Functional importance of polymerization and localization of calsequestrin in *C. elegans*

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Summary

Dual roles of calsequestrin (CSQ-1) being the Ca^{2+} donor and Ca^{2+} acceptor make it an excellent Ca^{2+}-buffering protein within the sarcoplasmic reticulum (SR). We have isolated and characterized a calsequestrin (*csq-1*)-null mutant in *Caenorhabditis elegans*. To our surprise, this mutant *csq-1(jh109)* showed no gross defects in muscle development or function but, however, is highly sensitive to perturbation of Ca^{2+} homeostasis. By taking advantage of the viable null mutant, we investigated the domains of CSQ-1 that are important for polymerization and cellular localization, and required for its correct buffering functions. In transgenic animals rescued with various CSQ-1 constructs, the in vivo patterns of polymerization and localization of several mutated calsequestrins were observed to correlate with the structure-function relationship. Our results suggest that polymerization of CSQ-1 is essential but not sufficient for correct cellular localization and function of CSQ-1. In addition, direct interaction between CSQ-1 and the ryanodine receptor (RyR) was found for the first time, suggesting that the cellular localization of CSQ-1 in *C. elegans* is indeed modulated by RyR through a physical interaction.

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Key words: Calsequestrin, Ca^{2+} buffering, Polymerization, Localization, RyR

Introduction

Calsequestrin is a high-capacity (40-50 mol Ca^{2+} mol^{-1} calsequestrin) and low-affinity Ca^{2+}-binding protein (K_d=40 μM) localized in the lumen of the junctional sarcoplasmic reticulum (SR) of skeletal and cardiac muscles (Cozens and Reithmeier, 1984; Ikemoto et al., 1972; MacLennan and Wong, 1971). It stores Ca^{2+} in sufficient quantities to allow repetitive contractions. Albeit its ability to sequester a large amount of Ca^{2+}, being a low-affinity Ca^{2+}-binding protein ensures the rapid delivery of Ca^{2+} when the need arises. Thus calsequestrin efficiently functions as a Ca^{2+}-buffering protein. Overexpression of calsequestrin results in severe hypertrophy and heart failure in mice which is accompanied with impaired Ca^{2+} loading (Jones et al., 1998; Sato et al., 1998; Wang et al., 2000). A missense mutation in calsequestrin resulting in a single amino acid substitution (D307H) results in a genetic disease called catecholaminergic polymorphic ventricular tachycardia (Lahat et al., 2001).

In mammals, calsequestrin protein is in physical proximity with the ryanodine receptor (RyR) through its interaction with triadin (Guo and Campbell, 1995) and junctin (Zhang et al., 1997). In our previous paper, we reported that the RyR protein UNC-68 appeared to affect the correct localization of CSQ-1 in vivo (Cho et al., 2000).

The crystal structure of rabbit skeletal calsequestrin shows that calsequestrin consists of three domains (I, II, and III), each with a topology similar to that of *E. coli* thioredoxin (Wang et al., 1998). The individual domains fold into an α-β structure with five β-strands in the core, flanked by four-α-helices, two on each side of the sheet (Wang et al., 1998). The folding and compaction of calsequestrin is dependent on Ca^{2+} and the ionic strength, and occurs optimally with Ca^{2+} and K^+ concentrations close to those in the lumen of the SR in vivo (~1 mM Ca^{2+} and ~100 mM K^+). Crystal structures show that calsequestrin forms two different dimers: front-to-front and back-to-back (Gatti et al., 2001; Wang et al., 1998). In the back-to-back interaction, the C-terminal segments form a negatively charged pocket within the dimer that favors Ca^{2+} binding. The front-to-front interaction also generates a negatively charged pocket that supports Ca^{2+} binding and, like the back-to-back dimer, is likely to be favored by Ca^{2+} binding. With these two types of interactions, calsequestrin is predicted to form a continuous, linear polymer when Ca^{2+} concentration is increased. In addition, it has been confirmed that both back-to-back (C-terminal) and front-to-front (N-terminal) interactions are necessary for calsequestrin polymerization (Park et al., 2003).

However, in vivo roles of either of the two interactions of calsequestrin have yet to be investigated in any of the conventional model organisms. In our previous study, we reported RNAi mediated knockdown phenotypes in *C. elegans* (Cho et al., 2000). Here, we report the isolation and characterization of the *csq-1* deletion mutant *csq-1(jh109)* that

provides a valuable tool to study the structure-function relationship of CSQ-1 in vivo. Our results demonstrate that CSQ-1 polymerization in *C. elegans* is essential, but not sufficient for its correct localization and function in SR.

Results

Characterization of the calsequestrin knockout mutant *csq-1(jh109)* in *C. elegans*

We have isolated the *csq-1* deletion mutant *csq-1(jh109)* by removing several exons in the middle of the gene (Fig. 1A). When genomic DNA from a single worm was used as template, the PCR amplification product confirmed the isolation of a homozygote deletion mutant (Fig. 1B). To further evaluate the nature of this allele, we performed western blot analysis of protein extracts from wild-type (N2) worms and *csq-1(jh109)*. As evidenced from Fig. 1C, no CSQ-1 protein was detected in *C. elegans* carrying the *csq-1(jh109)* mutation, verifying the isolated allele is functionally null.

To our surprise, *csq-1(jh109)* mutants were viable and showed no obvious muscle defects. This is consistent with our previous characterization of RNAi (RNA interference) knockdown phenotypes. We have shown that animals deficient of calsequestrin exhibit normal movement in body-wall muscle (Cho et al., 2000). Since CSQ-1 is abundantly expressed in body-wall muscles of *C. elegans*, we further investigated potential muscle-specific function of CSQ-1 by characterizing the *csq-1(jh109)* mutant. We found no obvious alteration in muscle functioning, as evidenced by the fact that both the wild-type and *csq-1(jh109)* animals moved with equal efficiency (Fig. 2A). As expected, body-wall muscle structures examined by electron microscope showed normal sarcomere structures compared with wild-type animals (Fig. 2F,G). However, Ca^{2+} signaling plays a crucial role in fertilization, embryonic development, post-embryonic development and contraction of vulval muscles, a prerequisite for the expulsion of embryos.

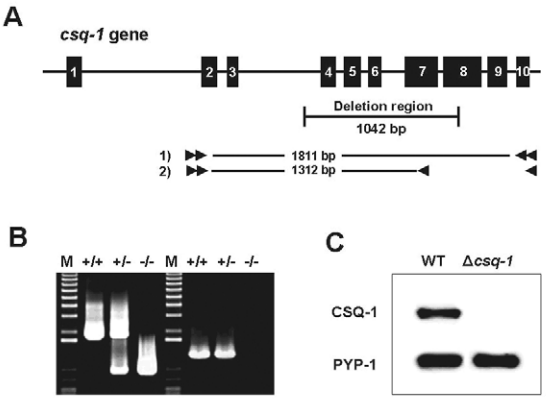


Fig. 1. Schema of the *csq-1*-null mutant *csq-1 (jh109)*. (A) The 1042 bp deletion, which removed several exons, is shown by a horizontal bar. (1) and (2) indicate primer sets used for nested PCR (indicated by arrows) performed for mutant isolation. (B) Agarose gel of PCR fragments showing bands obtained from single worm PCR. Wild type (+/+), heterozygote (+/–), and homozygote (–/–). Absence of a band in lane labelled (–/–) confirms that the worm is a homozygote. (C) Western blot showing the expression of CSQ-1 and PYP-1 (inorganic pyrophosphatase as control) in wild-type worms (lane 1) and *csq-1(jh109)* animals (lane 2).

Based on the strong expression of CSQ-1 in vulval muscle (Cho et al., 2000), which was resistant to RNAi treatment, we sought to know whether there is a defect associated with the reproductive function in *csq-1(jh109)* mutant animals. However, as shown in Fig. 2B, the brood size of *csq-1(jh109)* animals was indistinguishable from that of wild-type animals, precluding the possible role of CSQ-1 in reproduction and/or embryonic development in *C. elegans*.

Since calsequestrin has been attributed to be a major Ca^{2+} buffering protein in the SR (Fliegel et al., 1987; Scott et al., 1988), we reasoned that *csq-1*-null mutants might not acclimatize that well in an environment of either high or low Ca^{2+} concentration [Ca^{2+}]. As expected, the survival rate of *csq-1(jh109)* was reduced dramatically to 50% compared with wild-type worms, when grown on plates with nematode growth medium (NGM) containing high [Ca^{2+}] (Fig. 2C). Furthermore, the survival rate of *csq-1(jh109)* was reduced to 40% when grown on plates containing the Ca^{2+} chelator EGTA (Fig. 2D). Taken together, our results suggest that CSQ-1 functions as a major Ca^{2+}-buffering protein in *C. elegans*.

In addition to its primary effect on chelating and thereby depleting Ca^{2+}, EGTA is also known to elicit ER stress. Hence, our observation of decreased survivability of *csq-1(jh109)* on EGTA-containing plates could be due to either defective Ca^{2+} buffering or ER stress. To rigorously discern between these two possibilities, we grew the worms on plates containing 1 mM DTT, a substance known to cause ER stress. Survival rates of *csq-1(jh109)* animals were slightly reduced compared with those of wild-type animals (Fig. 2E). However, *crt-1(jh101)* animals deficient in calreticulin CRT-1, an ER-resident chaperon protein, showed dramatically decreased survival (Fig. 2E), but survived relatively well on plates with excess Ca^{2+} (Fig. 2C). Furthermore, the *csq-1(jh109);crt-1(jh101)* double mutant showed only additive defects under growing conditions with EGTA, but not when DTT was present. Taken together, our results suggest that the primary cause for decreased survivability of *csq-1* mutants on EGTA plates is not ER stress but ineffective Ca^{2+} buffering ability.

In vivo analysis of polymerization and cellular localization of CSQ-1 within the SR

The quaternary structure of calsequestrin changes from dimers to long, linear polymers as [Ca^{2+}] is increased. Two types of interaction between calsequestrin (front-to-front and back-to-back interactions) could be related to this polymerization (Wang et al., 1998). Recently, this idea was exploited further by Park et al. using several mutant versions of canine calsequestrin analyzed with respect to their property of polymerization in vitro (Park et al., 2003). Since a viable *C. elegans* knockout mutant of calsequestrin provides an excellent in vivo model, we were interested in how the subtle changes in the predicted intermolecular contact regions of CSQ-1 are translated into differential CSQ-1 polymerization and cellular localization of CSQ-1 in the SR compartment.

In rabbit skeletal-muscle calsequestrin, stable formation of the calsequestrin dimer is necessary for the subsequent polymerization process (Wang et al., 1998). From a structural point of view (Wang et al., 1998), an interaction between serine acidic hydrophobic (SAH) residues of one calsequestrin with the dibasic hydrophobic (DBH) of the other appears to be important for back-to-back interaction. To have a schematic

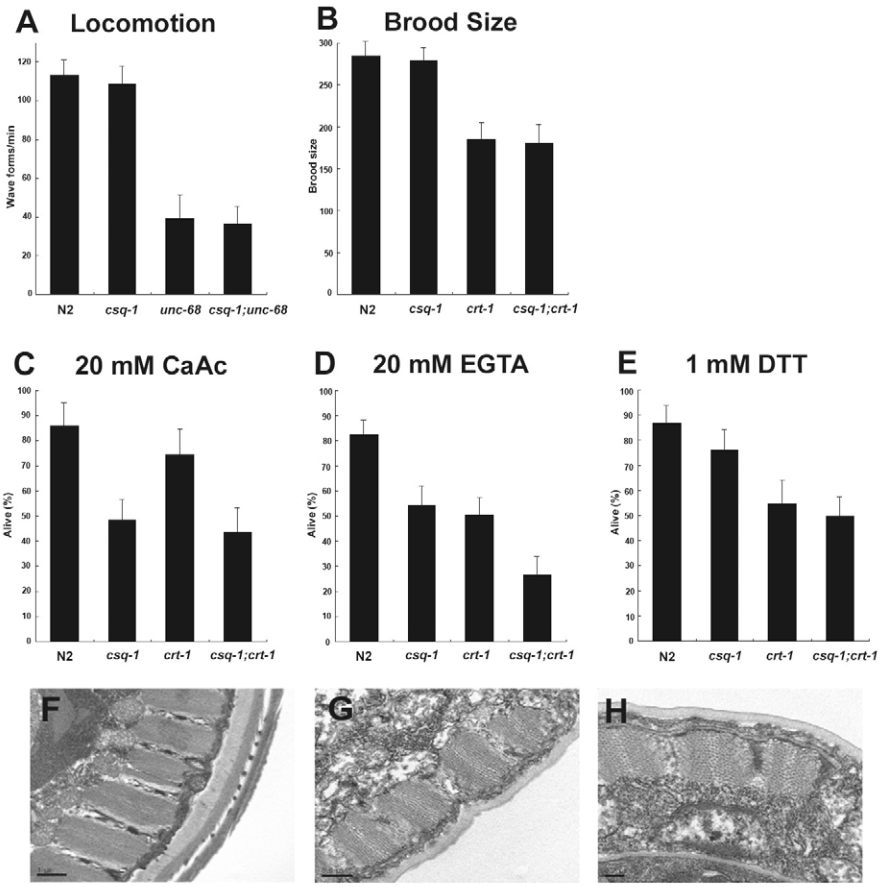


Fig. 2. Phenotypes of wild-type and *csq-1* mutant worms. (A) Analysis of motility assays in liquid medium showing the average number of waveforms measured in 1 minute (error bars give s.d., n=20). (B) Analysis of average brood size of the indicated strains (error bars give s.d., n=20). (C,D,E) Survival rate under high-[Ca^{2+}] stress conditions, low-[Ca^{2+}] stress conditions and DTT stress conditions. Young adult worms were grown on plates containing 20 mM calcium acetate, 20 mM EGTA or 1 mM DTT solution. Surviving worms of each strain were counted after 3 days. The *unc-68* gene encodes the RyR and *crt-1* encodes calreticulin. *csq-1;crt-1* is a double mutant of calsequestrin and calreticulin and *csq-1;unc-68* is double mutant of calsequestrin and RyR. (F-H) Electron micrographs of the transverse section showing body-wall muscle structure of wild-type worms (F), *csq-1(jh109)* mutant worms (G) and *csq-1(jh109)* mutant worms grown on EGTA plates (H).

model of *C. elegans* CSQ-1, we performed homology modeling of *C. elegans* CSQ-1 by using rabbit calsequestrin as a template (Fig. 3A). In this model, the back-to-back interface has a basic residue, Lys111, that is highly conserved among all three calsequestrins (Fig. 3B,C). The side chain of Lys111 was found to be protruding out towards the interfacial region. Interestingly, three acidic residues (Asp196, Glu247 and Glu251) of the interacting CSQ-1 were found to be in the vicinity of Lys111 (Fig. 3B). Our modeling suggests that the interaction of Lys111 of one CSQ-1 with some or all of the three acidic residues of the other CSQ-1 at the interface may be essential for the formation of a stable dimer. To test our hypothesis, we changed Lys111 to Ala by site-directed mutagenesis. This mutation completely changed the cellular localization of CSQ-1 and appears to be non-functional (Fig. 4G,I). To rule out general misfolding of the CSQ-1(K111A) mutant protein, we also expressed CSQ-1(K111Q) in the *csq-1(jh109)* mutant. It showed a diffused localization throughout the muscle similar to the localization of CSQ-1(K111A) (see Fig. S2C,D in supplementary material). Our result indicates that back-to-back interaction mediated by a possible salt bridge of Lys111 is essential for the dimer formation. Hence, the K111A mutation in CSQ-1 allowed neither polymerization nor proper localization (adjacent to RyR), as evidenced by its diffuse distribution. We therefore conclude that polymerization is essential for the correct localization of CSQ-1.

The C-terminal residues are not essential for polymerization but important for correct localization

The in vitro light-scattering experiment with canine mutant calsequestrin (ΔC27, deletion of C-terminal 27 residues) showed Ca^{2+}-dependent dimerization of ΔC27. However, they failed to form polymers like wild-type protein (Park et al., 2003). The exact mode of interaction (front-to-front vs back-to-back) has not been unequivocally determined (Wang et al., 1998). By contrast, we found no evidence for any defect in the oligomerization of the corresponding mutant in *C. elegans*. Instead, our results suggests that the C-terminal 26 amino acids are necessary for correct localization of CSQ-1 to the proximity of RyR. The staining pattern of *csq-1*-null mutants expressing either the ΔC26-mutant form or a ΔC17 mutant were indistinguishable from that of the RyR-null mutant [*unc-68(e540)*] stained with anti-CSQ-1 antibody (Fig. 4D,H). This result indicates that C-terminal residues, at least up to the last 26 amino acids, are not essential for polymerization per se; rather, they are important for correct localization of CSQ-1 to the proximity of RyR.

For the correct front-to-front interaction, 13 N-terminal residues were found to be essential in canine calsequestrin (Park et al., 2003). *C. elegans* CSQ-1 has a similar sequence (residues 32 to 43). Proximal to this region, we also found seven additional amino acids that seem to be unique to *C. elegans* CSQ-1 (see gap in sequence alignment of Fig. 3C). To resolve which of these two regions are essential for the front-to-front interaction, we made two mutant constructs: ΔN23-31 and ΔN32-43, lacking aa residues at positions 23 to 31 and 32 to 43, respectively. Immunostaining of *csq-1* mutants expressing construct ΔN23-31 was indistinguishable (data not shown) from that of wild-type animals (Fig. 4E): typical sieve-like patterns were observed on each case. This result indicates that the N-terminal residues 23-31 are not essential for polymerization or localization of CSQ-1. Interestingly, staining

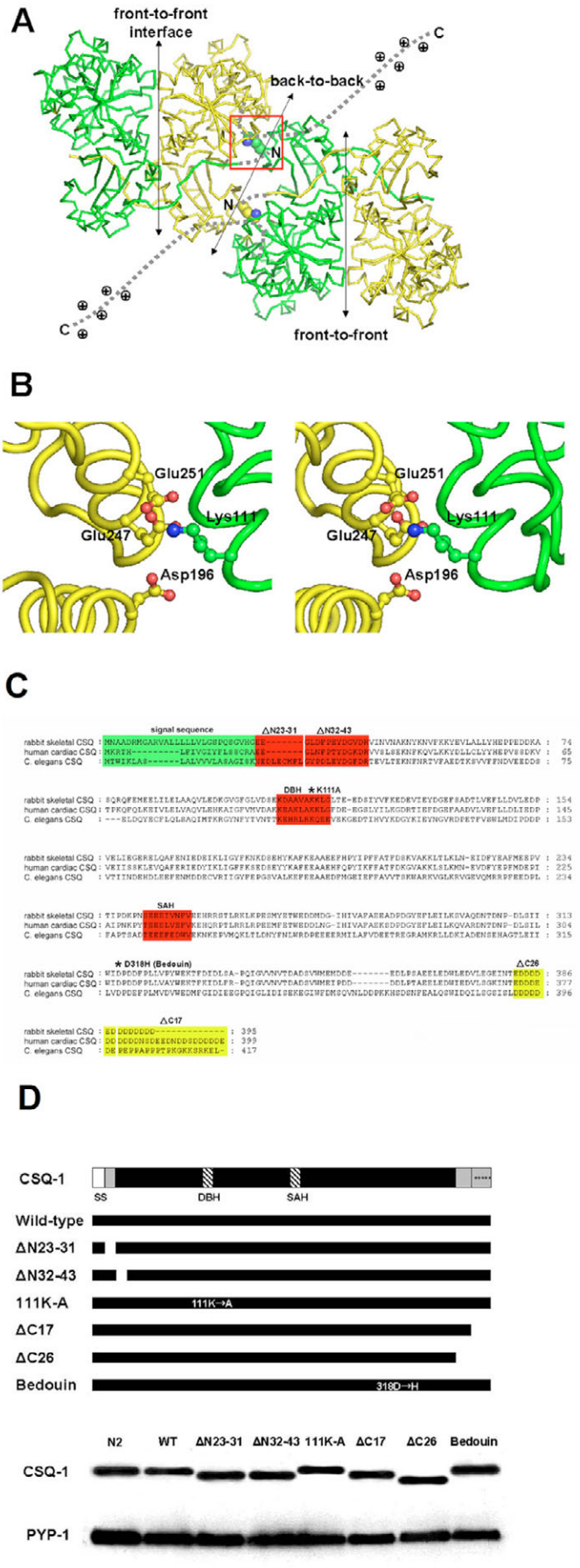


Fig. 3. Homology model and schematic diagram of mutated *C. elegans* calsequestrin. (A) Schematic prediction of polymerized CSQ-1. The monomeric model of CSQ-1 was built using the rabbit calsequestrin structure. The tetramer is shown as C^{α}-traces to display putative polymerizing interactions between monomers. Monomers are either green or yellow. N and C-termini of each molecule are shown as dashed lines because they are disordered in the template structure. Note the positively charged residues in the C-terminus specific to *C. elegans* calsequestrins. The location of the conserved back-to-back interface formed between SAH (Serine Acidic Hydrophbic) and DBH (Dibasic Hydrophobic) is boxed in red. Lys111 residues are shown as spheres. (B) Close-up view of the back-to-back interface of CSQ-1. The two interacting molecules are green and yellow. Three negatively charged residues (Glu247, Glu251 and Asp196) are placed in the vicinity of Lys111 and might participate in a salt bridge. (C) Amino acid sequence of *C. elegans* calsequestrin aligned with the rabbit skeletal calsequestrin and human cardiac calsequestrin CSQ2 amino acid sequences. Green box, signal sequence; first to fourth red and first and second yellow boxes indicate ΔC26 and ΔC17, ΔN23-31, ΔN32-43, DBH, SAH, respectively. (D) Each DNA construct represents wild-type (WT), N-terminal 9-amino-acid truncation, N-terminal 12-amino-acid truncation, C-terminal 17-amino-acid truncation and C-terminal 26-amino-acid truncation. Mutant CSQ-1 constructs K111A and Bedouin contain a Lys111 to Ala and Asp318 to His mutation, respectively. These constructs were introduced into the *csq-1(jh109)* mutant and their expression was detected by western blotting using anti-CSQ-1 and anti-PYP-1 (internal control) antibodies.

of the ΔN32-43 mutant CSQ-1 proteins appeared as random aggregates (Fig. 4F) that were not localized properly, suggesting CSQ-1 precipitation in the SR. Note that the observed precipitation is probably not due to the retention of CSQ-1 to the cytoplasm, because the mutant harbors an intact signal sequence. We conclude that correct linear polymerization is essential for correct localization of CSQ-1 in vivo.

Functional relevance of CSQ-1 polymerization and localization

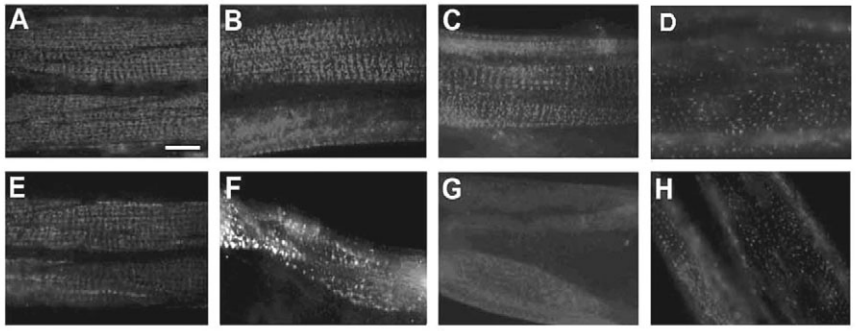
To better correlate the differences in the polymerization and localization of CSQ-1 with the efficiency of protein-activity, we injected different mutant constructs into *csq-1(jh109)* animals that were instrumental in studying the structure-function relationship of calsequestrin. Sensitivity of the *csq-1(jh109)* mutant worm towards slight perturbations in $[Ca^{2+}]$ was fully reversed by injection of the wild-type *csq-1* gene (Fig. 4I). Similarly, constructs ΔN23-31 and the Bedouin mutations (missense mutations of human *CASQ2*) also fully rescued CSQ-1 function in the *csq-1*-null mutant phenotype, which was not surprising because these mutated proteins still polymerized and localized correctly. Interestingly, mutant constructs ΔC17 as well as ΔC26 failed to rescue CSQ-1 function in the *csq-1*-null mutant phenotype (Fig. 4I). These two mutant proteins, although polymerized correctly, failed to display wild-type localization. As mentioned above, polymerization is essential but not sufficient for correct localization. We, therefore, suggest that polymerization, although necessary, is not sufficient for correct functioning of CSQ-1. The K111A mutant is futile in terms of its rescuing ability, underscoring the importance of back-to-back interaction in polymerization, localization and function.

Conservation of Lys111 across the calsequestrins from different species indicates the existence of high selection-pressure over this residue.

Direct interaction between the C-terminus of CSQ-1 and intraluminal loop regions of RyR in *C. elegans*

Previously, we have reported that RyR affects the localization of CSQ-1 in *C. elegans* (Cho et al., 2000). Calsequestrin in mammals has been shown to regulate the RyR through triadin and junctin (Guo and Campbell, 1995; Zhang et al., 1997). But no homolog of triadin and junctin has (so far) been found in *C. elegans*. According to our homology modeling, the C-terminal region of CSQ-1 that contains positive charges might directly interact electrostatically with the intraluminal loops of RyR. Therefore, to investigate the nature of interaction between CSQ-1 and RyR, an in vitro binding assay was performed at different concentrations (0-2 mM) of Ca^{2+}. As shown in Fig. 5A, we found that CSQ-1 directly interacted with loop I and loop II of the RyR.

To identify the regions of CSQ that interact with the loop regions of the RyR, in vitro binding assay were performed using mutated CSQ-1 protein in which the C-terminal 17 amino acids were deleted. As shown Fig. 5B, the CSQ-1 deletion mutant (ΔC17-CSQ-1) failed to bind RyR loop regions, suggesting that the C-terminal (17 amino acids) region of CSQ-1 is essential for binding these loop regions. Since the RyR loop regions have contain negatively charged amino acids, positive charges in the C-terminal region of CSQ-1 might interact electrostatically with RyR loop regions and, therefore, stabilize any binding between CSQ-1 and RyR (as schematically presented in Fig. 5C).



	Strain	Antibody	Staining pattern	
A	WT	α-CSQ-1	Punctate and mesh-like pattern	
B	WT	α-UNC-68	Punctate and mesh-like pattern	
C	*csq-1(jh109)*	α-UNC-68	Punctate and mesh-like pattern	
D	*unc-68(e540)*	α-CSQ-1	Dispersed pattern	
	Strain	**Antibody**	**Rescue Construct**	**Rescue pattern**
E	*csq-1(jh109)*	α-CSQ-1	WT	Mesh-like pattern
F	*csq-1(jh109)*	α-CSQ-1	ΔN32-43	Aggregate pattern
G	*csq-1(jh109)*	α-CSQ-1	K111A	Diffused pattern
H	*csq-1(jh109)*	α-CSQ-1	ΔC26/ΔC17	Dispersed pattern

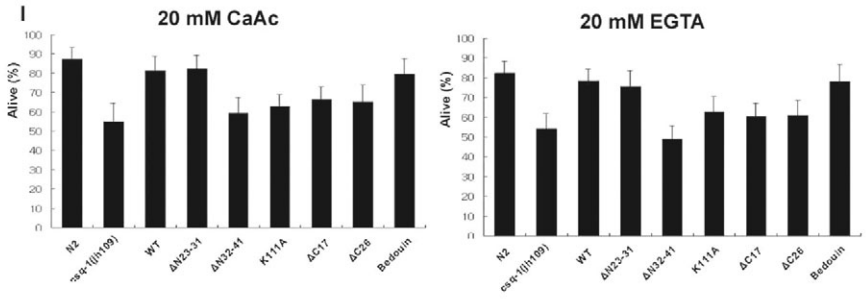


Fig. 4. Cellular localization and functional rescue of calsequestrin. (A,B) Wild-type worms stained with (A) anti-CSQ-1 antibody and (B) anti-UNC-68 (RyR) antibody show punctate and mesh-like staining in body-wall muscles. (C) The *csq-1(jh109)* mutant stained with anti-UNC-68 antibody shows similar punctuate and mesh-like staining pattern. (D) The *unc-68(e540)* mutant stained with anti-CSQ-1 antibody show a dispersed punctate pattern; no mesh-like pattern was observed. (E) *csq-1*-null mutant *csq-1(jh109)* transformed by constructs encoding WT, ΔN23-31 and Bedouin CSQ-1 show mesh-like pattern. (F) ΔN32-43 shows aggregated pattern. (G) K111A shows diffused pattern. (H) ΔC17 and ΔC26 show dispersed pattern similar to that shown in D. (I) Survival rates of wild-type and transgenic animals expressing mutated CSQ-1 under high-[Ca^{2+}] and low-[Ca^{2+}] conditions. Young F1 transgenic worms were grown on NGM plates with 20 mM EGTA and 20 mM CaAc. Survival rates of worms with rescued CSQ-1 function was compared with that of wild-type animals at 20°C. Over 50 animals were tested for each data point of a single set of experiments, and each experiment was repeated three times.

Discussion

Consistent with RNAi-mediated knockdown phenotypes previously studied by us (Cho et al., 2000), *csq-1*-null mutants did not show an obvious phenotype. In our previous study, body-wall muscle expression of *csq-1* was completely abolished by RNAi treatment. However, ineffective silencing of *csq-1* transcripts in the vulval and pharyngeal muscle left the open question of potential vulva-specific or pharynx-specific functions we might have missed. We observed that the brood size of *csq-1*-null mutants (Fig. 2B), as well as pharyngeal pumping (data not shown), were comparable with those of wild-type animals. Furthermore, we did not observe any other defects associated with egg-laying or pharyngeal pumping. Our current data based on *csq-1*-knockout animals suggests that CSQ-1 is not required for vulval or pharyngeal muscle function. This is not surprising and, actually, similar to the recent finding that cardiac calsequestrin is not essential for providing sufficient Ca^{2+} storage for normal function of cardiac muscle (Knollmann et al., 2006). Despite absent cardiac calsequestrin, calsequestrin-knockout mice maintain relatively normal Ca^{2+}

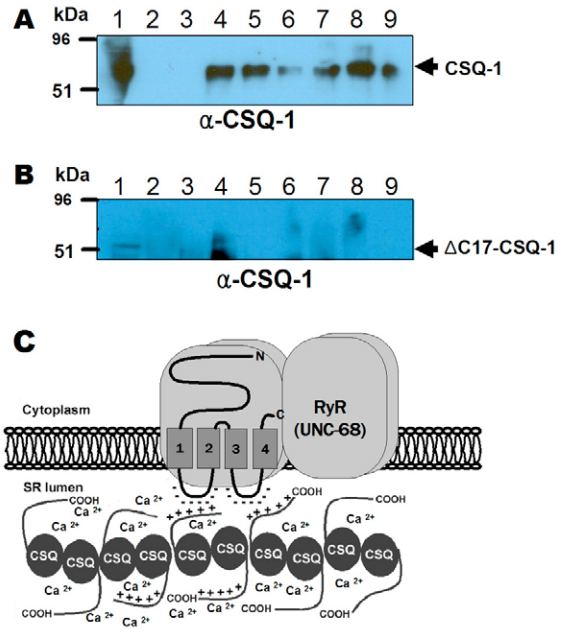


Fig. 5. Direct interaction between the C-terminal region of calsequestrin and the intraluminal loops of RyR. (A,B) In vitro binding assays were performed using (A) full-length CSQ-1 and (B) C-terminally deleted CSQ-1 (ΔC17-CSQ-1). Intraluminal loops of RyRs [GST-RyR loop I (lanes 4, 5, 6) and GST-RyR loop II (lanes 7, 8, 9)] were tested for direct interaction in the presence of Ca^{2+} (lanes 4, 7; 0.5 mM Ca^{2+}, lane 5, 8; 2 mM Ca^{2+}) or absence of Ca^{2+} (lanes 6, 9; 1 mM EGTA). Lane 1, supernatant of CSQ-1 (A) or that of ΔC17-CSQ-1 (B); lane 2, affinity beads alone; lane 3, GST control. (C) Simplified model of calsequestrin localization in *C. elegans.* Positively charged residues at the C-terminal end of *C. elegans* calsequestrin may directly interact with the negatively charged residues of the loops of RyRs to localize calsequestrin into the vicinity of the SR membrane. This could allow fast and localized release of Ca^{2+} through RyRs once the receptor is activated.

release and contractile function. Calreticulin is another endoplsmic reticulum (ER)-resident Ca^{2+}-binding protein, and our study indicates that *C. elegans* calreticulin (*crt-1*) is also expressed both in body-wall muscle as well as in pharyngeal muscle (Park et al., 2001). Therefore, we investigated the expression levels of other Ca^{2+}-binding proteins, such as calreticulin (CRT-1) and calnexin (CNX-1), in *csq-1(jh109)* mutants to determine whether a deficiency in CSQ-1 protein induces a compensatory overexpression of the other Ca^{2+}-binding proteins. Protein levels of CRT-1 and CNX-1 were unchanged (data not shown). More interestingly, the levels CSQ-1 itself was unchanged in wild-type animals stressed by low [Ca^{2+}]. Taken together, CRT-1 and CNX-1 are probably not overexpressd to compensate for CSQ-1 function in the *csq-1*-null mutant. This is consistent with the study of calsequestrin knockout mice, where no apparent upregulation of other Ca^{2+}-binding protein was detected (Knollmann et al., 2006).

However, the expression of SERCA1 protein has been reported to be reduced by up to 50% in CSQ-2 and CSQ-1–CSQ-2 knockdown myotubes (Wang et al., 2006). So we investigated the expression levels of the sarcoplasmic reticulum Ca^{2+} ATPase homolog *sca-1* (SCA-1; mammalian homolog: SERCA1) in *csq-1*-null mutants. To measure mRNA expression levels of *sca-1* in *csq-1*-null mutants, we performed reverse transcriptase (RT)-PCR using two different *C. elegans* strains (N2 and *csq-1(jh109)*) grown on plates with NGM and NGM supplemented with EGTA. mRNA levels of *sca-1* were significantly reduced in *csq-1(jh109)* mutants (supplementary material Fig. S1), which is consistent with a recent report (Wang et al., 2006). But, interestingly, the *sca-1* mRNA levels in *csq-1(jh109)* grown on EGTA-supplemented plates were not significantly different (supplementary material Fig. S1). However, the mechanism which leads to the reduction of SERCA1 expression remains unexplained, and genetic dissection of *C. elegans* mutants might provide some clues in the future.

In vitro, calsequestrin forms a long, linear polymer as dimers (back-to-back and front-to-front dimer) (Park et al., 2003). However, whether such polymerization does indeed happen in vivo has so far not been investigated. We exploited the *csq-1*-null mutant to directly visualize the status of CSQ-1 polymerization in *C. elegans*. We took two main parameters into consideration: (1) status of polymerization and, (2) RyR-dependent localization of CSQ-1. We then correlated these with their functional property. In wild-type worms, CSQ-1 polymerized correctly (punctate and mesh-like pattern). However, in *unc-68(e540)* mutants, the typical sieve-like pattern was lost and CSQ-1 distribution appeared as random spots, suggesting a role for RyRs in CSQ-1 localization.

Mouse, rat and rabbit calsequestrins are known to bind to junctin and triadin. The C-terminal of triadin (especially the KEKE motif) binds with the second luminal loop of RyRs (Lee et al., 2004), thus bringing calsequestrin into the proximity of RyRs. Thus, RyRs define the localization of CSQ-1. The similar immunostaining pattern of CSQ-1 corroborates with our result. Since wild-type CSQ-1 failed to form the typical sieve-like pattern in RyR mutants, the appearance of random spots indicates successful polymerization but failed correct localization. It is worthwhile to note that polymerization does not depend upon any other protein; purified calsequestrin successfully forms polymers at high [Ca^{2+}] in vitro (Park et al., 2003) and intraluminal [Ca^{2+}] is around 1 mM. Hence, wild-type CSQ-1 must have succeeded in polymerization and our observation of CSQ-1 appearing as random spots in RyR mutants is solely indicative of defective localization of CSQ-1. Furthermore, polymerization does not assure correct localization. In other words, polymerization and localization appear to be independent processes.

Immunostaining patterns of ΔC26 and ΔC17 CSQ-1 are similar to that of wild-type CSQ-1 in *unc-68(e540)* mutants, indicating that both ΔC27 and ΔC17 mutant proteins have succeeded to polymerize; however, they have failed to localize adjacent to RyRs. In vitro studies, though, carried out by Park et al. (Park et al., 2003) indicate that the canine calsequestrin mutants with C-terminal deletions failed to form polymers. The apparent discrepancy could be the manifestation of differences in the structure of the two calsequestrins. Alternatively, because the C-terminal region of the *C. elegans* CSQ-1 is unique, the function of this region may not be generalized to the mechanism of mammalian calsequestrin. Therefore, the importance of the C-terminal region for the polymerization of calsequestrin remains to be studied further. In addition, the C-terminus of *C. elegans* CSQ-1 does not contain as many

negatively charged amino acids as mammalian calsequestrins; indeed, the C-terminus of CSQ-1 contains many positively charged amino acids. Hence, an overall negative charge, hypothesized to be essential for binding Ca^{2+} and for Ca^{2+}-mediated polymerization might not be relevant to *C. elegans* CSQ-1 owing to the existence of a relatively large number of positively charged residues. Based on our homology modeling, we have found that the C-terminal region of CSQ-1, which contains positive charges, interacts with intraluminal loops of RyR as seen in our in vitro binding assay. Therefore, Ca^{2+} release from RyRs could be modulated by calsequestrin through a physical interaction in *C. elegans*.

Interaction between the SAH motif and the DBH motif is found in many protein-protein interactions (He et al., 1993; Mitchell et al., 1988). For instance, binding of calmodulin to its target is mediated by SAH-DBH interaction (Ikura et al., 1992; Meador et al., 1992; Meador et al., 1993). Structural analysis of rabbit calsequestrin shows the interaction of the SAH domain of one calsequestrin and the DBH domain of another calsequestrin molecule. Likewise, our homology modeling hinted to the possibility that the interaction between the SAH motif and the DBH motif is essential to stabilize back-to-back interactions. The highly conserved Lys111 in the DBH motif was found to be the point of interaction with some or all three of the negatively charged residues in the back-to-back interaction. When mutant CSQ-1 (K111A point mutation) was expressed, a diffuse localization throughout the muscle was seen instead of a punctate appearance like in the wild-type polymerized CSQ-1 in *unc-68* mutants. Thus, we believe that *csq-1*(K111A) mutants neither polymerized nor localized properly. However, to rule out a general misfolding of the CSQ-1(K111A) mutant protein, we also expressed the CSQ-1(K111Q) mutant protein in *csq-1(jh109)* worms. As shown in supplementary material Fig. S2C,D, the mutant protein CSQ-1(K111Q) showed a diffused localization throughout the muscle, similar to the localization of CSQ-1(K111A). Additionally, we predicted that the highly conserved Lys111 in the DBH motif interacts with the three negatively charged residues (Asp197, Glu247 and Glu251) in our homology model. To determine whether these negatively charged amino acids are – like Lys-111 – important for polymerization and localization of calsequestrin, we expressed CSQ-1(E247Q), CSQ-1(E251Q) and doubly mutated protein CSQ-1(E247Q/E251Q) in *csq-1*-null mutants. As shown in supplementary material Fig. S2E-J, CSQ-1(E247Q) expression resulted in a diffused protein pattern, mostly similar to that of the CSQ-1(K111A) mutant protein (supplementary material Fig. S2E), sometimes showing a weak dispersed pattern (supplementary material Fig. S2D). Sometimes, the staining for CSQ-1(E247Q) was brighter than that of CSQ-1(K111A), but the pattern was generally consistent with the one shown in supplementary material Fig. S2A. Like CSQ-1(E247Q), mutant protein CSQ-1(E251Q) mostly showed a diffused pattern (supplementary material Fig. S2G) and much less frequently a weak dispersed pattern (supplementary material Fig. S2H). Interestingly, the doubly mutated protein CSQ-1(E247Q/E251Q) showed completely diffused localization (supplementary material Fig. S2I,J). It appears that the single mutations (E247Q or E251Q) affect the interaction between Glu247 or Glu251 and Lys111. Furthermore, the doubly mutated protein CSQ-1(E247Q/E251Q) disrupted this interaction completely and, therefore, failed to both polymerize and localize. Therefore, Glu247 and Glu251 are, like Lys111, also important for polymerization of calsequestrin.

Similar to canine calsequestrin, the N-terminal region of CSQ-1 (residues 32 to 43) is essential for the formation of a physiologically relevant linear polymer. Deletion of this region led to the formation of aggregates that were not localized appropriately. We infer from this that only long, linear polymers – and not random aggregates – are localized adjacent to RyR. Interestingly, the structural defects were directly reflected at the functional level. Only those mutant constructs that polymerized properly (ΔN23-31, Bedouin) rescued CSQ-1 function in *csq-1(jh109)* animals. By contrast, mutant proteins that polymerized correctly but failed to localize (constructs ΔC17 and ΔC26), formed random aggregates (construct ΔN32-43) or both failed to polymerize and localize [mutant protein CSQ-1(K111A)] did not rescue CSQ-1 function in the *csq-1(jh109)* animals. In addition, mammalian calsequestrin (rabbit skeletal) could rescue CSQ-1 function in the *csq-1(jh109)* animals (supplementary material Fig. S3). Even though the C-terminus of *C. elegans* CSQ-1 is different from that in mammals, these data suggest that calsequestrin functions are conserved from *C. elegans* to mammals. The possible reason why the C-terminus of *C. elegans* CSQ-1 has the unique feature of its many positively charged residues is to facilitate the direct interaction between CSQ-1 and RyR.

In summary, we found the following: (1) polymerization and localization of CSQ-1 are independent processes in *C. elegans*. Additionally, we observed a clear correlation between polymerization-status and correct localization of CSQ-1 in the proximity of RyRs. (2) Polymerization is essential but not sufficient for the correct localization of CSQ-1. (3) Back-to-back inter-CSQ-1 interaction involving Lys111 is important for the polymerization. (4) Formation of long, linear polymers – not aggregation – accounts for correct localization. (5) Correct polymerization followed by RyR-dependent localization of CSQ-1 is essential for its function as an efficient Ca^{2+}-buffering protein.

Materials and Methods

C. elegans strains and maintenance

The *C. elegans* Bristol N2 strain was obtained from the *Caenorhabditis* Genetics Center (CGC) at the University of Minnesota, MN. The knockout calsequestrin (*csq-1*) mutant *csq-1(jh109)* was isolated using a reverse genetics method (this study) and *crt-1(jh101)* was previously isolated in our laboratory (Park et al., 2001). Worm breeding and handling were conducted as previously described (Brenner, 1974).

Construction of double mutant strain and phenotype analysis

Double mutantion animals *csq-1(jh109);unc-68(r1161)* and *csq-1(jh109);crt-1(jh101)* were constructed by standard genetic methods. PCR was used to detect the *unc-68(r1161)*, *csq-1(jh109)* and *crt-1(jh101)* mutants. The *csq-1(jh109)* deletion mutant was characterized microscopically by phenotypic defects. The brood size, survival rate and motility of both the wild-type and mutant worms were estimated at 20°C. To study sensitivity to EGTA and Ca^{2+} acetate (CaAc), gravid adults were allowed to lay eggs for 4 hours on plates containing 20 mM EGTA or 20 mM CaAc and were then removed from the plates. Survival rates of worms were determined 3 days later.

Generation of CSQ mutant constructs

To generate the site-specific mutations K111A, K111Q, E247Q, E251Q and the double mutation E247Q/E257Q, we used overlap extension method. Overlapping PCR fragments were mixed, denatured and annealed to generate hetero-duplexes that were extended and amplified into full-length DNA using two primers. This PCR product was cloned into the pPD49.83 vector containing the upstream region of *csq-1*. A similar strategy was applied to make deletions constructs (ΔN23-32, ΔN32-43, ΔC26, and ΔC17).

Microinjection of mutant constructs and immunofluorescence microscopy

The mutated CSQ-1 constructs were microinjected into *csq-1*-null mutant *csq-1(jh109)* animals to obtain germline transformants as described by Mello and Fire (Mello and Fire, 1995). As a transformation marker, plasmid pRF4 containing a dominant gene roller [*rol-6(su1006)*] was co-injected. Mutant constructs (150 μg/μl) were injected in the gonads of the adult hermaphrodites as described (Mello and Fire, 1995). After recovery in bacteria-seeded worm agar plates, the injected worms were transferred to fresh worm plates at 16-hour intervals. The progeny of injected animals was selected for roller phenotype and were collected for immunostaining or EGTA sensitivity test.

C. elegans was immunostained as described previously (Ahnn and Fire, 1994; Miller and Shakes, 1995). Stained specimens were observed under a fluorescence microscope (Olympus BX50). Anti-CSQ-1 antibody (Cho et al., 2000) and anti-UNC-68 antibody (Cho et al., 2000) were used as described.

In vitro binding assay

In vitro binding assays were performed as described previously (Lee et al., 2004). An affinity column was prepared by immobilizing GST-RyR loop I and GST-RyR loop II fusion proteins on glutathione-Sepharose beads for 2 hours at 4°C (Amersham Biosciences). Solubilized proteins CSQ-1 and CSQ-1(ΔC17) (CSQ-1 lacking the 17 C-terminal amino acids) were obtained by centrifugation, mixed with buffer (20 mM Tris-Cl pH 7.4, 1 mM DTT and protein inhibitor mixture) and incubated with the purified GST-RyR loop I and GST-RyR loop II protein Sepharose complex for 4 hours at 4°C. After incubation, the fusion protein-Sepharose complexes were washed three times with 20 mM Tris-Cl pH 7.4, 0.15 M NaCl, 0.2% Triton X-100. The bound proteins were eluted by boiling in SDS-PAGE sample buffer, separated by SDS-PAGE and immunoblotted using anti-CSQ antibody.

Comparative modeling of CSQ-1

A homology model of *C. elegans* CSQ-1 was built by using the X-ray structure of rabbit calsequestrin (PDB id 1A8Y) (Wang et al., 1998). The alignment and modeling building was performed using the program MODELER version 6 (Sali and Blundell, 1993). The model covers residues Gly32 to Ser371. The best model with the lowest MODELLER restraint energy was kept and the side chains of the model were further refined by energy minimization using the GROMOS96 implementation of Swiss-PdbViewer (Guex and Peitsch, 1997). Construction of the CSQ-1 polymer was done by superposition of each monomer to the separate crystallographic symmetry position of the rabbit calsequestrin structure. Figures were prepared using PyMOL (DeLano, 2002).

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References

Ahnn, J. and Fire, A. (1994). A screen for genetic loci required for body-wall muscle development during embryogenesis in Caenorhabditis elegans. *Genetics* **137**, 483-498.

Brenner, S. (1974). The genetics of Caenorhabditis elegans. *Genetics* **77**, 71-94.

Cho, J. H., Oh, Y. S., Park, K. W., Yu, J., Choi, K. Y., Shin, J. Y., Kim, D. H., Park, W. J., Hamada, T., Kagawa, H. et al. (2000). Calsequestrin, a calcium sequestering protein localized at the sarcoplasmic reticulum, is not essential for body-wall muscle function in Caenorhabditis elegans. *J. Cell Sci.* **113**, 3947-3958.

Cozens, B. and Reithmeier, R. A. (1984). Size and shape of rabbit skeletal muscle calsequestrin. *J. Biol. Chem.* **259**, 6248-6252.

DeLano, W. L. (2002). *The PyMOL User's Manual*. San Carlos, CA: DeLano Scientific.

Fliegel, L., Ohnishi, M., Carpenter, M. R., Khanna, V. K., Reithmeier, R. A. and MacLennan, D. H. (1987). Amino acid sequence of rabbit fast-twitch skeletal muscle calsequestrin deduced from cDNA and peptide sequencing. *Proc. Natl. Acad. Sci. USA* **84**, 1167-1171.

Gatti, G., Trifari, S., Mesaeli, N., Parker, J. M., Michalak, M. and Meldolesi, J. (2001). Head-to-tail oligomerization of calsequestrin: a novel mechanism for heterogeneous distribution of endoplasmic reticulum luminal proteins. *J. Cell Biol.* **154**, 525-534.

Guex, N. and Peitsch, M. C. (1997). SWISS-MODEL and the Swiss-PdbViewer: an environment for comparative protein modeling. *Electrophoresis* **18**, 2714-2723.

Guo, W. and Campbell, K. P. (1995). Association of triadin with the ryanodine receptor and calsequestrin in the lumen of the sarcoplasmic reticulum. *J. Biol. Chem.* **270**, 9027-9030.

He, Z., Dunker, A. K., Wesson, C. R. and Trumble, W. R. (1993). Ca(2+)-induced folding and aggregation of skeletal muscle sarcoplasmic reticulum calsequestrin. The involvement of the trifluoperazine-binding site. *J. Biol. Chem.* **268**, 24635-24641.

Ikemoto, N., Bhatnagar, G. M., Nagy, B. and Gergely, J. (1972). Interaction of divalent cations with the 55,000-dalton protein component of the sarcoplasmic reticulum. Studies of fluorescence and circular dichroism. *J. Biol. Chem.* **247**, 7835-7837.

Ikura, M., Clore, G. M., Gronenborn, A. M., Zhu, G., Klee, C. B. and Bax, A. (1992). Solution structure of a calmodulin-target peptide complex by multidimensional NMR. *Science* **256**, 632-638.

Jones, L. R., Suzuki, Y. J., Wang, W., Kobayashi, Y. M., Ramesh, V., Franzini-Armstrong, C., Cleemann, L. and Morad, M. (1998). Regulation of Ca2+ signaling in transgenic mouse cardiac myocytes overexpressing calsequestrin. *J. Clin. Invest.* **101**, 1385-1393.

Knollmann, B. C., Chopra, N., Hlaing, T., Akin, B., Yang, T., Ettensohn, K., Knollmann, B. E., Horton, K. D., Weissman, N. J., Holinstat, I. et al. (2006). Casq2 deletion causes sarcoplasmic reticulum volume increase, premature Ca2+ release, and catecholaminergic polymorphic ventricular tachycardia. *J. Clin. Invest.* **116**, 2510-2520.

Lahat, H., Pras, E., Olender, T., Avidan, N., Ben-Asher, E., Man, O., Levy-Nissenbaum, E., Khoury, A., Lorber, A., Goldman, B. et al. (2001). A missense mutation in a highly conserved region of CASQ2 is associated with autosomal recessive catecholamine-induced polymorphic ventricular tachycardia in Bedouin families from Israel. *Am. J. Hum. Genet.* **69**, 1378-1384.

Lee, J. M., Rho, S. H., Shin, D. W., Cho, C., Park, W. J., Eom, S. H., Ma, J. and Kim, D. H. (2004). Negatively charged amino acids within the intraluminal loop of ryanodine receptor are involved in the interaction with triadin. *J. Biol. Chem.* **279**, 6994-7000.

MacLennan, D. H. and Wong, P. T. (1971). Isolation of a calcium-sequestering protein from sarcoplasmic reticulum. *Proc. Natl. Acad. Sci. USA* **68**, 1231-1235.

Meador, W. E., Means, A. R. and Quiocho, F. A. (1992). Target enzyme recognition by calmodulin: 2.4 A structure of a calmodulin-peptide complex. *Science* **257**, 1251-1255.

Meador, W. E., Means, A. R. and Quiocho, F. A. (1993). Modulation of calmodulin plasticity in molecular recognition on the basis of x-ray structures. *Science* **262**, 1718-1721.

Mello, C. and Fire, A. (1995). DNA transformation. *Methods Cell Biol.* **48**, 451-482.

Miller, D. M. and Shakes, D. C. (1995). Immunofluorescence microscopy. *Methods Cell Biol.* **48**, 365-394.

Mitchell, R. D., Simmerman, H. K. and Jones, L. R. (1988). Ca2+ binding effects on protein conformation and protein interactions of canine cardiac calsequestrin. *J. Biol. Chem.* **263**, 1376-1381.

Park, B. J., Lee, D. G., Yu, J. R., Jung, S. K., Choi, K., Lee, J., Lee, J., Kim, Y. S., Lee, J. I., Kwon, J. Y. et al. (2001). Calreticulin, a calcium-binding molecular chaperone, is required for stress response and fertility in Caenorhabditis elegans. *Mol. Biol. Cell* **12**, 2835-2845.

Park, H., Wu, S., Dunker, A. K. and Kang, C. (2003). Polymerization of calsequestrin. Implications for Ca2+ regulation. *J. Biol. Chem.* **278**, 16176-16182.

Sali, A. and Blundell, T. L. (1993). Comparative protein modelling by satisfaction of spatial restraints. *J. Mol. Biol.* **234**, 779-815.

Sato, Y., Ferguson, D. G., Sako, H., Dorn, G. W., 2nd, Kadambi, V. J., Yatani, A., Hoit, B. D., Walsh, R. A. and Kranias, E. G. (1998). Cardiac-specific overexpression of mouse cardiac calsequestrin is associated with depressed cardiovascular function and hypertrophy in transgenic mice. *J. Biol. Chem.* **273**, 28470-28477.

Scott, B. T., Simmerman, H. K., Collins, J. H., Nadal-Ginard, B. and Jones, L. R. (1988). Complete amino acid sequence of canine cardiac calsequestrin deduced by cDNA cloning. *J. Biol. Chem.* **263**, 8958-8964.

Wang, S., Trumble, W. R., Liao, H., Wesson, C. R., Dunker, A. K. and Kang, C. H. (1998). Crystal structure of calsequestrin from rabbit skeletal muscle sarcoplasmic reticulum. *Nat. Struct. Biol.* **5**, 476-483.

Wang, W., Cleemann, L., Jones, L. R. and Morad, M. (2000). Modulation of focal and global Ca2+ release in calsequestrin-overexpressing mouse cardiomyocytes. *J. Physiol.* **524**, 399-414.

Wang, Y., Xu, L., Duan, H., Pasek, D. A., Eu, J. P. and Meissner, G. (2006). Knocking down type 2 but not type 1 calsequestrin reduces calcium sequestration and release in C2C12 skeletal muscle myotubes. *J. Biol. Chem.* **281**, 15572-15581.

Zhang, L., Kelley, J., Schmeisser, G., Kobayashi, Y. M. and Jones, L. R. (1997). Complex formation between junctin, triadin, calsequestrin, and the ryanodine receptor. Proteins of the cardiac junctional sarcoplasmic reticulum membrane. *J. Biol. Chem.* **272**, 23389-23397.