Homo-dimerization of RyR1 C-terminus *via* charged residues in random coils or in an α -helix

Eun Hui Lee^{1,3} and Paul D. Allen²

¹Department of Physiology, College of Medicine
The Catholic University of Korea
Seoul 137-701, Korea

²Department of Anesthesiology, Perioperative and Pain Medicine
Brigham and Women's Hospital, Harvard Medical School
Boston, MA 02115, USA

³Corresponding author: Tel, 82-2-590-4990;
Fax, 82-2-532-9575; E-mail, EHUI@catholic.ac.kr

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Abbreviations: RyR, ryanodine receptor; *wt*, wild type

Abstract

To investigate the mechanism by which the C-terminus (4,938-5,037) of the ryanodine receptor 1 (RyR1) homo-tetramerizes, forming a functional Ca^{2+} -release channel, the structural requirements for the tetramerization were studied using site-directed mutagenesis. Alanine-substitutions at five charged residues, E4976, H5003, D5026, E5033 and D5034, significantly decreased the formation of homo-dimers (reduced by > 50%). Interaction between the C-terminus and cytoplasmic loop I (4,821-4,835) required two positively charged residues, H4832 and K4835. Based on the predicted protein secondary structures, all seven charged residues are located in random coils. Paired alanine-substitutions at six negatively charged residues (E4942A/D4953A, D4945A/E4952A and E4948A/E4955A) of the α -helix (4,940-4,956) in the C-terminus increased homo-dimerization. Therefore, the homo-tetramerization of RyR1 may be mediated by intra- and/or inter-monomer electrostatic interactions among the C-terminal charged residues in random coils or in an α -helix.

Keywords: mutagenesis, site-directed; ryanodine receptor calcium release channel; structure-activity relationship

Introduction

The ryanodine receptors (Ca^{2+} -release channels,

RyRs) in the sarcoplasmic reticulum (SR) are homo-tetrameric proteins composed of four individual monomers (Sandow, 1965). Three RyR isoforms arising from three separate genes have been identified: RyR1 (skeletal isoform), RyR2 (cardiac isoform) and RyR3 (brain isoform). RyR1 and RyR2 are essential proteins for excitation-contraction coupling in striated muscles, such as skeletal and cardiac muscle, functioning as Ca^{2+} -release channels in the SR. RyR3 is a ubiquitous protein with much lower expression levels relative to those of RyR1 and RyR2; the functional role of RyR3 in the cells in which it is expressed remains undefined (Ogawa *et al.*, 2000).

In rabbits, the three RyR isoforms show 67% (RyR1-RyR2), 67% (RyR1-RyR3) and 70% (RyR2-RyR3) amino acid sequence identity (Hakamata *et al.*, 1992), and there is a similar degree of identity among other mammalian isoforms (Sandow, 1965; Zorzato *et al.*, 1990; Williams *et al.*, 2001; Samso *et al.*, 2005). RyR is assumed to be composed of a large cytoplasmic foot assembly (N-terminal 3/4~4/5), a smaller transmembrane (TM) assembly (C-terminal 1/4~1/5) and a short cytoplasmic C-terminus domain (100 amino acids). The amino acid sequences of the C-terminus of the three rabbit RyR isoforms show extremely high homology ($\geq 94\%$).

The C-terminus of RyR2 appears to be important for the homo-tetramerization of RyR2 (Stewart *et al.*, 2003), and the deletion of the last 15 amino acids from a full-length RyR1 cDNA produces a non-functional protein (Gao *et al.*, 1997). In support of the hypothesis that the C-terminus is important in the homo-tetramerization of RyR1 and RyR2, the critical domains for homo-tetramerization of IP_3 receptors (IP_3Rs) – sister ER Ca^{2+} -release channels to RyRs – are located both in the C-terminus and in the fifth and sixth TM domains (Galvan *et al.*, 1999). In addition, self-interaction is observed in the cytoplasmic region preceding the first TM domain by yeast two-hybrid studies (Magnino *et al.*, 2001). In the case of *shaker*- and *shaw*-type K^+ channels, homo-tetramerization is mediated by Zn^{2+} ions coordinated with cysteine and histidine residues of the T1 domain localized in the hydrophilic N-terminal region (Bixby *et al.*, 1999; Jahng *et al.*, 2002). Therefore, based on observation of these very different mechanisms, it is possible that each homo-tetrameric channel family evolved a

unique way to assemble functional oligomeric channels and these differences have been preserved.

In the current study, the mechanisms governing homo-tetramerization of RyR1 by its C-terminus were examined using site-directed mutagenesis coupled with biochemical assays. The charged residues located in random coils or in the α -helix of C-terminal RyR1 were critical to homo-dimerization of the C-terminus, suggesting that RyR1 homo-tetramerization via its C-terminus was possibly mediated by intra- and/or inter-electrostatic interactions among the charged residues located in the C-terminal random coils and the α -helix.

Materials and Methods

Materials

Isopropyl- α -D-thiogalacto-pyranoside (IPTG) and ZnSO_4 were obtained from Sigma-Aldrich (St. Louis, MO). Monoclonal anti-RyR1 antibody (34C) was provided by Drs. J. Airey and J. Sutko (Developmental Studies Hybridoma Bank, Iowa City, IA). Anti-His and anti-GST antibody were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Glutathione-Sepharose 4B (GST) and protein G-Sepharose 4 Fast Flow affinity bead were obtained from Amersham Biosciences (Piscataway, NJ).

Mutation and expression of the RyR1 C-terminal fragments

Using rabbit RyR1 cDNA, C-terminal portions depicted in Figure 4 were sub-cloned into a GST vector (pGEX, Amersham Biosciences, Piscataway, NJ) and/or a His-tag vector, (pRSET A, Invitrogen Corporation, Carlsbad, CA) between *Bam*HI and *Xho*I sites. Mutations of GST- or His-tagged constructs were carried out using site-directed mutagenesis system (QuickChange site-directed mutagenesis kit, Stratagene, La Jolla, CA) with desired mis-matched oligonucleotides sets. Production of GST- or His-tagged proteins were induced in log phase *E. coli* by the addition of 0.1 mM IPTG for 8 h at 37°C, and each bacterial cell lysate was prepared by sonication in PBS (2.71 mM Na_2HPO_4 , 1.54 mM KH_2PO_4 , 155 mM NaCl and pH 7.2) with protease inhibitors (1 μM pepstatin, 1 μM leupeptin, 100 μM PMSF and 1 μM trypsin inhibitor). After centrifugation of the bacterial cell lysate at 7,700 *g* for 1 min, protein concentration of the supernatant was determined by Bradford method and used for further experiments.

Co-immunoprecipitation and GST pull-down assays

For the co-immunoprecipitation assay, heavy SR vesicles were prepared from rabbit fast-twitch muscle (back and leg), using a procedure described elsewhere (Lee *et al.*, 2004), and were solubilized in a buffer solution (1% Triton X-100, 10 mM Tris, 150 mM NaCl, 5 mM EDTA, 1 mM Na_3VO_4 , 10% glycerol, protease inhibitors, pH 7.4). The solubilized SR (500 μg total protein) were incubated with bacterial lysate containing wild type His-tag C-terminus (*wtHis-A*) or GST-tag cytoplasmic loop I (*wtGST-C*) for 4 h at 4°C, and incubated with 30 μl of anti-RyR1 antibody overnight at 4°C, followed by further incubation with protein G-Sepharose 4 Fast Flow affinity beads (Amersham Biosciences) for 4 h at 4°C. For the GST pull-down assay, bacterial lysate, expressing one of the GST-tag proteins, was incubated with bacterial lysate, expressing one of the His-tag proteins, for 4 h at 4°C, and incubated with GST beads (Amersham Biosciences) for 4 h at 4°C. For both assays, the bead-complexes were then washed three times with PBS to remove non-specifically bound proteins, then loaded on 8 or 15% polyacrylamide gels and analyzed by immunoblot analysis.

Native and modified-native PAGE

A Laemmli gel system (Laemmli, 1970) without SDS was used for native PAGE. For modified-native PAGE, the amount of reducing reagent was decreased by 60% or 80% compared with a standard SDS-PAGE (0.04% SDS in polyacrylamide gel and tris-glycine running buffer, 0.2% SDS and 2% 2-mercaptoethanol in sample buffer).

Prediction of secondary protein structure

Secondary structures were predicted using a computer software program, PSIPred (v2.5), from Brunel University (McGuffin *et al.*, 2000). The confidence of each residue for the predicted secondary structure was scored using confidence-values ranging from 0 (low) to 9 (high).

Statistical analysis

Results are given as means $\pm$ S.E. of at least three independent experiments. Significant differences were analyzed using the paired *t*-test (GraphPad InStat, v2.04). Differences were considered to be significant at $P < 0.05$.

C-terminal cysteine and histidine (indicated by asterisks in Figure 1A) were substituted with alanines. The mutants were expressed in bacteria and then subjected to modified-native PAGE, followed by immunoblot analysis (Figure 1D). To eliminate the formation of His-tag oligomeric complexes, expression of His-tag only was used as a negative control (Vector in Figure 1D and E). Among the eight cysteine or histidine mutants, H5003A nearly abolished the ability of the C-terminus to homo-dimerize. The other seven mutants showed a degree of homo-dimerization similar to that of *wtHis-C-terminus* (Wild in Figure 1D and E). In addition, the presence or absence of Zn^{2+} ions (100 μM ZnSO_4) did not affect the degree of homo-dimerization of either *wtHis-C-terminus* or any of the other mutants (Figure 1E), suggesting that, unlike K^+ channels, the homo-tetramerization of RyR1 C-terminus is mediated by factors other than inter-coordination by Zn^{2+} ions at cysteine and histidine residues.

Because histidine can be positively charged at neutral pH, H5003 may have participated in homo-dimerization of the C-terminus through electrostatic interactions between or among C-terminal negatively charged residues. To test this hypothesis, negatively charged residues in the C-terminus (9 aspartic acids (D) and 12 glutamic acids (E)) indicated by short bars in Figure 1A) were sub-

stituted with alanines and the ability of the mutants to homo-dimerize was examined (Figure 2A). As before, the His-tag (Vector in Figure 2A) was expressed as a negative control. Ratios of the monomer- to dimer-band-intensities were normalized to that of *wtHis-C-terminus* (Wild) and were presented as bar graphs (Figure 2B). Similar to H5003A, the E4976A, D5026A, E5033A and D5034A mutants showed more than a 50% reduction in homo-dimerization ability compared with *wtHis-C-terminus* (E4976A by $56.2 \pm 9.9\%$, H5003A by $94.2 \pm 1.6\%$, D5026A by $54.7 \pm 7.4\%$, E5033A by $95.6 \pm 1.4\%$ and D5034A by $65.3 \pm 7.8\%$). Therefore it is likely that, along with H5003, these four negatively charged residues in the C-terminus of RyR1 play important roles in RyR1 homo-tetramerization via intra- and/or inter-monomer electrostatic interactions. In support of this, Del-15, which lacks the last 15 amino acids of the C-terminus, including D5026, E5033 and D5034, also showed a $87.3 \pm 3.9\%$ reduction in homo-dimerization ability compared with *wtHis-C-terminus*. This finding suggests that the loss of function previously reported in truncated RyR1s (deletion of the last 15 amino acids) (Gao *et al.*, 1997) may have been caused by the inability of the truncated monomers to properly assemble and homo-tetramerize. According to the sequence alignment of RyR C-termini from different species

Figure 2. Homo-dimerization of a deletion mutant and single mutants of the negatively charged C-terminal residues. (A) Modified-native PAGE and immunoblot analysis of a deletion mutant (Del-15, deletion of the final 15 residues of the C-terminus) and single mutants of the 21 negatively charged aspartic or glutamic acids. (B) Normalized ratios between monomer- and dimer-band-intensities by that of *wtHis-C-terminus* in (A) is presented as bar graphs. Mutants showing a reduction in homo-dimerization ability of $> 50\%$ are indicated by white asterisks. Wild is *wtHis-C-terminus*, as a positive control. Vector is His-tag only, as a negative control.

(Supplemental data 3) and all three rabbit RyR isoforms (Figure 1A), negatively charged residues (D or E) at 5026, 5033 and 5034 are well-conserved in the RyR C-termini. In contrast, IP₃R C-termini vary in size and amino acid sequence. Therefore, despite similarities between the two sister Ca²⁺-release channels, the homo-dimerization and/or homo-tetramerization mechanisms of RyR1 are likely to be unique in at least some respects.

The role of Helix I on the homo-dimerization of the C-terminus

We used PSIPred from Brunel University to predict the secondary structure of the C-terminus. Using this program, two α -helices (Helix I and II) and a short β -strand were predicted for the wtC-terminus, with high confidence values (Figure 3A). According to the prediction, all five charged residues (E4976, D5003, D5026, E5033 and D5034) that caused a significant decrease in homo-dimerization ability

when mutated to alanine (Figure 2) are located in random coils. This result suggests that the intra- and/or inter-monomer electrostatic interactions at these residues have a degree of flexibility. The opening of RyR1s is associated with a 4-degree rotation of the TM assembly relative to the cytoplasmic assembly compared with the closed state (Orlova *et al.*, 1996). As a result, the C-terminus just beyond the TM assembly of RyR1 may undergo re-location or conformational changes due to channel opening and closing. Therefore, because the charged residues are located in flexible random coils, they may either tolerate or facilitate the switching between channel opening and closing.

The effect of mutating all five charged residues was examined using the secondary structure prediction program (Supplemental data 2A) and the confidence-values changes caused by these mutations were checked. Interestingly, mutating all 5 residues induced confidence-value changes both in the 14 amino acids (4,949-4,962) that comprised

Figure 3. Prediction of the secondary structure of the C-terminus and double mutations at Helix I (A) According to the secondary structure prediction program described in Materials and methods, there were two predicted α -helices (Helix I and II, indicated by cylinders) and a short β -strand (indicated by a blunt arrow) in the C-terminus of RyR1. The dotted vertical line indicates the C-terminal end of Del-15. (B) N-terminal top view of Helix I. All six negatively charged residues of Helix I were located in one half of Helix I. (C) The homo-dimerization ability of double mutants at the negatively charged residues of the Helix I. Ratios between monomer- and dimer-band-intensities were normalized to that of the wtHis-C-terminus and were presented as bar graphs. Wild is wtHis-C-terminus, as a positive control. Vector is His-tag only, as a negative control.

half of Helix I and in the mutated sites of the C-terminus, suggesting that, in addition to random coil interactions, Helix I also was involved in C-terminal homo-dimerization of RyRs.

The top view of right-handed Helix I containing six negatively charged residues is depicted in Figure 3B. Interestingly, all six residues (E4942, D4945, E4948, E4952, D4953 and E4955) face one half of Helix I and are paired in their directions (E4942/D4953 in the right, D4945/E4952 in the middle and E4948/E4955 in the left). When they mutated with a single alanine-substitution, their

homo-dimerization ability was only marginally reduced (Figure 2). To determine if double alanine-substitutions of the paired negatively charged residues had a greater effect, two negatively charged residues facing the same direction were substituted with alanines (Figure 3C). Unexpectedly, all three double-mutants showed an increase in homo-dimerization ability (> 2 -fold), rather than decreased ability, compared with *wtHis*-C-terminus. According to secondary structure prediction (Supplemental data 2B), all three double-mutants showed a decrease in the confidence-value of

Figure 4. Interaction of the C-terminus with adjacent regions. (A) The pore-loop of RyR1 with the C-terminus was depicted based on a model proposed by Chen *et al.* (2002). The predicted last, second-last and third-last TM segments are represented as TM_n, TM_{n-1} and TM_{n-2}, respectively. A, C-terminus; B, putative pore-loop; C, cytosolic loop I; D, cytoplasmic loop I to C-terminus; E, putative pore-loop to C-terminus; F, cytoplasmic loop I to putative pore-loop. (B) Interaction between *wtHis*-A (*wtHis*-C-terminus, the same as Wild in Figures 1, 2 and 3) and each of the GST-tag proteins (GST-A, -B, -C, -D, -E and -F) was examined using a GST-pull down assay. Each band intensity ratio was normalized to that of *wtGST*-A, *wtGST*-C or *wtHis*-A and was presented as bar graphs in the right side of (B), (C) and (D), respectively. (C) Interactions between *wtHis*-A and single mutants at four positively charged amino acids in cytosolic loop I (GST-C mutants) were examined as described in (B). (D) GST-pull down assay of *wtGST*-C with each C-terminal mutant (His-A mutants) that showed a reduction in dimerization ability of $> 50\%$ (Figure 2). GST or His is GST-tag or His-tag only, as a negative control. *Significant difference compared with *wtGST*-C or *wtHis*-A ($P < 0.05$). (E) Interaction between *wtHis*-A or *wtGST*-C and full-length RyR1 from solubilized rabbit skeletal SR was examined by co-immunoprecipitation with anti-RyR1 antibody and then by immunoblot analysis with anti-His or anti-GST antibody. Input (without RyR1 antibody) and GST (GST-tag only) were used as negative controls.

Helix I, but an increase in the confidence-value of a random coil sequence (4,984-NLANYMFFFLMYLIN-4,997). This result indicates that the random coil sequence was stabilized by the double mutations in Helix I and, by so doing, enhanced the homo-dimerization ability of the C-terminus. This hypothesis agrees well with the fact that Del-15, which had a reduction in homo-dimerization ability > 87%, had a decrease in confidence-value of the same random coil region (Supplemental data 2A). Therefore, in addition to the five charged residues in Figure 2, Helix I also must play an important role in the homo-tetramerization of RyR1 via homo-dimerization of the C-terminus.

Interaction between the C-terminus and cytosolic loop I

The C-terminus is well equipped to interact with neighboring regions of RyR1 due to its location at the free end of the RyR1 and its predicted domi-

nant flexible random coils. To test this possibility, proteins longer or shorter than the C-terminus were cloned as N-terminal GST-tag proteins (Figure 4A): GST-A, C-terminus (D4,938-S5,037, the same as His-C-terminus except for GST-tag); GST-B, putative pore-loop (R4,860-D4,917, a SR luminal portion between TM_{n-1} and TM_n); GST-C, cytosolic loop I (K4,821-K4,835, a short cytoplasmic loop between TM_{n-2} and TM_{n-1}); GST-D, cytoplasmic loop I to C-terminus (K4,821-S5,037); GST-E, putative pore-loop to C-terminus (R4,860-S5,037); GST-F, cytoplasmic loop I to putative pore-loop (K4,821-D4,917).

Interaction between *w*tHis-A (*w*tHis-C-terminus, the same as Wild in Figures 1, 2 and 3) and one of the individual GST-tag proteins was examined using the GST pull-down assay (Figure 4B). GST-A, GST-C, GST-E and GST-F showed strong interactions with *w*tHis-A. Interaction of His or the GST tag itself with the control proteins was not detected (His and GST in Figure 4B, C, and D). As

Figure 4. Continued.

expected from previous results, there were robust interactions between His-A and GST-A or between His-A and GST-E, and that most likely was due to interactions between the 'A' portions (C-termini) of the proteins. Interestingly, a robust interaction between His-A and GST-C or between His-A and GST-F also was observed. Based on the makeup of these two constructs, this interaction may best be explained by an interaction between the 'A' portion of His-A and the 'C' portion (cytosolic loop I) of GST-C and GST-F because the GST-C and GST-F share this structural feature. Cytoplasmic loop I is part of Region 3 (3,916-4,973), one of the mutational hot-spots associated with malignant hyperthermia and central core disease (Lyfenko *et al.*, 2004). Mutations at Region 3 are pathological because they presumably alter the assembly of the pore-forming region and consequently change the selectivity or gating properties of RyR1. The shift of GST-D, which contained both 'A' and 'C' portions, was likely caused by self-folding within a single GST-D molecule. This self-folding would prevent interaction between His-A and GST-D, as was observed in the current experiment. In terms of full-length RyRs, either self (intra)-folding between 'A' and 'C' portions in a single RyR1 or inter-folding between 'A' and 'C' portions from two RyR1s could be possible. There was no significant interaction between His-A and GST-B.

Cytosolic loop I contains four charged residues and all of them are positively charged. To identify residues in cytoplasmic loop I that are critical for interaction with the C-terminus (*wtHis-A*), each of the four positively charged residues was substituted with alanine and then subjected to the GST pull-down assay with *wtHis-A* (Figure 4C). Two of the mutants, H4832A and K4835A, showed a significantly decreased ability to interact with *wtHis-A*. Conversely, *wt* cytoplasmic loop I (*wtGST-C*) was subjected to GST-pull down assays with each of the five His-A mutants that showed more than a 50% reduction in homo-dimerization ability, as shown in Figure 2 (Figure 4D). E5033A and D5034A had a significant reduction in their ability to interact with *wtGST-C*. Therefore, it appears that positively charged H4832 and K4835 could be electrostatic partners with negatively charged E5033 and D5034, forming salt bridges, in the interaction between cytoplasmic loop I and the C-terminus.

According to the secondary structure prediction for cytoplasmic loop I (Supplemental data 4), E5033 and D5034 also were located in a random coil, as were the five critical charged residues shown in Figure 3A. Because the proposed mechanism involves all seven charged residues in

'flexible random coils' and because of the effect of mutation in Helix I on the 'flexible random coil', the homo-tetramerization of RyR1 via its C-terminal electrostatic interactions among RyR1 monomers is probably governed by an 'induced-fit model'. In this model, C-terminus has complete structural arrangements when they are gathered and stand in a knot (a tetrameric RyR1). In this context, if the mechanism for RyR1 homo-tetramerization is accomplished by dimerization of two dimers, creation of the tetramer requires more than homo-dimerization of two monomers, probably due to volume effects and steric hindrances. Therefore, the involvement of other RyR1 regions, such as N-terminal foot regions, cannot be completely excluded as playing a role in the homo-tetramerization of RyR1.

Because the homo-tetramerization mechanism of RyR1 was examined using only its C-terminus and cytoplasmic loop I fragments, it is possible that the structures of these fragments are different than the corresponding regions of full-length RyR1 and may negate the findings of the present study. To rule out this possibility, co-immunoprecipitation experiments of full-length RyR1 were performed with both fragments – confirming the interaction of both the C-terminus (*wtHis-A*) and cytoplasmic loop I (*wtGST-C*) with full-length RyR1 (Figure 4E), thus supporting the original hypothesis and more possibility for inter-folding by 'A' and 'C' portions from two full-length RyR1s instead of self (intra)-folding by 'A' and 'C' portions in a single full-length RyR1.

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