Complex Formation between Junctin, Triadin, Calsequestrin, and the Ryanodine Receptor

PROTEINS OF THE CARDIAC JUNCTIONAL SARCOPLASMIC RETICULUM MEMBRANE

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Several key proteins have been localized to junctional sarcoplasmic reticulum which are important for Ca\(^{2+}\) release. These include the ryanodine receptor, triadin, and calsequestrin, which may associate into a stable complex at the junctional membrane. We recently purified and cloned a fourth component of this complex, junctin, which exhibits homology with triadin and is the major 26-kDa-calsequestrin-binding protein detected in cardiac sarcoplasmic reticulum vesicles (Jones, L. R., Zhang, L., Sanborn, K., Jorgensen, A. O., and Kelley, J. (1995) J. Biol. Chem. 270, 30787–30796). In the present study, we have examined the binding interactions between the cardiac forms of these four proteins with emphasis placed on the role of junctin. By a combination of approaches including calsequestrin-affinity chromatography, filter overlay, immunoprecipitation assays, and fusion protein binding analyses, we find that junctin binds directly to calsequestrin, triadin, and the ryanodine receptor. This binding interaction is localized to the luminal domain of junctin, which is highly enriched in charged amino acids organized into “KEKE” motifs. KEKE repeats are also found in the common luminal domain of triadin, which likewise is capable of binding to calsequestrin and the ryanodine receptor (Guo, W., and Campbell, K. P. (1995) J. Biol. Chem. 270, 9027–9030). It appears that junctin and triadin interact directly in the junctional sarcoplasmic reticulum membrane and stabilize a complex that anchors calsequestrin to the ryanodine receptor. Taken together, these results suggest that junctin, calsequestrin, triadin, and the ryanodine receptor form a quaternary complex that may be required for normal operation of Ca\(^{2+}\) release.

Ultrastructural and biochemical evidence suggests that a protein complex exists at the junctional SR\(^1\) membrane in cardiac and skeletal muscle to facilitate the release of Ca\(^{2+}\) which occurs during muscle contraction (1–4). Components of this protein complex identified to date include the ryanodine receptor or Ca\(^{2+}\) release channel, which is visualized by electron microscopy as projecting feet on the cytoplasmic surface of the junctional membrane (2, 4); calsequestrin, a high capacity Ca\(^{2+}\)-binding protein located in the junctional SR lumen, which buffers the calcium that is released during muscle contraction (5–8); and triadin (9–11) and junctin (8, 12), putative “anchoring” proteins, which appear to stabilize calsequestrin at the inner face of the junctional SR membrane. Calsequestrin is seen by electron microscopy as an electron-dense matrix in the SR lumen, where the protein appears to be physically connected to ryanodine receptors by “anchoring strands” (13) or “rope-like fibers” (14). Biochemical evidence suggests that calsequestrin actively participates in muscle contraction by regulating the amount of Ca\(^{2+}\) released by the ryanodine receptor (15–18). This regulatory effect may be mediated by calsequestrin-anchoring proteins such as triadin (19, 20) and junctin (8, 12).

Junctin (8, 12) and triadin (11, 20) are integral membrane proteins sharing structural and amino acid sequence similarity which co-localize with the ryanodine receptor and calsequestrin at the junctional SR membrane in cardiac and skeletal muscle. Junctin was first identified as a 26-kDa calsequestrin-binding protein in cardiac and skeletal muscle junctional SR membranes with use of an \(^{125}\)I-calsequestrin filter overlay assay, where it was the main calsequestrin-binding protein detected (8). Based on this result, it was proposed that junctin was one of the proteins anchoring calsequestrin to the SR membrane. Subsequently, junctin was purified and cloned and shown to be identically expressed in skeletal and cardiac muscle (12). Triadin was first identified as a 95-kDa protein in skeletal muscle junctional SR vesicles (9, 10). Subsequently, one skeletal muscle (11) and three cardiac (20) triadin isoforms were cloned. The cardiac triadin isoforms exhibit apparent molecular weights of 92,000 (triadin 3), 40,000 (triadin 2), and 35,000 (triadin 1) on SDS-PAGE. All triadin isoforms share identical sequences from residues 1–264. Although junctin and triadin are the products of different genes, they exhibit intriguing similarities. Both proteins have single membrane spanning domains that are 62% identical, short N-terminal segments located in the cytoplasm, and long C-terminal tails, which project intraluminally and are highly charged and basic (11, 12, 20). Notable in the luminal domains of both proteins is the frequent occurrence of long runs of alternating positively and negatively charged residues, which are frequently enriched in lysine and glutamic acid. Recurring sequences of this type have been described by others as “KEKE” association motifs and are thought to be involved in the promotion of several types of protein-protein binding interactions (21–23). A GST fusion protein containing the common luminal domain of triadin, composed of residues 69–264, and incorporating the KEKE regions was recently

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‡The abbreviations used are: SR, sarcoplasmic reticulum; PAGE, polyacrylamide gel electrophoresis; MOPS, 3-(N-morpholino)propanesulfonic acid; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid; GST, glutathione S-transferase; N-junctin, GST fusion protein containing N-terminal, cytoplasmic domain of junctin; C-junctin, GST fusion protein containing C-terminal, luminal domain of junctin; H-triadin, GST fusion protein containing homologous luminal domain of triadin.
showed to interact directly with calsequestrin and the ryanodine receptor in detergent extracts prepared from skeletal muscle homogenates (19) and cardiac microsomes (20). These results provided evidence for an anchoring role for triadin, in which it connects calsequestrin to the ryanodine receptor at the junctional SR membrane in cardiac and skeletal muscle (19, 20).

The close structural similarities between junctin and triadin suggest that both proteins have related functions. However, to date little is known about the biological role of junctin and whether, in particular, junctin, like triadin, is capable of interacting directly with the ryanodine receptor, as well as with calsequestrin (8, 12). Moreover, for triadin itself, formation of a complex between the ryanodine receptor and calsequestrin has been demonstrated solely by use of fusion protein binding assays but not for native triadin or with use of purified proteins.

In the work presented here, we have tested for the ability of junctin to bind to junctional SR proteins important for Ca2+ release, with focus on analysis of cardiac membrane proteins. Using several different approaches, we demonstrate that junctin binds to both calsequestrin and the ryanodine receptor and, remarkably, also interacts directly with triadin. Our results provide strong support for a model in which a quaternary protein complex exists between junctin, triadin, calsequestrin, and the ryanodine receptor at the junctional SR membrane. This complex may be important for operation of Ca2+ release during excitation-contraction coupling in cardiac and skeletal muscle.

EXPERIMENTAL PROCEDURES

Isolation of Cardiac Junctional SR Vesicles—Subfraction D microsomes enriched in junctional SR vesicles were isolated from canine left ventricles by sucrose gradient centrifugation as described previously (24). Protein concentrations were determined by the method of Lowry et al. (25).

Calsequestrin-Affinity Chromatography—5 mg of canine cardiac calsequestrin purified by phenyl-Sepharose chromatography (26) were covalently coupled to 1 ml of Affi-Gel 15 (Bio-Rad) according to the manufacturer’s instructions. For calsequestrin-affinity chromatography, 15 mg of canine cardiac junctional SR vesicles were solubilized for 20 min at room temperature in 2 ml of medium containing 23 mM HEPES, 0.1 M NaCl, 0.5 M NaCl, and 2% Triton X-100 (pH 7.2). The sample was centrifuged for 10 min at 100,000 rpm in a Beckman TL-100.3 rotor. The supernatant was diluted 1:5 in 20 mM MOPS, 1 mM EGTA, and passed through 1 ml of the calsequestrin-avidity matrix. The column was then eluted with four 1-ml washes of 20 mM MOPS, 150 mM NaCl, 1 mM EGTA, and 0.1% Triton X-100 (pH 7.2), followed by four 1-ml washes of the same buffer with 500 mM NaCl, 1 mM EGTA, and finally with three 1-ml washes of the Ca2+-containing buffer with the NaCl concentration increased to 0.5 M. The column fractions were then analyzed by SDS-PAGE followed by immunoblotting with antibodies to junctin and triadin. For protein staining with Coomassie Blue, the samples were incubated for 20 min at room temperature in 2 ml of medium containing 23 mM HEPES, 15 mg of canine cardiac junctional SR vesicles were solubilized for 1 hour at a protein concentration of 4 mg/ml in buffer containing 3% CHAPS, 1.0 M NaCl, 1 mM dithiothreitol, 20 mM Tris-HCl (pH 7.4), and protease inhibitors (10 mM leupeptin, 2 mM pepstatin, and 10 mM Pefabloc). Solubilized proteins were obtained by centrifugation with a Beckman TL-100 rotor at 100,000 rpm for 5 min.

Immunoprecipitation—CHAPS-solubilized cardiac junctional SR proteins, prepared as above, were diluted 10-fold in 20 mM Tris-HCl (pH 7.4), 1 mM dithiothreitol, and protease inhibitors to reduce the high salt and detergent concentrations. The dilution buffer also contained 1 mM EGTA or the CaCl2 concentrations indicated in the figure legends. 700 l of diluted supernatant was preincubated with 50 l of protein A-Sepharose CL-4B beads (Sigma) for 2 h at 4 °C with rotating shaking and then sedimented to eliminate nonspecific binding. Antibodies were added to 700-l aliquots of the precleared supernatants, and the samples were incubated for 2 h at 4 °C, followed by further incubation with 50 l of protein A-Sepharose CL-4B beads for 2 h. Immunoprecipitates were washed four times with buffer containing 20 mM Tris-HCl (pH 7.4) and 1.0 M NaCl, and 0.5% CHAPS including EGTA or CaCl2 as indicated. The proteins were eluted from the affinity beads by boiling in SDS-sample buffer containing 10% SDS and subjected to immunoblotting (12). [3H]Ryanodine binding to proteins adsorbed to affinity beads was measured as described below.

Construction of GST Fusion Proteins—Three GST fusion proteins were used in this work. N-junctin is the GST fusing protein containing the cytoplasmic domain of junctin; C-junctin is the GST fusion protein containing the C-terminal, luminal domain of junctin (residues 46–210); and H-triadin (20) is the GST fusion protein containing the homologous luminal domain of triadin (residues 69–264). The pGEX vector with the insert for H-triadin was generously supplied by K. Campbell (20). For preparation of N-junctin, DNA encoding residues 1–23 of junctin was generated by polymerase chain reaction and inserted in frame into the pGEX vector. H-triadin and N-junctin fusion proteins were expressed and purified from Escherichia coli BL-21 using glutathione-Sepharose chromatography (Pharmacia Biotech Inc.). We observed that C-junctin expressed poorly in Escherichia coli, necessitating its purification from SF21 cells. For preparation of C-junctin, DNA encoding residues 46–210 of canine junctin (12) was generated by polymerase chain reaction and inserted in frame into the EcoRI site of pGEX-4T, a baculovirus transfer vector containing a 6xHis tag and a GST tag upstream of the multiple cloning sites. C-junctin was expressed in SF21 insect cells and purified with use of Ni2+-nitrilotriacetic acid resin. All amplified fragments were confirmed by sequencing (31).

Fusion Protein-Sepharose Affinity Binding Assay—Fusion proteins were prepared by in vitro translation of the GST fusion protein at 37 °C and then purified on glutathione-Sepharose beads. The GST fusion proteins were incubated with glutathione-Sepharose beads for 2 h at 4 °C (Pharmacia). Diluted extracts containing CHAPS-solubilized junctional SR proteins (see above) were preincubated with glutathione-Sepharose beads for 2 h at 4 °C to eliminate nonspecific binding. Preincubated supernatants were then incubated with the affinity matrices for 2 h at 4 °C. Beads were washed in the same buffer used for immunoprecipitation assay, and bound

Baculovirus Protein Complex

The baculovirus transfer vector pVL1392. The protein was then expressed in insect were extracted with sodium carbonate (pH 11.4) to obtain a carbonase sequence recently described for phospholamban (28). Sf21 cells expressing junc-
proteins were eluted by boiling in SDS-sample buffer and then subjected to SDS-PAGE and immunoblotting. [3H]Ryanodine binding to proteins absorbed to affinity beads was measured as described above.

**Purification of the Cardiac Ryanodine Receptor**—15 mg of canine cardiac junctional SR vesicles were suspended on ice in 3 ml of medium containing 5% CHAPS, 20 mM MOPS (pH 7.2), 1 mM NaCl, 5 mM dithiothreitol, 0.2 mM CaCl₂, 0.25 mM sucrose, 5 mM MgCl₂, 50 μM Pefabloc, and 10 μM/ml leupeptin. The sample was centrifuged at 50,000 rpm for 30 min in a Beckman Ti70.1 rotor, and the supernatant was loaded on the top of a 35-ml, 10–25% linear sucrose gradient in a Beckman VC53 rotor. The sucrose gradient contained the same buffer as the CHAPS concentration reduced to 0.3% and the phosphatidylcholine concentration reduced to 1 mg/ml. Centrifugation was for 2 h at 50,000 rpm. Gradient fractions of 2 ml were collected, and aliquots were subjected to SDS-PAGE and analyzed for [3H]ryanodine binding (24). When the purified ryanodine receptor was used in the fusion protein binding assay, the sample from the sucrose gradient was diluted 1:10 in 20 mM Tris-HCl, 0.3% CHAPS to reduce the NaCl concentration to 0.1 M.

**[3H]Ryanodine Binding Assay**—[3H]Ryanodine binding to the solubilized ryanodine receptor or to receptors absorbed to affinity beads was assayed in 20 mM MOPS (pH 7.2), 1 mM NaCl, 5 mM dithiothreitol, 0.2 mM CaCl₂, 0.25 mM sucrose, and 15 mM [3H]ryanodine. Nonspecific binding was determined in the presence of 10 μM non-radioactive ryanodine. Solubilized receptors were precipitated with polyethylene glycol prior to filtration over glass-fiber filters (24). Affinity beads containing bound receptors were filtered directly.

**SDS-PAGE, Immunoblotting, and Antibodies—**SDS-PAGE was conducted according to Porzio and Pearson (27) or Laemmli (32). For detection of ryanodine receptors, 5% polyacrylamide was used, and for detection of all other proteins, 8% polyacrylamide was used. Immunoblotting with different antibodies was performed as described previously using 125I-protein A to detect antibody-binding proteins (12).

Triadin polyclonal antibody (GP58) used in this study was a generous gift from K. Campbell. This antibody recognizes all triadin isoforms (20). Antibodies to the unique C terminus of canine cardiac triadin 1 as well as to the purified recombinant protein were also raised in rabbits (20). Antibodies to the unique C terminus of canine cardiac triadin 1 as well as to the purified recombinant protein were also raised in rabbits (20). Antibodies to the unique C terminus of canine cardiac triadin 1 as well as to the purified recombinant protein were also raised in rabbits (20). Antibodies to the unique C terminus of canine cardiac triadin 1 as well as to the purified recombinant protein were also raised in rabbits (20). Antibodies to the unique C terminus of canine cardiac triadin 1 as well as to the purified recombinant protein were also raised in rabbits (20). Antibodies to the unique C terminus of canine cardiac triadin 1 as well as to the purified recombinant protein were also raised in rabbits (20). Antibodies to the unique C terminus of canine cardiac triadin 1 as well as to the purified recombinant protein were also raised in rabbits (20). Antibodies to the unique C terminus of canine cardiac triadin 1 as well as to the purified recombinant protein were also raised in rabbits (20). Antibodies to the unique C terminus of canine cardiac triadin 1 as well as to the purified recombinant protein were also raised in rabbits (20). Antibodies to the unique C terminus of canine cardiac triadin 1 as well as to the purified recombinant protein were also raised in rabbits (20). Antibodies to the unique C terminus of canine cardiac triadin 1 as well as to the purified recombinant protein were also raised in rabbits (20). Antibodies to the unique C terminus of canine cardiac triadin 1 as well as to the purified recombinant protein were also raised in rabbits (20). Antibodies to the unique C terminus of canine cardiac triadin 1 as well as to the purified recombinant protein were also raised in rabbits (20). Antibodies to the unique C terminus of canine cardiac triadin 1 as well as to the purified recombinant protein were also raised in rabbits (20). Antibodies to the unique C terminus of canine cardiac triadin 1 as well as to the purified recombinant protein were also raised in rabbits (20). Antibodies to the unique C terminus of canine cardiac triadin 1 as well as to the purified recombinant protein were also raised in rabbits (20). Antibodies to the unique C terminus of canine cardiac triadin 1 as well as to the purified recombinant protein were also raised in rabbits (20). Antibodies to the unique C terminus of canine cardiac triadin 1 as well as to the purified recombinant protein were also raised in rabbits (20). Antibodies to the unique C terminus of canine cardiac triadin 1 as well as to the purified recombinant protein were also raised in rabbits (20). Antibodies to the unique C terminus of canine cardiac triadin 1 as well as to the purified recombinant protein were also raised in rabbits (20). Antibodies to the unique C terminus of canine cardiac triadin 1 as well as to the purified recombinant protein were also raised in rabbits (20). Antibodies to the unique C terminus of canine cardiac triadin 1 as well as to the purified recombinant protein were also raised in rabbits (20). Antibodies to the unique C terminus of canine cardiac triadin 1 as well as to the purified recombinant protein were also raised in rabbits (20). Antibodies to the unique C terminus of canine cardiac triadin 1 as well as to the purified recombinant protein were also raised in rabbits (20). Antibodies to the unique C terminus of canine cardiac triadin 1 as well as to the purified recombinant protein were also raised in rabbits (20). Antibodies to the unique C terminus of canine cardiac triadin 1 as well as to the purified recombinant protein were also raised in rabbits (20). Antibodies to the unique C terminus of canine cardiac triadin 1 as well as to the purified recombinant protein were also raised in rabbits (20). Antibodies to the unique C terminus of canine cardiac triadin 1 as well as to the purified recombinant protein were also raised in rabbits (20).

**RESULTS**

**Calsequestrin-Affinity Chromatography**—In previous work we identified junctin as the major calsequestrin-binding protein in cardiac (and skeletal muscle) junctional SR vesicles by use of a filter overlay assay in which 125I-calsequestrin was incubated with SR proteins blotted to nitrocellulose (8, 12). The interaction between labeled calsequestrin and junctin was inhibited by Ca²⁺ binding to calsequestrin, with half-maximal inhibition occurring at 0.5–0.6 mM added CaCl₂. Conversely, immunoprecipitation with anti-junctin antibodies brought down calsequestrin, and the binding interaction was inhibited over a similar Ca²⁺ concentration range (Fig. 1B). These results further confirm that junctin binds to calsequestrin and that the binding interaction of calsequestrin with these two proteins is relatively specific.

**Co-immunoprecipitation of Calsequestrin and Junctin Solubilized from Canine Cardiac Junctional SR Vesicles**—To test for interactions between endogenous junctin and calsequestrin, immunoprecipitation with detergent-solubilized junctional SR proteins was employed using anti-junctin or anti-calsequestrin antibodies (Fig. 2). Junctin was immunoprecipitated from solubilized cardiac SR vesicles by anti-calsequestrin affinity purified antibodies (Fig. 2A). Quantitative immunoblotting (33) demonstrated that junctin and triadin in these fractions were purified 11.3- and 19.3-fold, respectively, from junctional SR vesicles. These results confirm that junctin and triadin are major calsequestrin-binding proteins and that the binding interaction of calsequestrin with these two proteins is relatively specific.
Identification of the Junctin Domain Associating with Calsequestrin—To identify the calsequestrin interaction domain of junctin, binding assays with GST fusion proteins were conducted. N-junctin (cytoplasmic domain) and C-junctin (lumenal domain) affinity beads were incubated with cardiac junctional SR proteins solubilized in CHAPS. Calsequestrin bound specifically to C-junctin affinity beads but did not bind to N-junctin affinity beads or GST beads alone (Fig. 3). These results demonstrated that the calsequestrin-binding domain of junctin is localized to the lumenal portion of the molecule. Consistent with immunoprecipitation results, the binding interaction between C-junctin and calsequestrin was strongest when assayed in the absence of Ca\(^{2+}\) but nonetheless remained significant even at millimolar Ca\(^{2+}\) concentration (Fig. 3). The two proteins co-precipitated by the C-junctin beads, migrating just above the 97-kDa molecular mass standard (Fig. 3), are the calsequestrin-like (CSQ Like) proteins (26, 37). The calsequestrin-like proteins were also immunoprecipitated in the experiments depicted in Figs. 2 and 6 but are not shown in these figures for brevity.

**Fig. 2.** Co-immunoprecipitation of junctin and calsequestrin from detergent extract (Input) of canine cardiac junctional SR vesicles. A shows calsequestrin immunoprecipitation (CSQ I.P.) results, and B shows junctin immunoprecipitation (Junctin I.P.) results. Blots were probed with calsequestrin (Anti-CSQ) or junctin (Anti-Junct.) antibodies. Immunoprecipitations were conducted in 1 mM EGTA or the added CaCl\(_2\) concentrations indicated. When preimmune serum (Preimm.) was used, 2 mM CaCl\(_2\) was included in the immunoprecipitation buffer, to control for the possibility for Ca\(^{2+}\)-induced precipitation of calsequestrin (42).

**Fig. 3.** Calsequestrin binding to junctin fusion proteins. Control GST beads (GST), N-junctin affinity beads (N-J.), and C-junctin affinity beads (C-J.) were incubated with the CHAPS extract from canine cardiac junctional SR vesicles (Input), and the bound proteins were probed with anti-calsequestrin antibody. Incubations were conducted in 1 mM EGTA or at the Ca\(^{2+}\) concentrations indicated (top underlined). Calsequestrin (CSQ) is indicated on the immunoblot migrating at approximately 55 kDa. The protein doublet visible just above the 97-kDa standard represents the calsequestrin-like (CSQ Like) proteins (26, 37).

**Fig. 4.** Purified recombinant canine junctin. 3.9 µg of purified recombinant junctin (Junc.) (lane 1) and protein standards (Std.) were subjected to SDS-PAGE and stained with Coomassie Blue (left panel). An autoradiogram of 0.6 µg of the \(^{125}\)I-labeled protein (lane 2) is shown in the middle panel. The purified recombinant protein (lane 3) and canine cardiac junctional SR vesicles (lane 4) were blotted and probed with junctin monoclonal antibody 5D8 (right panel).
hibited an identical mobility on SDS-PAGE as native junctin (lane 4) in cardiac SR vesicles.

To identify cardiac SR proteins interacting directly with junctin, junctional SR proteins were separated by SDS-PAGE, transferred to nitrocellulose, and overlaid with 125I-junctin. Fig. 5 shows that of the numerous proteins present in SR vesicles visualized by Amido Black staining (left panel), only a few proteins were labeled with 125I-junctin (right panel). Among them were calsequestrin, the calsequestrin-like protein, triadin, and junctin itself. The identities of these proteins were confirmed by immunoblotting with calsequestrin, triadin, and junctin antibodies (data not shown). The 125I-junctin-binding protein migrating just above the 31-kDa molecular mass standard (asterisk) is the same protein apparent in Fig. 1A which is recognized by the junctin antibody (asterisk).

Junctin and Triadin Interactions—The apparently specific binding of recombinant junctin to triadin detected by the 125I-junctin filter overlay assay suggested that endogenous junctin in SR membranes might interact directly with endogenous triadin. To test this idea, detergent-solubilized cardiac SR vesicle proteins were immunoprecipitated with the triadin antibody. This antibody immunoprecipitated junctin and calsequestrin, whereas the preimmune serum was without effect (Fig. 6). Like the junctin-calsequestrin interaction (Fig. 2), the triadin-calsequestrin interaction was also inhibited by millimolar Ca2+ (Fig. 6, middle panel). Immunoprecipitation of junctin by triadin antibodies, however, was Ca2+-insensitive (lower panel), suggesting that the binding interaction between junctin and triadin occurred independently of any requirement for calsequestrin.

To test for a direct interaction between junctin and triadin, H-triadin affinity beads were employed (Fig. 7). These affinity beads, containing the common luminal domain of triadin, effectively adsorbed junctin from CHAPS-solubilized cardiac SR vesicles (Fig. 7A). As observed with immunoprecipitation of the native proteins (Fig. 6), triadin (H-triadin) binding to junctin was unaffected by the Ca2+ concentration (Fig. 7A). H-triadin affinity beads also bound purified recombinant junctin (Fig. 7B), further confirming a direct interaction between these two proteins. Again, the binding interaction was Ca2+-independent.

Junctin Interactions with the Cardiac Ryanodine Receptor—To test for endogenous junctin binding to the cardiac ryanodine receptor, we immunoprecipitated cardiac junctional SR vesicle proteins solubilized in CHAPS with junctin monoclonal antibody 5D8 covalently coupled to protein A-agarose beads. The immunoprecipitate was then probed for ryanodine

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**FIG. 5.** 125I-Junctin overlay of canine cardiac junctional SR proteins. 50 μg of junctional SR proteins were electrophoresed in 8% polyacrylamide, transferred to nitrocellulose, and incubated with 125I-junctin. The left panel depicts the nitrocellulose strip stained with Amido Black (Stain), and the right panel shows the corresponding autoradiograph after incubation of the blot with 125I-junctin (Overlay). Right margin: calsequestrin-like Proteins (CSQ-like); calsequestrin (CSQ); triadin (Tri.); junctin (Junc.); approximately 31-kDa protein cross-reacting with junctin antibodies (asterisk).

**FIG. 6.** Triadin immunoprecipitation (I.P.) from detergent extract (Input) of canine cardiac junctional SR vesicles. Immunoprecipitation was conducted exactly as described in the legend to Fig. 2, except this time using affinity purified antibodies to canine cardiac triadin 1. Blots were probed with triadin (Anti-Tri.), calsequestrin (Anti-CSQ), and junctin (Anti-Junc.) antibodies.

**FIG. 7.** H-triadin (H-Tri.) precipitation of junctin. A, proteins from cardiac junctional SR vesicles solubilized in CHAPS (Input) were adsorbed to GST beads (GST) or H-triadin affinity beads (H-Tri.). B, purified recombinant junctin in Triton X-100 (Input) was adsorbed to GST or H-Tri. beads. Junctin binding to beads in A and B was monitored after four washes of beads in detergent-containing buffer followed by immunoblotting bound proteins with junctin monoclonal antibody 5D8. Binding and bead washing was conducted in buffer containing 2 mM Ca2+ (Ca) or 1 mM EGTA (EGTA).
receptors by immunoblotting with a ryanodine receptor monoclonal antibody and also by $[^{3}H]$ryanodine binding assay (Fig. 8). Immunoblotting revealed that a substantial fraction of the solubilized ryanodine receptors were immunoprecipitated with use of the junctin antibody beads (A, lane 4). Protein A beads without attached antibody did not precipitate any ryanodine receptors (A, lane 3). $[^{3}H]$Ryanodine binding assay indicated that 30% of the receptors were adsorbed by the junctin antibody beads (B). Control experiments demonstrated that sufficient junctin antibody beads were added to precipitate all of the endogenous junctin. These results suggest that a substantial fraction, but not all, of the cardiac ryanodine receptors were bound to junctin in the detergent extract. The junctin monoclonal antibody immunoprecipitated similar amounts of ryanodine receptors whether assayed in 1 mM EGTA or 2 mM Ca$^{2+}$ (data not shown).

To localize the region of junctin interacting with the cardiac ryanodine receptor, N-junctin and C-junctin affinity beads were incubated with cardiac junctional SR proteins solubilized in CHAPS (Fig. 9). The H-triadin fusion protein, which has previously been shown to bind to the ryanodine receptor (20), was included as a positive control. C-junctin and H-triadin affinity beads bound most of the solubilized ryanodine receptors, as detected by $[^{3}H]$ryanodine binding (upper) or by immunoblotting (lower) assays. N-junctin affinity beads or GST beads alone failed to interact with ryanodine receptors. The binding of the luminal domain of junctin to the ryanodine receptor was dose-dependent, saturating at approximately 250–500 µg of junctin fusion protein added per 300 µg of junctional SR protein analyzed (Fig. 10). This suggests that under the conditions of the immunoprecipitation assay depicted in Fig. 8, the inability of the endogenous junctin to bind all of the solubilized ryanodine receptors was probably a consequence of insufficient endogenous junctin.

To confirm that junctin binds directly to the ryanodine receptor and to exclude the involvement of intermediary binding proteins (calsequestrin, triadin, etc.), we purified the cardiac ryanodine receptor to homogeneity and tested for its ability to interact with C-junctin (Fig. 11). H-triadin binding to the purified ryanodine receptor was also tested. The purified cardiac ryanodine receptor exhibited a $[^{3}H]$ryanodine binding activity of 165 pmol/mg protein and migrated as single high molecular weight protein band on SDS-PAGE (Fig. 11A). No calsequestrin, junctin, or triadin was detected in the purified preparation by immunoblotting with antibodies recognizing these proteins. Approximately 75–80% of the total $[^{3}H]$ryanodine binding sites were precipitated by the C-junctin or H-triadin affinity beads (Fig. 11B), which was confirmed by immunoblotting with the
Junctin was originally identified as a major calsequestrin-binding protein of junctional SR vesicles by use of a filter overlay method (8, 12). In the present study we confirmed the binding of junctin to calsequestrin under more native conditions employing calsequestrin-affinity chromatography. Binding of junctin to the affinity matrix occurred in Ca$^{2+}$-regulated fashion, as observed previously with use of the $^{125}$I-calsequestrin overlay method (8, 38). Although all of the junctin solubilized from cardiac SR vesicles did not bind to the affinity column, whereas all the triadin solubilized was quantitatively retained (Fig. 1), it is difficult to deduce from this type of experiment whether junctin or triadin binds more tightly to calsequestrin. This is because all of the endogenous calsequestrin in cardiac SR vesicles passed freely through the affinity column along with the junctin that was not retained (data not shown). This unretained junctin could have been complexed with the endogenous calsequestrin. The broad elution of junctin from the calsequestrin-affinity column in Ca$^{2+}$-containing buffer compared with the sharp elution of triadin (Fig. 1) suggests that junctin may indeed bind more strongly to calsequestrin than triadin. However, experiments with the individually purified proteins will ultimately be required to establish the relative affinities of junctin versus triadin for calsequestrin. Although the absolute amount of junctin associated with calsequestrin at the junctional SR membrane was not determined in this study, co-immunoprecipitation assays with detergent extracts from whole SR vesicles suggested that the amount is substantial. Fusion protein analyses localized the calsequestrin-binding domain of junctin to amino acid residues 46–210, the intraluminal region containing 55% charged residues. Calsequestrin also contains a high density of charged residues. Calsequestrin also contains a high density of charged residues (7), suggesting that electrostatic forces between junctin and calsequestrin are responsible for its loss of interaction with junctin and triadin at very high Ca$^{2+}$ concentrations (≥10 mM). At the average physiological Ca$^{2+}$ concentration in the SR lumen (~1 mM) (36), however, considerable calsequestrin remains bound to junctin and triadin even when the proteins are substantially diluted into immunoprecipitation or fusion protein binding buffers.
Calsequestrin contains clusters of both positively and negatively charged amino acids at the N-terminal end of the molecule (5), and experiments are currently in progress to test if this region participates in its binding to junctin (and triadin).

An unexpected finding from this study is that junctin binds directly to triadin. Three different approaches were used to confirm this finding. In the first approach, using the 125I-junctin overlay method, we observed that junctin bound directly to the triadin doublet. In the second approach, triadin antibodies immunoprecipitated junctin from detergent-solubilized junctional SR proteins and this interaction occurred independently of any requirement for calsequestrin. In the third approach, H-triadin affinity beads were shown to precipitate junctin solubilized from SR vesicles as well as purified recombinant junctin. In contrast to junctin and triadin interactions with calsequestrin, the junctin interaction with triadin was Ca2+ independent. The junctin binding domain was localized to residues 69–264 of triadin, the common luminal domain also interacting with calsequestrin and the ryanodine receptor (20). Thus it appears that the highly charged luminal domains of junctin and triadin are equally capable of binding to calsequestrin, to the ryanodine receptor (see below), and, surprisingly, even to each other. The unusual feature common to the luminal domains of triadin and junctin is their highly charged nature (~50% charged residues) with frequent runs of alternating positively and negatively charged amino acids. Realini et al. (21, 22) have noted that such KEKE motifs are present in a number of Ca2+ binding proteins and have proposed that such charged regions represent protein association domains. Similar protein association domains, described as “polar zippers,” have been described by Perutz (23). We hypothesize that the KEKE motifs shared by junctin and triadin are required for their specific interactions with each other, as well as with other junctional SR proteins that contain a high density of charged residues. Note that Fig. 5 demonstrates that 125I-junctin is even capable of binding to itself immobilized on nitrocellulose as well as to the protein of approximately 31 kDa (asterisk), which is thought to be a junctin isoform (12). This suggests the possibility for the formation of protein complexes in the plane of the SR membrane where junctin and triadin are present in relatively high concentration; these protein complexes may target calsequestrin to the membrane in proximity to the ryanodine receptor.

The similarities in protein structure and subcellular localization between triadin and junctin led us to investigate whether junctin, like triadin (19, 20), is capable of binding to the ryanodine receptor. We indeed observed this interaction and, moreover, demonstrated that junctin and triadin bind directly to the purified ryanodine receptor with no intervening proteins required. Immunoprecipitation data obtained with the junctin monoclonal antibody confirmed that endogenous junctin in cardiac SR vesicles binds a considerable fraction of the total ryanodine receptors solubilized from the membranes by detergent (Fig. 8). Failure to immunoprecipitate all of the ryanodine receptors with the junctin antibody could be due to a fraction of the receptors containing bound triadin competing for the junctin binding sites or to other factors, such as a lowered binding affinity of the ryanodine receptor for junctin when the proteins are solubilized from the membrane and diluted into detergent solution. Since the luminal domains of junctin and triadin are sufficient for binding to the ryanodine receptor, it appears that in intact muscle these proteins must interact with the ryanodine receptor from the luminal surface of the SR membrane. The second intraluminal loop of the cardiac and skeletal muscle ryanodine receptors, connecting transmembrane segments 3 and 4, is highly conserved and is enriched in charged amino acids (39). It is possible that intraluminal loop 2 of the ryanodine receptor binds to KEKE motifs common to triadin and junctin. Based on our biochemical assays with purified proteins, junctin and triadin interactions with the ryanodine receptor are predicted to be Ca2+ independent.

In summary, a simple model for the protein interactions occurring at the junctional membrane has junctin and triadin highly concentrated in the plane of the membrane and interacting directly with each other (Fig. 12). The C-terminal, luminal domains of junctin and triadin contribute equally to the binding of calsequestrin, which by itself is incapable of binding to the ryanodine receptor. The luminal domains of junctin and triadin also bind directly to the ryanodine receptor and by this fashion anchor calsequestrin to the membrane in proximity to the Ca2+ release channel. Junctin and triadin interactions with the ryanodine receptor are Ca2+ independent, whereas junctin and triadin binding to calsequestrin may be Ca2+ regulated, for example as the Ca2+ concentration within the junctional SR rises and falls during muscle relaxation and contraction. Cooperativity of binding between the four proteins of the complex may be facilitated by electrostatic interactions between KEKE domains of junctin and triadin and the propensities of these proteins to aggregate by charged interactions. Junctin is a prominent protein component of both skeletal and cardiac muscle junctional SR membranes (12), and it may be that this protein acts as scaffold to collect and tether diverse molecules at the junctional interface. Resolution of the issue of whether junctin simply serves as a structural agent to retain calsequestrin at the SR junction or also plays a more active role in regulating Ca2+ release will require further investigation. However, it should be noted that several investigators have proposed that Ca2+ binding to calsequestrin directly regulates the amount of Ca2+ released by the ryanodine receptor (15–18) and that three studies have implicated junctin and 29- to 30-kDa proteins, which may be junctin isoforms, in mediating this regulatory mechanism (15, 40, 41).

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REFERENCES
1. Johnson, E. A., and Sommer, J. R. (1967) J. Cell Biol. 33, 103–129
2. Fleischer, S., and Inui, M. (1989) Annu. Rev. Biophys. Biophys. Chem. 18, 333–364
3. Meissner, G. (1994) Annu. Rev. Physiol. 56, 485–508
4. Franzini-Armstrong, C., and Jorgensen, A. O. (1994) Annu. Rev. Physiol. 56, 509–534
5. Yan, K., and Zarain-Herzberg, A. (1994) Mol. Cell. Biochem. 135, 61–70
6. Fliegel, L., Ohnishi, M., Carpenter, M. R., Khanna, V. K., Reithmeier, R. A. F., and MacLennan, D. H. (1987) Proc. Natl. Acad. Sci. U. S. A. 84, 1167–1171
7. Scott, B. T., Simmerman, H. K. B., Collins, J. H., Nadal-Ginard, B., and Jones, L. R. (1988) J. Biol. Chem. 263, 8958–8964
8. Mitchell, R. D., Simmerman, H. K. B., and Jones, L. R. (1988) J. Biol. Chem. 263, 1376–1381
9. Caswell, A. H., Brandt, N. R., Brunswig, J. P., and Purkerson, S. (1991) Biochemistry 30, 7507–7513
10. Knudson, C. M., Stagg, K. J., and Krog, K. A. (1990) J. Biol. Chem. 265, 12673–12678
11. Knudson, C. M., Stagg, K. J., and Krog, K. A. (1990) J. Biol. Chem. 265, 12646–12654
12. Jones, L. R., Zhang, L., Sanborn, K., Jorgensen, A. O., and Kelley, J. (1995) J. Biol. Chem. 270, 37078–37096
13. Franzini-Armstrong, C., Kenney, J. L., and Varianne-Marston, E. (1987) J. Cell Biol. 105, 49–56
14. Costello, B., Chadwick, S., Saito, A., Chu, A., Maurer, A., and Fleischer, S. (1986) J. Cell Biol. 103, 741–753
15. Ikemoto, N., Ronjat, M., Meszaros, L. G., and Kashita, M. (1989) Biochemistry 28, 6764–6771
16. Ikemoto, N., Ronjat, M., Meszaros, L. G., and Kashita, M. (1989) Biochemistry 28, 6764–6771
17. Kawazaki, T., and Kosoi, M. (1994) Biochem. Biophys. Res. Commun. 199, 1120–1127
18. Donoso, P., Beltran, M., and Hidalgo, C. (1996) Biochemistry 35, 13419–13425
19. Guo, W., and Campbell, K. P. (1995) J. Biol. Chem. 270, 9027–9031
20. Guo, W., Jorgensen, A. O., Jones, L. R., and Campbell, K. P. (1996) J. Biol. Chem. 271, 448–455
21. Realini, C., Rogers, S. W., and Rechsteiner, M. (1994) FEBS Lett. 348, 109–113
22. Realini, C., and Rechsteiner, M. (1995) J. Biol. Chem. 270, 29644–29667
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23. Perutz, M. (1994) Protein Sci. 3, 1629–1637
24. Rardon, D. P., Cefali, D. C., Mitchell, R. D., Seiler, S. M., and Jones, L. R. (1989) Circ. Res. 64, 779–789
25. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) J. Biol. Chem. 183, 265–275
26. Cala, S. E., and Jones, L. R. (1983) J. Biol. Chem. 258, 11992–11996
27. Porzio, M. A., and Pearson, A. M. (1977) Biochim. Biophys. Acta 490, 27–34
28. Zimmerman, H. K. B., Kobayashi, Y. M., Autry, J. M., and Jones, L. R. (1996) J. Biol. Chem. 271, 5941–5946
29. Reddy, L. G., Jones, L. R., Cala, S. E., O’Brien, J. J., Tatulian, S. A., and Stokes, D. L. (1995) J. Biol. Chem. 270, 9390–9397
30. Harlow, E., and Lane, D. (1988) Antibodies: A Laboratory Manual, pp. 521–523, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
31. Sanger, F., Nicklen, S., and Coulson, A. R. (1977) Proc. Natl. Acad. Sci. U. S. A. 74, 5463–5467
32. Laemmli, U. K. (1970) Nature 227, 680–685
33. Mahoney, L., and Jones, L. R. (1986) J. Biol. Chem. 261, 15257–15265
34. Olmsted, J. B. (1981) J. Biol. Chem. 256, 11955–11957
35. Al-Seikhan, B. A., Strasburg G. M., and Jones, L. R. (1997) Biophys. J. 72, A171
36. Shen, W., Steenbergen, C., Levy, L. A., Vance, J., London, R. E., and Murphy, E. (1996) J. Biol. Chem. 271, 7398–7403
37. Cala, S. E., Scott, B. T., and Jones, L. R. (1990) Semin. Cell Biol. 1, 265–275
38. Damiani, E., and Margreth, A. (1990) Biochem. Biophys. Res. Commun. 172, 1253–1259
39. Nakai, J., Imagawa, T., Hakamata, Y., Shigekawa, M., Takeshima, H., and Numa, S. (1990) FEBS Lett. 271, 169–177
40. Yamaguchi, N., Kawasaki, T., and Kasai, M. (1995) Biochem. Biophys. Res. Commun. 210, 648–653
41. Kangari, T., Yamaguchi, N., and Kasai, M. (1996) Biochem. Biophys. Res. Commun. 227, 700–706
42. Ikemoto, N., Bhatnagar, G. M., and Gergely, J. (1971) Biochem. Biophys. Res. Commun. 44, 1510–1517