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Communication

Association of Triadin with the Ryanodine Receptor and Calsequestrin in the Lumen of the Sarcoplasmic Reticulum*

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Triadin is a major membrane protein that is specifically localized in the junctional sarcoplasmic reticulum of skeletal muscle and is thought to play an important role in muscle excitation-contraction coupling. In order to identify the proteins in the skeletal muscle that interact with triadin, the cytoplasmic and luminal domains of triadin were expressed as glutathione *S*-transferase fusion proteins and immobilized to glutathione-Sepharose to form affinity columns. Using these affinity columns, we find that triadin binds specifically to the ryanodine receptor/Ca^{2+} release channel and the Ca^{2+}-binding protein calsequestrin from CHAPS (3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid)-solubilized skeletal muscle homogenates. The luminal but not the cytoplasmic domain of triadin-glutathione *S*-transferase fusion protein binds [^{3}H]ryanodine receptor, whereas neither the cytoplasmic nor the luminal portion of triadin binds [^{3}H]PN-200-100-labeled dihydropyridine receptor. In addition, the luminal domain of triadin interacts with calsequestrin in a Ca^{2+}-dependent manner and is capable of inhibiting the reassociation of calsequestrin to the junctional face membrane. These results suggest that triadin is the previously unidentified transmembrane protein that anchors calsequestrin to the junctional region of the sarcoplasmic reticulum, and is involved in the functional coupling between calsequestrin and the ryanodine receptor/Ca^{2+} release channel.

In muscle cells, depolarization of the transverse tubules (T-tubules)[1] results in Ca^{2+} release from the sarcoplasmic reticulum and the elevation of the cytoplasmic Ca^{2+} leads to muscle contraction. Considerable research has been focused on identifying and characterizing the protein components that are important in the regulation of calcium storage and release from the sarcoplasmic reticulum. Several proteins that are specifically localized to the triad junction play essential roles in excitation-contraction coupling. The T-tubular dihydropyridine receptor senses the voltage across the membrane, and activation of this receptor leads to the release of Ca^{2+} from the sarcoplasmic reticulum (1–4). The ryanodine receptor/Ca^{2+} release channel is localized in the junctional sarcoplasmic reticulum and is responsible for the Ca^{2+} release from the Ca^{2+} store (5–8). In the lumen of the sarcoplasmic reticulum, the major protein is calsequestrin, an acidic protein that binds to calcium with moderate affinity and high capacity (9). It is anchored to the luminal face of the junctional sarcoplasmic reticulum (10) and is thought to sequester and concentrate calcium near its release site. Calsequestrin and the ryanodine receptor/Ca^{2+} release channel are functionally coupled. Activation of the ryanodine receptor induces calcium dissociation from calsequestrin, allowing the free Ca^{2+} to be released (11); calsequestrin also mediates the intraluminal Ca^{2+} control over the activation of the ryanodine receptor/Ca^{2+} release channel (12–14). Triadin is an abundant membrane protein in the junctional sarcoplasmic reticulum, where it co-localizes with the ryanodine receptor/Ca^{2+} release channel (15–17). It contains a single transmembrane domain that separates this protein into cytoplasmic and luminal segments (16). Notably, only 47 amino acids of triadin are cytoplasmic, with the bulk of this protein including the carboxyl terminus being located in the lumen of the sarcoplasmic reticulum, which contains a high concentration of positively charged amino acids (16). This membrane topology suggests that it plays an important role in the lumen of the sarcoplasmic reticulum.

Here, we report that triadin associates with both the ryanodine receptor/Ca^{2+} release channel and calsequestrin in the lumen of the sarcoplasmic reticulum. Our results indicate that triadin is the transmembrane protein that anchors calsequestrin to the junctional face membrane. In addition, our data, along with previous biophysical studies, suggest that triadin mediates the functional coupling between the ryanodine receptor/Ca^{2+} release channel and calsequestrin in the lumen of the sarcoplasmic reticulum.

EXPERIMENTAL PROCEDURES

Generation of Triadin-GST Fusion Proteins—Triadin cDNA fragments (16) corresponding to amino adds 100-706 (luminal sequence) and amino acids 1–47 (cytoplasmic sequence) were amplified using polymerase chain reaction and were subcloned into EcoRI sites of pGEX vectors. The plasmid constructs were confirmed by sequencing. The GST fusion proteins were expressed in Escherichia coli DH5α cells and purified using glutathione-Sepharose 4B affinity column. The expressed proteins can be recognized by all the available triadin antibodies (15).

Muscle Homogenization and Fusion Protein Affinity Sepharose Binding Assay—Rabbit skeletal muscle was homogenized in a 1:3 ratio in the homogenization buffer containing 20 mm Tris-HCl, pH 7.5, 1 M Nad, 1.5% CHAPS, and protease inhibitors: aprotinin (76.8 nM), benzamidine (0.83 mM), iodoacetamide (1 mM), leupeptin (1.1 (μM), pepstatin A (0.7 μM), and phenylmethylsulfonyl fluoride (0.23 mM). The homogenate was incubated on ice for 2 h and then centrifuged at 30,000 rpm in a Beckman ultracentrifuge using the Ti-45 rotor for 35 min. The supernatant was collected and diluted 1:10 in the dilution buffer containing 20 mM Tris-HCl, pH 7.5, 1 mM $CaCl_2$, 1 mM dithiothreitol, and protease inhibitors as described above. The diluted homogenate was further incubated at 4°C for 5 h and centrifuged again to remove precipitated materials (mainly myosin). The solubilized skeletal muscle homogenate was precleared using glutathione-Sepharose 4B for 3 h and then incubated with the fusion protein-Sepharose for 4 h. After incubation, the Sepharose was washed sequentially using Buffer I (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM $CaCl_2$, 1 mM, 0.5% CHAPS), and Buffer II (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM $CaCl_2$, 0.15%

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[1] The abbreviations used are: T-tubule, transverse tubule; GST, glutathione *S*-transferase; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-l-propanesulfonic acid; PAGE, polyacrylamide gel electrophoresis; C- and L-triadin, cytoplasmic and luminal domains of triadin.

CHAPS). Bound proteins were eluted using 20 mM Tris-HCl, pH 7.4, 0.15% CHAPS, 0.75 M Nad, 20 mM EDTA and analyzed by SDS-PAGE. Anti-calsequestrin monoclonal antibody VIIID1-2 (18) and sheep anti-rabbit skeletal muscle ryanodine receptor antibody (19) were used in the immunoblot assay.

[^{3}H]Ryanodine and [^{3}H]PN-200-100 Binding Assay—The skeletal muscle homogenate was pre-equilibrated with 20 nM [^{3}H]ryanodine at 37 °C for 60 min in a buffer containing 20 mM Tris-HCl, pH 7.4, 0.15% CHAPS, 200 mM KC1, 10 mM ATP, and 20 μM free Ca^{2+}. The Sepharose was incubated with the labeled muscle homogenates as described above, and the [^{3}H]ryanodine bound to the Sepharose was counted. As a positive control, Sepharose conjugated with anti-ryanodine receptor monoclonal antibody XA7 (20) was used. For [^{3}H]PN-200-100 binding experiments, the skeletal muscle homogenate was pre-equilibrated with 20 nM [^{3}H]PN-200-100 at 37 °C for 60 min. in a buffer containing 20 mM Tris-HCl, pH 7.4, 0.15% CHAPS, 150 mM NaCl, and 200 μM diltiazem. The Sepharose was incubated with the labeled muscle homogenate as described above. After washing, the amount of [^{3}H]PN-200-100 bound to the Sepharose was counted. For nonspecific binding, 10 μM mtrendipine was added to the incubation buffer. As a positive control, Protein G-Sepharose (Pharmacia) that was coupled with the anti-α1 subunit of the dihydropyridine receptor monoclonal antibody IIC12 (21) was used.

Inhibition of the Reassociation of Calsequestrin with Junctional Face Membrane by Triadin Fusion Protein—Junctional face membrane was prepared by 0.1% Triton X-100 and 0.75 M NaCl extraction of the rabbit skeletal muscle triads (11). To reconstitute the calsequestrin/junctional face membrane complex, the Junctional face membrane was incubated with Calsequestrin that had been purified using phenyl-Sepharose (22) in a buffer containing 10 mM Tris-HCl, pH 7.5, 0.1% Triton X-100, 1 mM $CaCl_2$, and 150 mM NaCl. After incubation at 4 °C for 4 h, the sample was centrifuged using a Beckman TL-100 ultracentrifuge at 100,000 rpm for 15 min. The insoluble pellet, which contained calsequestrin attached to junctional face membrane, was collected and analyzed using SDS-PAGE. For triadin fusion protein competition assay, various amounts of fusion protein were added to the above incubation mixture. After incubation and centrifugation, the pellets were separated on SDS-PAGE and transferred to nitrocellulose for immunoblot assay using anti-calsequestrin antibody VIIID1-2. The relative amount of calsequestrin on the immunoblot was estimated using Molecular Dynamics 300S densitometer.

The reassociated calsequestrin was calculated by subtracting the residual calsequestrin in the Junctional face membrane from the total amount of calsequestrin in the reassociated calsequestrin/junctional face membrane complex. The reassociated calsequestrin in the absence of triadin fusion protein was designated as 100%.

RESULTS AND DISCUSSION

To identify the proteins that interact with triadin, domains of triadin that are localized in the cytoplasm and the lumen of the sarcoplasmic reticulum (C-triadin and L-triadin, respectively) were expressed as glutathione *S*-transferase (GST) fusion proteins and immobilized on glutathione-Sepharose to form affinity columns. Homogenates were prepared from rabbit skeletal muscle and solubilized with 1.5% CHAPS. Detergent-solubilized material was diluted 10-fold and applied to the column, washed, and eluted with a buffer containing 0.8 m NaCI and 20 mm EDTA. Two proteins with molecular masses -63 and -550 kDa (Fig. 1*A*, *arrowheads*) were eluted from the Sepharose that conjugated with the luminal domain of triadin (L-triadin-Sepharose). The 63-kDa protein was identified as Calsequestrin, the calcium-binding protein in the lumen of the sarcoplasmic reticulum; the high molecular mass protein was identified as the ryanodine receptor/Ca^{2+} release channel (Fig. 1*A*). Control experiments were also performed. As shown in Fig. 1*B*, glutathione-Sepharose, glutathione-Sepharose that contains GST alone (GST-Sepharose), or the cytoplasmic domain of triadin (C-triadin-Sepharose), all failed to bind the ryanodine receptor and Calsequestrin (Fig. 1*B*). Only L-triadin-Sepharose was able to bind to these two proteins. The ryanodine receptor and Calsequestrin are minor components in the solubilized whole muscle homogenates; however, they are the only major proteins from the homogenates that bind to the

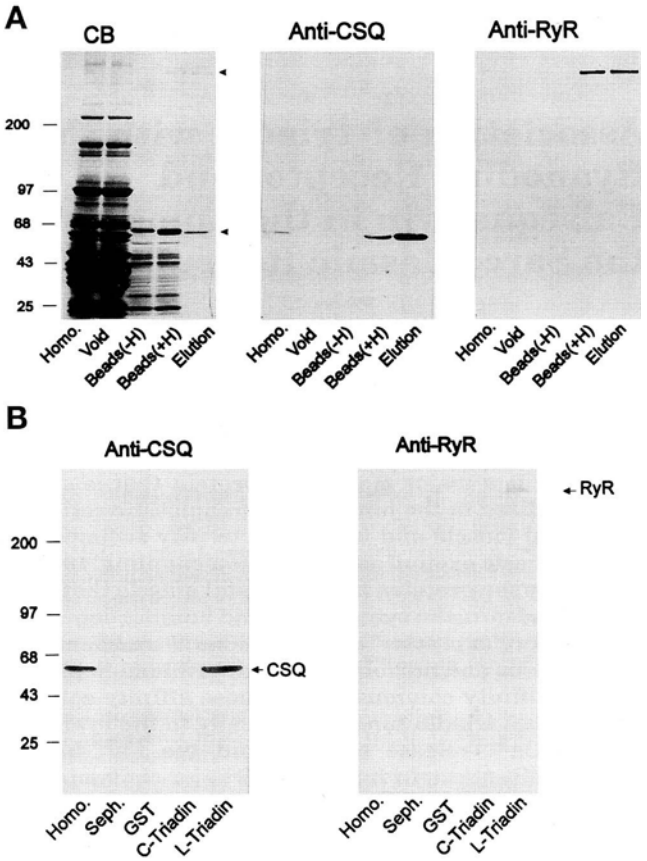

FIG. 1. **Association of the ryanodine receptor and calsequestrin with the luminal portion of triadin.** *A*, after predearance using glutathione-Sepharose, the CHAPS-solubilized skeletal muscle homogenate was subjected to affinity chromatography on L-triadin-Sepharose (30 μl). The starting material (75 μg), skeletal muscle homogenate (*Homo.*), flow-through (*Void*), L-triadin-Sepharose without incubation with the homogenate (*Beads(-H)*), fusion protein-Sepharose after incubating with muscle homogenates (*Beads(+H)*), and the sample eluted from the Sepharose (*Elution*) were analyzed on SDS-PAGE. The gels were either stained with Coomassie Blue (*CB*) or transferred to nitrocellulose and stained with anti-calsequestrin (*Anti-CSQ*) or anti-ryanodine receptor antibody (*Anti-RyR*). *B*, specific interaction of the luminal portion of triadin with the ryanodine receptor and calsequestrin. Chromatography was performed on various affinity columns including the glutathione-Sepharose (*Seph.*), GST-Sepharose (*GST*), C-triadin-Sepharose (*C-triadin*), and L-triadin-Sepharose (*L-triadin*). The ryanodine receptor and calsequestrin that bound to the Sepharose were analyzed by immunoblot assay.

L-triadin-Sepharose as detected in the Coomassie Blue-stained gel (Fig. 1*A*). This further demonstrates the specificity of the interactions. Similar results have been obtained with detergent-solubilized skeletal muscle triads. Furthermore, we find that L-triadin-Sepharose binds to the purified ryanodine receptor or purified calsequestrin (data not shown). Also, the triadin fusion protein was able to bind to calsequestrin in a protein overlay assay (data not shown). Thus, triadin interacts independently with both the ryanodine receptor and calsequestrin. The association of the ryanodine receptor with triadin was demonstrated further in [^{3}H]ryanodine receptor binding experiments. The solubilized skeletal muscle homogenate was prelabeled with [^{3}H]ryanodine and incubated with L-triadin-Sepharose. The amount of bound ryanodine receptor was determined by counting the [^{3}H]ryanodine on the L-triadin-Sepharose. As shown in Fig. 2*A*, the luminal portion fusion protein bound the labeled ryanodine receptor in a dose-dependent manner. The specificity of the binding was also examined. In a manner similar to the ryanodine receptor monoclonal antibody XA7-Sepharose (20), L-triadin-Sepharose specifically

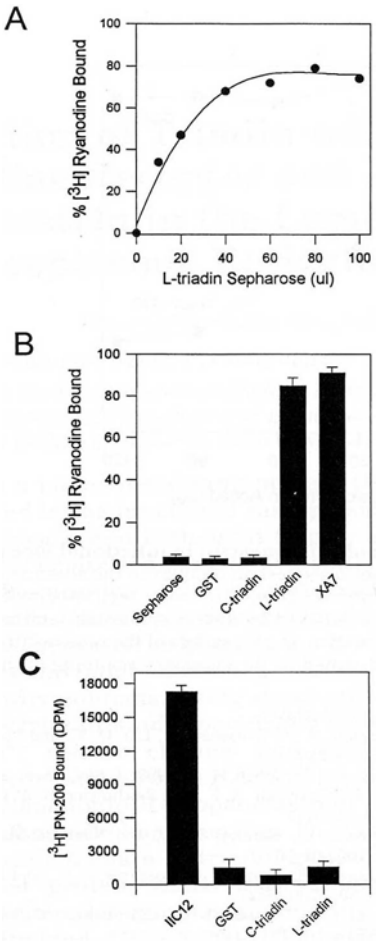

FIG. 2. **Binding of the prelabeled rabbit skeletal muscle ryanodine receptor by the luminal domain of triadin.** *A*, L-triadin-Sepharose binds [^{3}H]ryanodine-labeled receptor in a dose-dependent manner. *B*, specificity of the binding by the luminal domain of triadin is shown. Sepharose containing different fusion proteins were used in the experiment. Anti-ryanodine receptor monoclonal antibody XA7-Sepharose was used as positive control in the assay. *C*, binding of the [^{3}H]PN200-100-labeled dihydropyridine receptor by GST fusion proteins is shown. Anti-α1 subunit of the dihydropyridine receptor monoclonal antibody IIC12 Sepharose was used as positive control in the assay.

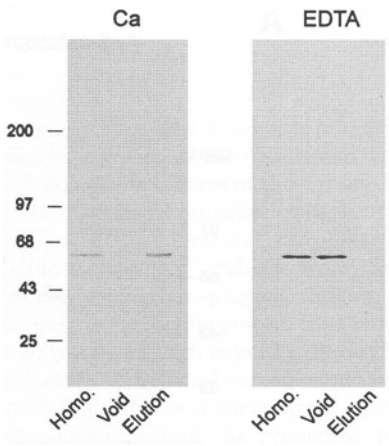

FIG. 3. **The interaction between calsequestrin and triadin is Ca^{2+}-dependent.** L-Triadin-Sepharose was incubated with solubilized skeletal muscle homogenate in the presence of 1 mM $CaCl_2$ or 20 mM EDTA. The starting material (*Homo.*), flow-through (*Void*), and the eluted proteins (*Elution*) were analyzed by immunoblot staining with anti-calsequestrin monoclonal antibody VIIID1-2.

bound the labeled receptor, whereas the GST-Sepharose or C-triadin-Sepharose was not able to bind to the labeled ryanodine receptor (Fig. 2*B*). Using the same bead assay, we examined the possible interaction of triadin-Sepharose with the [^{3}H]PN-200-100-labeled dihydropyridine receptor. However, neither the cytoplasmic nor the luminal portion of triadin was able to bind to the [^{3}H]PN-200-100-labeled receptor in the muscle homogenate (Fig. 2*C*). Our results demonstrate a specific interaction between triadin and the ryanodine receptor that occurs in the lumen of the sarcoplasmic reticulum. Clusters of negatively charged residues in the ryanodine receptor located in the lumen of the sarcoplasmic reticulum (23-25) are likely to be involved in the interaction with the positively charged triadin (16).

Calsequestrin binds Ca^{2+} with moderate affinity and high capacity (9), and the binding of Ca^{2+} leads to dramatic conformational change of this luminal protein (26, 27). We therefore examined the effect of Ca^{2+} on the interaction between calsequestrin and triadin. In the presence of $CaCl_2$, L-triadin-Sepharose was able to bind calsequestrin and remove calsequestrin from muscle homogenate (Fig. 3, *left panel*). However, when EDTA was present, the interaction was inhibited. Thus, calsequestrin binds to the luminal domain of triadin in a Ca^{2+}-dependent manner.

Calsequestrin is a soluble protein that remains associated with the luminal side of the junctional membrane of the sarcoplasmic reticulum through its interaction with a previously unidentified membrane protein (10). Calsequestrin can be extracted from the junctional face membrane by treatment with EDTA or high concentrations of NaCl (11, 29), indicating that the interaction between calsequestrin and its membrane anchoring protein can be inhibited by EDTA and is disruptable by high ionic strength. Notably, the association between calsequestrin and triadin-GST fusion protein is also inhibited by EDTA, and high salt concentration can elute calsequestrin from L-triadin-Sepharose. These binding properties resemble those of the interaction of calsequestrin with its anchoring protein in the junctional face membrane. In addition, like calsequestrin, triadin is an abundant protein that is specifically localized in the junctional region, with the bulk of this protein in the lumen of the sarcoplasmic reticulum (15, 16). Together, these characteristics strongly suggest that triadin is the physiological anchoring protein for calsequestrin at the junctional face of sarcoplasmic reticulum. To further test this hypothesis, we examined whether the triadin luminal domain-GST fusion protein was capable of inhibiting the reassociation of calsequestrin with the junctional face membrane. As shown in Fig. 4, most of the calsequestrin was removed from the junctional face membrane by treating triads vesicles twice with a buffer containing 20 mM Tris-HCl, pH 7.4, 0.1% Triton X-100, 0.75 M Nad (Fig. 4*A*, *lane 1*). When calsequestrin was incubated with the junctional face membrane in a buffer containing 150 mM NaCl and 1 mM $CaCl_2$, calsequestrin reassociated with the membrane fraction (Fig. 4*A*, *lane 2*). However, when increasing amounts of triadin-GST fusion protein were added to the incubation buffer, less calsequestrin reassociated to the junctional face membrane (Fig. 4*A*, *lanes 3-6*). The native triadin did not decrease during the competition process (Fig. 4*A*, *bottom*), and GST alone did not inhibit the reassociation (Fig. 4*B*). This result suggests that the soluble triadin luminal portion fusion protein interacts with calsequestrin and inhibits the reassociation of calsequestrin to the membrane by competing with the native triadin in the junctional face membrane. This study suggests that one functional role of triadin is to serve as the transmembrane protein that anchors calsequestrin to the luminal side of the junctional sarcoplasmic reticulum near the "SR feet" (ryanodine receptor) (10, 28), where calsequestrin exerts its physiological function.

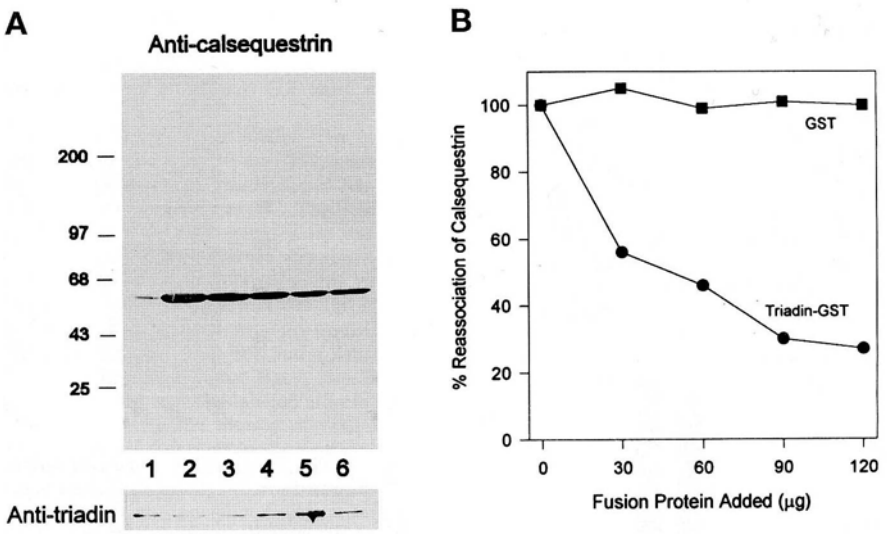

FIG. 4. **Effect of triadin luminal portion-GST fusion protein on the reassociation of calsequestrin to junctional face membrane.** *A*, proteins of the junctional face membrane (*lane 1*), and reassociated junctional face membrane/calsequestrin complex in the absence of triadin fusion protein (*lane 2*) and presence of increasing amounts (30, 60, 90, and 120 μg) of the fusion protein (*lanes 3-6*) were separated by SDS-PAGE and transferred to nitrocellulose. Calsequestrin attached to the junctional face membrane was detected by anti-calsequestrin antibody. The native triadin in the junctional face membrane detected by anti-triadin antibody was shown at the bottom. *B*, percentage of the reassociated calsequestrin in the presence of various amounts of triadin luminal portion fusion protein or GST as estimated by densitometry scanning of the immunoblot.

It is known that the intraluminal Ca^{2+}-binding protein calsequestrin and the ryanodine receptor/Ca^{2+} release channel are functionally coupled (11-14). The ryanodine receptor, when activated by Ca^{2+} release agents, such as caffeine, induces dissociation of calcium from calsequestrin (11). Conversely, changes in luminal Ca^{2+} concentration lead to conformational changes in calsequestrin (26, 27), and this information can be transmitted to the ryanodine receptor, thus affecting the activation of the ryanodine receptor/Ca^{2+} release channel (12-14). However, despite the biophysical evidence for functional coupling between the ryanodine receptor and calsequestrin, there is no evidence for direct interaction between the two proteins. It is thought that the interaction is mediated by a third protein. Our data and previous studies suggest that triadin binds to the ryanodine receptor (17, 30), and the luminal domain of triadin also interacts with calsequestrin. Together, these results suggest that triadin anchors calsequestrin to the junctional face membrane, and thus may be involved in the functional coupling between calsequestrin and the ryanodine receptor/Ca^{2+} release channel in the lumen of the sarcoplasmic reticulum.

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