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The *asp*-rich region at the carboxyl-terminus of calsequestrin binds to Ca^{2+} and interacts with triadin

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Abstract Calsequestrin (CSQ) is a high capacity Ca^{2+} binding protein in the junctional sarcoplasmic reticulum of striated muscles, and has been shown to regulate the ryanodine receptor (RyR) through triadin and junctin. In order to identify the functional roles of specific regions on CSQ, several CSQ deletion mutants were prepared by molecular cloning and *Escherichia coli* expression. $^{45}Ca^{2+}$ overlay assay using a native gel system revealed that the major Ca^{2+} binding motif of CSQ resides in the *asp*-rich region (amino acids 354–367). In an in vitro binding assay using a glutathione-*S*-transferase affinity column, the interaction between CSQ and triadin was found to be Ca^{2+}-dependent, and the site of interaction was confined to the *asp*-rich region of CSQ. Our results suggest that the *asp*-rich region of CSQ could participate in the RyR-mediated Ca^{2+} release process by offering a direct binding site to luminal Ca^{2+} as well as triadin.

Key words: Sarcoplasmic reticulum; Ryanodine receptor; Excitation–contraction coupling; In vitro binding assay; Junctin

1. Introduction

The sarcoplasmic reticulum (SR) represents the major storage site for intracellular Ca^{2+}, essential for contraction of striated muscle cells. The ryanodine receptor (RyR) located in the terminal cisternae of SR membrane constitutes the channel for rapid Ca^{2+} release in response to depolarization of the surface membrane [1]. It is believed that in skeletal muscles, the dihydropyridine receptor (DHPR) located in the transverse tubules senses the arrival of an action potential and directs opening of the Ca^{2+} release channel through a direct interaction with RyR [2–4]. The activities of RyRs are known to be modulated by proteins such as FK-506 binding protein and calsequestrin (CSQ) [5–7]. CSQ is localized in the lumen of SR and binds Ca^{2+} with high capacity and moderate affinity [8]. It is a highly hydrophilic protein, containing clusters of positively and negatively charged residues dispersed throughout its sequence with a particular region enriched in aspartate (*asp*) residues at its carboxyl-terminus [9]. Triadin and junctin are structurally similar integral membrane proteins having a short amino-terminal cytoplasmic domain and a long stretch exposed to the luminal side of the SR [10,11]. The luminal region is especially enriched in multiple clusters of alternating lysine and glutamic acid residues, named as the KEKE motif [11–13]. Recent reports suggest that triadin can inhibit the activity of RyR in skeletal muscle by decreasing open probability of the Ca^{2+} release channel [14,15]. CSQ may also be involved in the regulation of RyR indirectly through its interaction with triadin and junctin [16,17]. However, the binding regions for triadin and junctin in CSQ have not been identified. The interaction between CSQ and triadin (and junctin) inside the SR lumen may be due to the particular charged domains in these proteins. In this study, we prepared various CSQ deletion mutants to identify the regions for binding of Ca^{2+} and triadin. We found that the *asp*-rich region of CSQ not only binds to triadin, but also binds to Ca^{2+}. This dual function of the *asp*-rich region is likely to be responsible for the Ca^{2+}-dependent interaction between triadin and CSQ.

2. Materials and methods

2.1. Materials

Glutathione–Sepharose 4B column and anti-glutathione *S*-transferase (GST) antibody were purchased from Amersham Pharmacia (Piscataway, NJ, USA). The restriction enzymes were purchased from New England BioLabs (Beverly, MA, USA). $^{45}CaCl_2$ (specific activity, 10 mCi/mmol) was obtained from NEN (Boston, MA, USA). Protease inhibitors and anti-triadin antibody were purchased from Boehringer Mannheim (Indianapolis, IN, USA) and Affinity Bio Reagents (Golden, CO, USA), respectively.

2.2. Generation and purification of GST–CSQ fusion proteins

To examine the role of the putative junctional domain (86–191 residues) [18] and the *asp*-rich region (354–367 residues) at C-terminus of CSQ, several deletion mutants of CSQ were produced as GST fusion proteins (see Fig. 1A). cDNA fragments amplified by polymerase chain reaction (PCR) were subcloned into pGEX-4T-1 vectors and confirmed by DNA sequencing. The GST fusion proteins were expressed in *Escherichia coli* BL21 (DE3) cells by addition of 0.1 mM isopropyl β-D thiogalactopyranoside (IPTG) and the bacterial cell lysates were prepared by sonication. After centrifugation at 15000 rpm, the supernatants were incubated with a glutathione–Sepharose 4B affinity column for 2 h at 4°C. After washing several times with phosphate-buffered saline, the bound proteins were eluted by reduced glutathione elution buffer according to the manufacturer's protocol

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Abbreviations: SR, sarcoplasmic reticulum; CSQ, calsequestrin; RyR, ryanodine receptor; DHPR, dihydropyridine receptor; wt, wild type; *asp*, aspartate; junc, junctional; GST, glutathione *S*-transferase; PCR, polymerase chain reaction; EGTA, ethylene glycol bis (β-aminoethylether)-*N*,*N*′ tetraacetic acid; DTT, dithiothreitol; IPTG, isopropyl β-D thiogalactopyranoside; PMSF, phenylmethylsulfonyl fluoride

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(Amersham Pharmacia). The purified proteins were examined by Coomassie blue staining.

2.3. Western blot and $^{45}Ca^{2+}$ overlay assay

SDS–PAGE was performed with the buffer system of Laemmli et al. [19] with an 8 or 12% polyacrylamide gel. The separated proteins were electrophoretically transferred onto nitrocellulose membrane. Immunoblotting was conducted using either anti-GST, anti-CSQ, anti-triadin or anti-junctin antibody. $^{45}Ca^{2+}$ overlay assay was performed as described previously [20]. The membrane was incubated with the overlay buffer containing 60 mM KCl, 5 mM $MgCl_2$ and 10 mM imidazole, pH 6.8 for 10 min at room temperature. The overlay was performed using 0.1 mCi of $^{45}Ca^{2+}$/blot. Unbound $^{45}Ca^{2+}$ was removed by washing with 50% ethanol solution, and bound radioactivity was visualized by exposure to X-ray film.

A 6% native gel electrophoresis was also performed as described previously [21]. The gels were transferred onto nitrocellulose membrane for immunoblotting and $^{45}Ca^{2+}$ overlay assay.

2.4. Preparation of SR vesicles from rabbit skeletal muscle

A heavy fraction of SR was prepared from rabbit back and leg fast twitch muscle as described previously [22]. The final pellets were suspended in a solution containing 0.15 M KCl, 20 mM MOPS (pH 6.8) and 0.3 M sucrose in the presence of protease inhibitors (1 μM pepstatin, 1 μM leupeptin, 100 μM phenylmethylsulfonyl fluoride (PMSF) and 1 μM trypsin inhibitor). The protein concentrations were determined by the Bradford method using bovine serum albumin as the standard. The SR vesicles were quickly frozen in liquid nitrogen and stored at −70°C until use.

2.5. In vitro binding assay using glutathione–Sepharose 4B column

In vitro binding assay was performed as described previously [17]. An affinity column was prepared by immobilizing 100–150 μg of GST–CSQ fusion proteins on 50 μl of glutathione–Sepharose beads for 2 h at 4°C (Pharmacia). The SR vesicles from rabbit skeletal muscles (10 mg/ml) were solubilized in a lysis buffer containing 2% Triton X-100, 20 mM Tris–HCl, pH 7.4, 1 mM dithiothreitol (DTT), 1 M NaCl and protease inhibitors (1 μM pepstatin, 1 μM leupeptin, 100 μM PMSF, and 1 μM trypsin inhibitor). The solubilized membranes were centrifuged at 65 000 rpm in a Beckman ultracentrifuge using the TLA-100.3 rotor for 45 min. The supernatant was collected and diluted 1:10 with the dilution buffer containing 20 mM Tris–HCl, pH 7.4, 1 mM DTT and protease inhibitors (1 μM pepstatin, 1 μM leupeptin, 100 μM PMSF, and 1 μM trypsin inhibitor), and incubated for 4 h with the purified GST–CSQ fusion protein–Sepharose 4B. After the incubation, the fusion protein–Sepharose complexes were washed with the buffer containing 0.2% Triton X-100, 20 mM Tris–HCl, pH 7.4, and 0.15 M NaCl. The bound proteins were eluted by boiling in the SDS sample buffer [19] or eluted by 20 mM $CaCl_2$ in the washing buffer. The eluates were subjected to immunoblotting with anti-triadin or anti-junctin antibody.

3. Results

3.1. Preparation of GST-CSQ deletion mutants

CSQ contains a putative junctional domain (amino acids (aa) 86–191) [18] and an *asp*-rich region at the carboxyl-terminus (aa 354–367). To investigate the function of these domains, five deletion mutants of CSQ were generated by using PCR from rabbit skeletal CSQ cDNA: N-CSQ (aa 1–86), C-CSQ (aa 193–367), Δjunc-CSQ (lacking aa 86–191), Δ*asp*-CSQ (lacking aa 354–367) and Δjunc-Δasp-CSQ (Fig. 1A). For generation of the Δjunc-CSQ construct, the amino-terminus of CSQ (N-CSQ, 1–258 bp) and carboxyl-terminus of CSQ (C-CSQ, 579–1101 bp) were amplified by PCR, and ligated together in a bacterial expression vector, pGEX-4T-1. For convenience of protein purification, these CSQ mutants were expressed in *E. coli* (BL21-DE3) as GST fusion proteins. Following an induction with 0.1 mM IPTG for 6 h, the various GST–CSQ fusion proteins were purified using a glutathione–Sepharose 4B column. Large amounts of the fusion proteins were eluted from the column as detected with Coomassie blue staining (Fig. 1B).

To confirm the expression of GST–CSQ mutant proteins, Western blot assays were performed. The GST–CSQ fusion proteins with the expected molecular weights were recognized by the anti-GST antibody (Fig. 2A). The identities of the CSQ mutants were also confirmed by Western blot with polyclonal antibody raised against CSQ (data not shown).

3.2. The asp-rich region of CSQ is a Ca^{2+} binding domain

To identify the putative Ca^{2+} binding motifs in CSQ, $^{45}Ca^{2+}$ overlay assay was performed with the purified GST–CSQ deletion mutants. As shown in Fig. 2B, the Δ*asp*-CSQ mutant could bind to $^{45}Ca^{2+}$ (lane 5), when the protein was run in a SDS-denaturing gel for blotting. Similar $^{45}Ca^{2+}$ binding was observed with wild type (wt)-CSQ (lane 3), Δjunc-CSQ (lane 4) and Δjunc-Δ*asp*-CSQ (lane 6). The X-ray crystal structure of the full-length CSQ revealed that most of the negatively charged residues are located inside the protein,

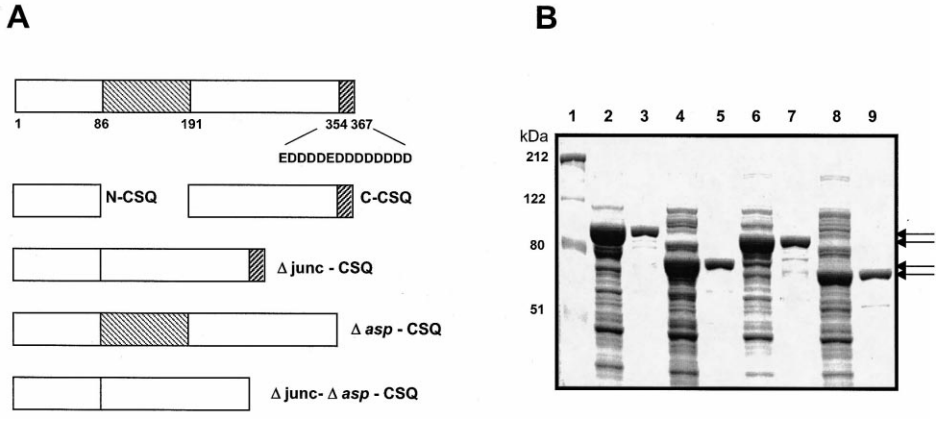

Fig. 1. Preparation of wild type and deletion mutated CSQ using glutathione–Sepharose 4B affinity column. A: A schematic diagram illustrating the different domains of CSQ, and the prepared various deletion mutants used for the present study. B: Purified GST-fusion wt-CSQ and various mutant CSQ expressed in *E. coli* (BL21(DE3)) were separated in an 8% SDS–PAGE gel. The arrows indicate the positions of each GST fusion proteins. Lane 1, protein size marker; lane 2, cell supernatant including GST–wt-CSQ; lane 3, GST–wt-CSQ; lane 4, cell supernatant including GST–Δjunc-CSQ; lane 5, GST–Δjunc-CSQ; lane 6, cell supernatant including GST–Δ*asp*-CSQ; lane 7, GST–Δ*asp*-CSQ; lane 8, cell supernatant including GST–Δjunc-Δ*asp*-CSQ; lane 9, GST–Δjunc-Δ*asp*-CSQ.

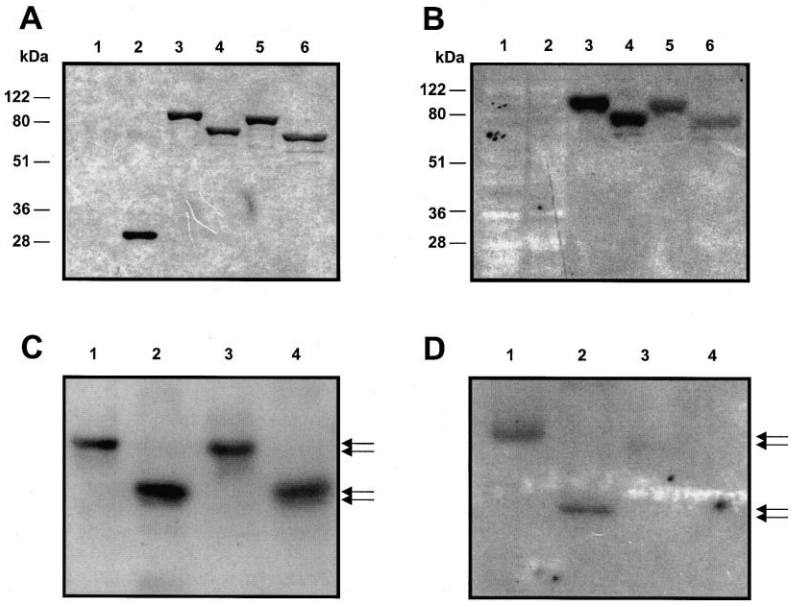

Fig. 2. Identification of Ca^{2+} binding domains in CSQ using $^{45}Ca^{2+}$ overlay assay under denaturing and native conditions. Purified GST–CSQ mutants (10 μg) were subjected to 8% SDS (A, B) or 6% native PAGE (20) (C, D). The immunoblotting was carried out with anti-GST antibody (A) or anti-CSQ antibody (C). Autoradiographs of the gels after overlaying with $^{45}Ca^{2+}$ (B, D). Lane 1, control cell lysates; lane 2, GST; lane 3, GST–wt-CSQ; lane 4, GST–Δjunc-CSQ; lane 5, GST–Δ*asp*-CSQ; lane 6, GST–Δjunc-Δ*asp*-CSQ (A, B). Lane 1, GST–wt-CSQ; lane 2, GST–Δjunc-CSQ; lane 3, GST–Δ*asp*-CSQ; lane 4, GST–Δjunc-Δ*asp*-CSQ (C, D).

which may participate in intra- and intermolecular oligomerization of CSQ [23]. Since the $^{45}Ca^{2+}$ overlay assay shown in Fig. 2B was performed with a denaturing gel containing SDS, it was possible that these negatively charged internal residues were exposed to $^{45}Ca^{2+}$ and caused the binding shown in Fig. 2B.

To test Ca^{2+} binding to the CSQ mutants in native conformation, the purified CSQ mutants were subjected to a native gel electrophoresis. For quantitative purpose, an equal amount of protein was loaded to the gel, as revealed by Western blot (Fig. 2C). A $^{45}Ca^{2+}$ overlay assay using the blot from the native gel revealed little or no binding of $^{45}Ca^{2+}$ to the Δ*asp*-CSQ and Δjunc-Δ*asp*-CSQ proteins which lack the *asp* motif of CSQ (Fig. 2D, lanes 3 and 4). In contrast, the $^{45}Ca^{2+}$ binding property was retained in the Δjunc-CSQ mutant (Fig. 2D, lane 2), similar to wt-CSQ (Fig. 2D, lane 1). This result suggests that the *asp*-rich region at the C-terminus is a major Ca^{2+} binding motif on CSQ.

3.3. *The asp-rich region of CSQ interacts with triadin or junctin*

To investigate the nature of interaction between CSQ and triadin, an in vitro binding assay was performed in the presence of various [Ca^{2+}] (0–2 mM). The junctional SR vesicles

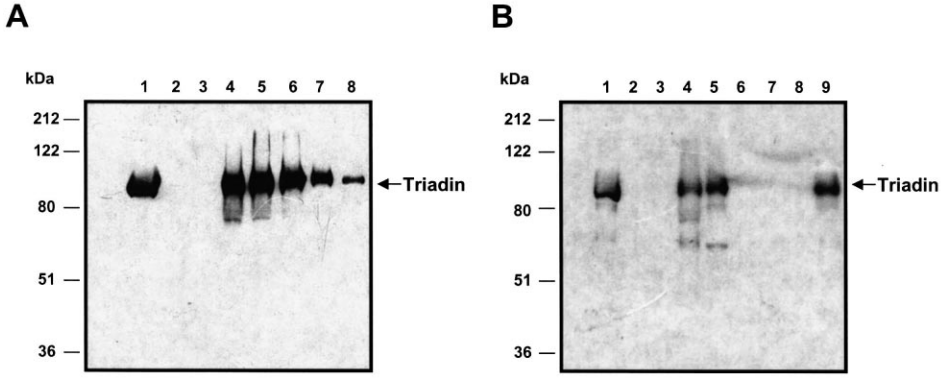

Fig. 3. Ca^{2+}-dependency of triadin–CSQ interaction (A), and an identification of triadin binding domain on CSQ (B). In vitro binding assay of triadin was performed with purified wt-CSQ associated GST affinity column (A). The purified wt-CSQ affinity beads were incubated with Triton X-100 solubilized rabbit skeletal heavy SR (500 μg) in various Ca^{2+} concentrations. Lane 1, SR; lane 2, control affinity beads without sample; lane 3, GST affinity beads without sample; lane 4, 1 mM EGTA; 5, 0.1 mM Ca^{2+}; lane 6, 0.5 mM Ca^{2+}; lane 7, 1.0 mM Ca^{2+}; lane 8, 2.0 mM Ca^{2+}. After washing several times, the bound proteins were eluted by SDS-sample buffer, and were subjected to 8% SDS–PAGE, and immunoblotting was done with anti-triadin antibody. (B) The same in vitro binding assay of triadin was performed with several CSQ deletion mutants in the absence of Ca^{2+}. Lane 1, SR; lane 2, control affinity beads; lane 3, GST affinity beads; lane 4, wt-CSQ affinity column; lane 5, Δjunc-CSQ affinity beads; lane 6, Δ*asp*-CSQ affinity beads; lane 7, Δjunc-Δ*asp*-CSQ affinity beads; lane 8, N-CSQ affinity beads; lane 9, C-CSQ affinity beads.

from rabbit skeletal muscle were solubilized with 2% Triton X-100, and applied to GST–wt-CSQ bound glutathione–Sepharose 4B beads. Proteins that interact with GST–wt-CSQ were separated on an 8% SDS–PAGE after boiling the protein complexes in the SDS loading buffer. These proteins were probed with an anti-skeletal triadin antibody in a Western blot assay (Fig. 3). As shown in Fig. 3A, increasing the concentration of Ca^{2+} in the incubation medium resulted in a progressive loss of interaction between triadin and CSQ, suggesting that the interaction is Ca^{2+} concentration-dependent. A similar result was reported earlier by a co-immunoprecipitation assay [17].

To search for the domains of CSQ that could serve for the interaction with triadin, in vitro binding assays with the various CSQ mutants were conducted in the presence of 1 mM ethylene glycol bis (β-aminoethylether)-*N*,*N*′ tetraacetic acid (EGTA) to enhance the interaction between CSQ and triadin (Fig. 3B). The carboxyl terminal portion of CSQ (C-CSQ) interacted with triadin (lane 9), whereas the amino-terminus of CSQ (N-CSQ) did not bind to triadin (lane 8). CSQ lacking the putative junctional domain (Δjunc-CSQ) also exhibited significant binding to triadin (lane 5). The interaction between CSQ and triadin was completely abolished in the CSQ mutants lacking the *asp*-rich region (lanes 6 and 7).

The interaction between C-CSQ and triadin also exhibited a strong Ca^{2+} dependence, in such a way that increasing Ca^{2+} concentration (from 0 to 5 mM) resulted in a gradual dissociation between triadin and C-CSQ (Fig. 4A). In light of the evidence that junctin plays a similar role to triadin in striated muscles [13,17], we also examined the possible interaction between junctin and C-CSQ. It was found that junctin also showed a Ca^{2+}-dependent interaction with C-CSQ (Fig. 4B). These results suggest that the *asp*-rich region of CSQ could be the binding site for Ca^{2+} as well as triadin and junctin.

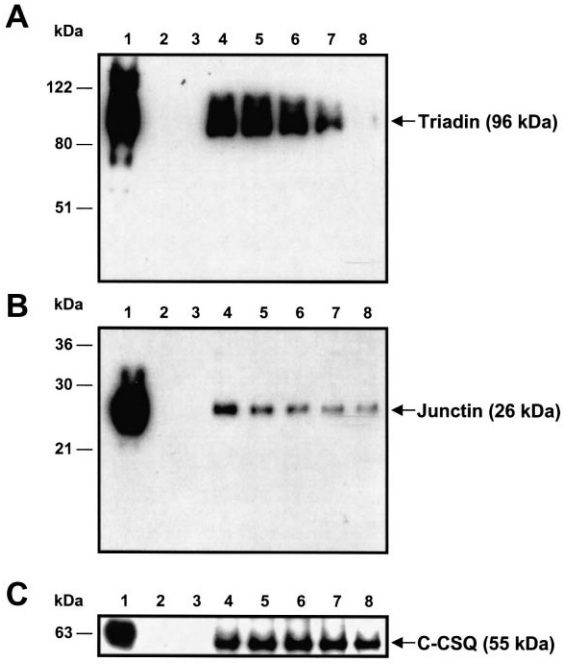

Fig. 4. In vitro binding of triadin (A) and junctin (B) with C-terminus CSQ in various Ca^{2+} concentrations. In vitro binding of triadin (A) or junctin (B) was performed with purified GST-C-terminus (C-CSQ) associated GST affinity beads. The purified C-CSQ affinity beads were incubated with Triton X-100 solubilized rabbit skeletal heavy SR (500 μg) in various Ca^{2+} concentrations. After washing several times, the bound proteins were eluted by 20 mM $CaCl_2$ buffer containing 0.2% Triton X-100, 150 mM NaCl, and 20 mM Tris–HCl (pH 7.4), and subjected to 12% SDS–PAGE, and immunoblotting was done with anti-triadin antibody or anti-junctin antibody. As a control, C-CSQ in each sample was eluted by SDS sample buffer, and then immunoblotting was done by anti-CSQ antibody (C). Lane 1, SR; lane 2, control affinity beads; lane 3, GST affinity beads; lane 4, 1 mM EGTA; lane 5, 0.5 mM Ca^{2+}; lane 6, 1.0 mM Ca^{2+}; lane 7, 2.0 mM Ca^{2+}; lane 8, 5.0 mM Ca^{2+}.

4. Discussion

CSQ, a major Ca^{2+} binding protein located in the junctional SR lumen in striated muscles [24,25] undergoes a conformational change upon Ca^{2+} binding [29] and has an intrinsic property of oligomerization [30]. There is growing evidence that CSQ may actively participate in regulating the amounts of Ca^{2+} release through the RyR/Ca^{2+} release channel [6,26–28]. The regulatory effect of CSQ may be mediated by the CSQ-anchoring proteins such as triadin and junctin [16,17]. Triadin and junctin are single transmembrane proteins with a short amino-terminal segment located in the cytoplasm, and a large charged carboxyl-terminal segment in the luminal side of SR [10,11]. These anchoring proteins could be implicated in the intermolecular interactions between CSQ and the RyR [16,17].

In the present study, we found that the interaction between CSQ and triadin was abolished when the *asp*-rich region was deleted from CSQ, suggesting that the *asp*-rich region is the interaction site for CSQ and triadin. This result is in disagreement with the proposal by Wang et al. [23] who argued that the amino-terminal arm of CSQ is responsible for its localization to RyR through interactions with triadin and junctin.

A recent report by Kobayashi et al. [13] showed that a specific KEKE motif (200–224) in the luminal side of cardiac triadin is responsible for interaction with CSQ. Amino acid sequences (aa 1–257) containing the specific KEKE motif are predicted to be identical in all triadin isoforms [31]. Thus, regardless of different isoforms, it is possible that an *asp*-rich region of CSQ interacts with the KEKE motif of triadin electrostatically, and therefore stabilizes the binding between CSQ and triadin.

A recent study by Nori et al. [32] showed that a CSQ mutant lacking the *asp*-rich region segregated to terminal cisternae of skeletal muscles as shown by immunofluorescence technique, suggesting that in vivo targeting of CSQ to terminal cisternae may depend on unknown or more complex mechanism.

Using a $^{45}Ca^{2+}$ overlay assay, we found that the Ca^{2+} binding capability of CSQ in the native condition was almost completely eliminated when the *asp*-rich motif was deleted from the protein (Fig. 2D), although all CSQ mutants could bind to Ca^{2+} in the denaturing condition. This result indicates that the specific Ca^{2+} binding capacity of CSQ is highly dependent on the proper conformation of the protein, and the *asp*-rich region of CSQ could serve as the major Ca^{2+} binding motif. Therefore, it is likely that triadin and Ca^{2+} may share the common binding domain that is the *asp*-rich region.

We also showed that the interaction between CSQ and triadin exhibits a strong Ca^{2+}-dependency. This is in agreement with the previous report by Zhang et al. [17], who demonstrated that the association between triadin (junctin) and CSQ

was strongest in the absence of Ca^{2+}. Here, using an in vitro binding assay with C-CSQ mutant (aa 192–367) containing *asp*-rich region, we showed that the association between triadin (junctin) and C-CSQ still had a strong dependence on Ca^{2+}, such that millimolar concentrations of Ca^{2+} can dissociate the interaction between CSQ and triadin (junctin). These results would explain the apparent Ca^{2+}-dependent interaction between triadin (junctin) and CSQ. It is possible that the elevated Ca^{2+} could bind to the *asp*-rich motif on CSQ, and cause a conformational change in CSQ allowing dissociation of triadin or junctin from CSQ. Alternatively, Ca^{2+} could directly compete with triadin molecules for the same binding site.

Our results add new insights to the intermolecular interactions among the junctional proteins present in the SR membrane and also the functional roles of CSQ and triadin (junctin) in the Ca^{2+} release process during the excitation–contraction coupling in striated muscles. When muscle is at the resting state, the majority of CSQ will be occupied with luminal Ca^{2+} forming oligomer or polymer [23,29,30]; triadin is free to interact with RyR which could have an inhibitory role on the Ca^{2+} release channel activity [14,15]. In response to an excitation signal from DHPR in the transverse tubule, opening of the RyR/Ca^{2+} release channels causes a transient decrease in the luminal Ca^{2+} concentration. This decrease in Ca^{2+} may lead to an association of CSQ with triadin and, therefore, dissociation of triadin from RyR. The RyR proteins, once free from binding to triadin (and therefore the removal of the partial inhibitory action of triadin), will enter a state with elevated Ca^{2+} release activity. In principle, this could serve as a positive feedback mechanism allowing for further release of Ca^{2+} into the myoplasm.

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