requirement for a rigorously defined KEKE motif, at least as originally proposed (13), as necessary for triadin binding to calsequestrin. For example, fusion peptides 6 and 7, which had charged residues on the opposite side of the putative β-strand changed to alanine, bound calsequestrin as well as intact triadin 1. However, these two fusion peptides no longer contained a KEKE motif between residues 200 and 224, because they retained only 56% charged residues here. Similarly, fusion peptide 2 in Fig. 10, which had four closely spaced KEKE-charged residues replaced by alanine (residues 202, 204, 206, and 208), actually bound 35S-calsequestrin more strongly than did full-length triadin 1. Only fusion peptides 4 and 5, which had blocks of even-numbered charged residues removed between residues 210 and 224, failed to bind 35S-calsequestrin. Although the atomic structure of triadin 1 is...
not known, the results of Fig. 10 strongly suggest that the
calsequestrin-binding domain of triadin is a β-strand.

Junctin is another calsequestrin-binding protein that is ho-
mologous to triadin and has similar KEKE association motifs
scattered throughout its luminal domain (6, 10). However,
when similar deletional mutagenesis-analysis was done with
junctin, we observed that deletion of any one of several widely
separated segments of its luminal domain was sufficient to
abolish calsequestrin binding (data not shown). It thus appears
that junctin does not have a single discrete binding site for
calsequestrin, which distinguishes it from triadin. The region
of calsequestrin binding to triadin (and junctin) has not been
identified. Cardiac calsequestrin does not have any consensus
KEKE motifs but does have numerous small clusters of lysine
and glutamic acid residues dispersed throughout its sequence
(17). Thus, the concept of triadin binding to calsequestrin,
stabilized by a polar zipper (15), seems a viable one. Recent
crystallization of skeletal muscle calsequestrin showed that
negatively charged sequences of calsequestrin are arranged
in platforms of negative charges (28). Since amino acids 200–
224 of triadin are sufficient for binding to calsequestrin, it
should be feasible to co-crystallize a triadin synthetic peptide of
these residues with purified recombinant calsequestrin, in or-
der to determine the three-dimensional structure of the bind-
ning complex. These experiments are currently in progress.

In other experiments we observed that the interaction be-
tween the calsequestrin-binding site of triadin and calseque-
strin was enhanced at low Ca²⁺ concentration, as has been
reported previously for intact triadin (6, 10, 18). Similar Ca²⁺
concentrations may occur intralumenally in junctional SR fol-
lowing Ca²⁺ release (29). One of the functions of triadin may be
to anchor calsequestrin to the junctional membrane in proxim-
ity to the RyR, such that during muscle relaxation, when Ca²⁺
levels in the SR are beginning to rise, Ca²⁺ will be concentrated
near the RyR where calsequestrin is tethered. When calseque-
strin becomes saturated with Ca²⁺, its propensity to aggregate
may help to keep it localized to the junctional SR luminal
space. Triadin 1 with the calsequestrin-binding domain deleted
(ACBD/triadin 1) would make an interesting construct for over-
expression in transgenic mouse hearts (11) to test for a possible
dominant-negative effect on excitation-contraction coupling.
Conversely, triadin 1 with enhanced calsequestrin binding, as
achieved by alanine mutagenesis of the proximal end of the
calsequestrin-binding domain, would be an interesting con-
struct to overexpress to test for a possible dominant-positive
effect. It remains to be investigated whether these mutations of
triadin alter its binding to the RyR (6, 7, 9).

Acknowledgment—We thank Mimi Sherman for excellent secretarial
work.

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* Y. M. Kobayashi and L. R. Jones, unpublished observations.