Journal of the Korean Magnetic Resonance Society **2015**, *19*, 74-82
DOI 10.6564/JKMRS.2015.19.2.074

Structural characterization of calmodulin like domain of ryanodine receptor type 1

Yonghyun Song[1†], Sunmi Kang[2†], and Sunghyouk Park[2*]

[1] Department of Biochemistry, Inha University Hospital, College of Medicine, Inha University, Incheon, Korea
[2] College of Pharmacy, Natural Product Research Institute, Seoul National University, San 56-1 Sillim-dong, Gwanak-gu, 151-742 Seoul, Korea

Received Aug 4, 2015; Revised Sep 17, 2015; Accepted Sep 25, 2015

Abstract Ryanodine receptor (RyR) is one of the two major Ca^{2+} channels in membranes of intracellular Ca^{2+} stores and is found in sarcoplasmic reticulum (SR), endoplasmic reticulum (ER). RyR1 is also the major calmodulin-binding protein of sarcoplasmic reticulum membranes.

Residues 4064-4210 in the RyR1 polypeptide chain has similar primary sequence with calmodulin (CaM) and was designated as CaM-like domain (CaMLD). When expressed as a recombinant peptide, CaMLD showed several CaM-like properties in previous studies. Still, previous studies of CaMLD were focused on protein-protein interactions rather than its own properties.

Here, we studied the expression of CaMLD and its sub-domains corresponding to each lobe of CaM in Escherichia coli. CaMLD could be obtained only as inclusion body, and it was refolded using urea solubilization followed by dialysis. Using spectroscopic approaches, such as NMR, circular dichroism, and gel filtration experiment, we found that the refolded CaMLD exists as nonspecific aggregate, even though it has alpha helical secondary structure. In comparison, the first half of CaMLD (R4061-4141) could be obtained as natively soluble protein with thioredoxin fusion. After the removal of the fusion tag, it exhibited folded and helical properties as shown by NMR and circular dichroism experiments. Its oligomeric status was different from CaMLD, existing as dimeric form in solution. However, the second half of the protein could not be obtained as soluble protein regardless of fusion tag. Based on these results, we believe that CaMLD, although similar to CaM in sequence, has quite different physicochemical properties and that the second half of the protein renders it the aggregative properties.

Keywords RyRs, Calmodulin-like domain, secondary structure

Introduction

The ryanodine receptor (RyR) is one of the two major Ca^{2+} channels in membranes of intracellular Ca^{2+} stores. It is found in the sarcoplasmic reticulum, and the endoplasmic reticulum [1]. RyR channels represent the primary pathway for Ca^{2+} release during the excitation-contraction coupling process. The activation of RyR channels leads to massive Ca^{2+} release from the sarcoplasmic reticulum, which in turn initiates contraction [2]. RyR is a single polypeptide of 560 kDa that normally exists in a

† These authors contributed equally to this work.
* Address correspondence to: **Sunghyouk Park,** College of Pharmacy, Natural Product Research Institute, Seoul National University, San 56-1 Sillim-dong, Gwanak-gu, 151-742 Seoul, Korea, Tel: 82-2-880-7831; E-mail: psh@snu.ac.kr

homotetrameric structure with two functional domains [3]. There are three isoforms: RyR1, RyR2, and RyR3 [4]. hRYR2 is a gene responsible for catecholaminergic polymorphic ventricular tachycardia [5]. RYR1 mutations have been reported in association with malignant hyperthermia and central core disease [6]. The activities of all three RyR isoforms are modulated by a large number of agents, including FKBP12, junctin, triadin, and calsequestrin [7]. RyRs also bind to calmodulin (CaM), which is itself a Ca^{2+} binding protein. RyR1 is the major CaM-binding protein of sarcoplasmic reticulum membranes [8]. At micro molar Ca^{2+} concentrations, CaM inhibits both RyR1 and RyR2. However, at lower concentrations, CaM activates RyR1 but inhibits RyR2 [9]. RyR3 also binds to CaM, and the effects of CaM on the channel are redox-sensitive [10]. The CaM-binding site on RyR1 involves amino acids (aa) 3614-3643 [11]. Calcium-free CaM and calcium-bound CaM bind with nano molar affinity to a synthetic peptide matching 3614-3643 of the ryanodine receptor [12]. The region encompassing residues 3583-3603 of RyR2 is highly homologous to the residues 3614-3643 of RyR1 [13]. Ca^{2+}-free CaM potentiates RYR1 opening, while Ca^{2+} CaM inhibits the channel [14]. A sequence which was identified within RyR1 (amino acids 4064-4210)and one within the carboxyl-terminal tail of the Cav1.1 a1 subunit (Cav1.1 1395-1540) have been predicted to fold like CaM and bind to Ca^{2+}. One possibility is that these CaM-like domains can interact with the CaM binding sites of these proteins, and that CaM will compete for this interaction. Intramolecular interaction between amino acids 4064-4210 and amino acids 3614-3643 in the native RyR1 can alter or regulate this interaction. R4064-4210 seems to have helical structure as shown by CD spectroscopy. Unlike CaM, Ca^{2+} produces only small changes in the a-helical content of R4064-4210, suggesting that the structural changes in this fragment upon binding to Ca^{2+} are probably not as extensive as those in CaM. R4064-4210 binds to CaM binding motifs (3614-3643). R4064-4210 also binds to the Cav1.1, as shown by pull down analysis [15]. The interaction of CaMBP-BADAN suggested a peptide region, R3534-4271, involved in CaM-RyR interaction [16]. Later experiments also showed that there might be an intramolecular interaction between the CaM-binding motif (R3614-3643) and R4114-4142 region (a region included in the CaM-like domain) that may serve as an intrinsic regulator of the RyR1 [17]. Still, these studies on CaM-like domain were rather focused on protein-protein interactions, and, therefore, its structural properties are not much known. Here we report the expression of R4061-4210 and its sub-domains in *Escherichia coli* and characterization of their structural features. We suggest that R4061-4210 is not similar to CaM in terms of oligomeric status and sub-domain characters.

Experimental Methods

Cloning and expression of CaMLD- Cloning was performed using a polymerase chain reaction (PCR)-based approach. For PCR amplification we used Taq DNA polymerase. For R4061-4210, the sense primer was GACAAGCATATGTTCTTCGACATGTTCCTGA AACTCAAGGAC. And the antisense primer was C CTCGAGGACCTGGGGCATCTCCCAC. The PCR product was purified, double digested with *Nde I* and *Xho I* enzymes and then ligated into pET30b vector. All the constructs were transformed into Rosetta (DE3) competent cells and the colonies were selected by colony PCR and expression tests. The correctness of clones was confirmed by DNA sequencing (Macrogen, Seoul, Korea).

Refolding and Purification of CaMLD- pET30b-R4061-4210 was expressed as insoluble inclusion body. To purify this peptide from inclusion bodies, R4061-4210 was extracted with 20 mM Tris-HCl pH 7.4 elution buffer containing 8 M urea. The solubilized R4061-4210 was dialyzed against 20 mM Tris-HCl pH 7.4 in a 7,000 Da Snakeskin™ Tubing (Pierce). The dialysis buffer was changed four times to remove the urea completely. Refolded

proteins were further purified by gel-filtration with Superdex^{TM} 75 HiLoad 16/60 (GE Healthcare) with a buffer containing 20 mM Tris-HCl (pH 7.4)

Circular dichroism spectroscopy- Circular dichroism (CD) spectroscopy was used to analyze the secondary structure of CaMLD. CD spectra were recorded in 20 mM Tris-HCl (pH 7.4) on a Jasco J-815 machine. The spectra were obtained with a 1 mm path cell, 1 nm bandwidth and 100 nm/min scan speed and 190 nm to 350 nm wavelength range. The spectral data were presented as mean residue ellipticity.

NMR spectroscopy- The NMR experiments were performed with ^{1}H, ^{1}H -^{15}N HSQC, and ^{1}H -^{15}N HSQC approaches on a Bruker 900 MHz NMR spectrometer equipped with cryogenic probes (Korea Basic Science Institute, Ochang, Korea). All experiments were performed at 25 °C in NMR sample buffer (20 mM Tris-HCl, pH 7.4). The final NMR sample had 5% D_2O for purpose of locking. All NMR data were processed and analyzed with nmrView software.

Cloning and expression of R4061-4141, R4141-4210- To study the partial structural features of CaMLD, we separately expressed the EF-Hand 1,2 (R4061-4141) and EF-Hand 3,4 (R4141-4210) parts. Cloning and expression were done in the same way as those described above for R4061-4210. For PCR amplification, we used Taq DNA polymerase. For R4061-4141, the sense primer was GACAAGTCA TGAGATTCTTCGACATGTTCCTGAAACTCAA GGAC and the antisense primer was TCGGTACCTCAGACCTGGGGCATCTCCCAC. The PCR product was purified, double digested with *Pag I* and *Kpn I* enzymes and then ligated into TRX vector. For R4141-4210, the sense primer was GACA AGGGATCCTTCAACGTGGCCGTGCTG and the antisense primer was CTCGAGGACCTGGGGCATCTCCCAC. The PCR product was purified, double digested with *BamH I* and *Xho I* enzymes and then ligated into GB1 vector. For the cloning of R4141-4210 in TRX vector, the sense primer was AGCCATGGCATTCAAC GTGGCCGTGCTG and the antisense primer was TCGGTACCTCAGACCTG GGGCATCTCCCAC. The PCR product was purified, double digested with *Nco I* and *Kpn I* enzymes and then ligated into TRX vector.
The all constructs were transformed into Rosetta (DE3) competent cells and the colonies were selected by colony PCR and expression. The correctness of clones was confirmed by DNA sequencing (Macrogen, Seoul, Korea).

Solubility test and Purification of constructs- The cells were grown in 10 ml LB media with 50 µg/ml of antibiotic at 37℃ until their OD_{600} absorbance reached 0.4. These were then separated into 5 ml samples, and incubated for another 10 hours at 37℃ and at 15℃ after adding 1 mM IPTG. The cells were centrifuged at 2600 x g for 10 minutes. The obtained pellet was dissolved in 600 µl of His-lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, pH 8.0), sonicated for 10 seconds on ice to burst the cell membrane and release the cellular contents, and finally centrifuged at 16100 x g for 10 minutes. The supernatant solution was removed into another centrifuge tube, and the pellet was re-dissolved in 600 µl of His-lysis buffer. Next, 120 µl of 6X SDS sample buffer was added and the sample was boiled for 5 minutes at 100℃, and analyzed with 18% SDS-PAGE.

Results

Expression and purification of R4061-4210- To get purified CaMLD of RyR1, we made constructs of R4061-4210, and it was expressed in Rosseta (DE3) cells. The R4061-4210 was found almost exclusively in inclusion bodies, which is consistent with a previous report [15]. However, R4061-4210 was poorly soluble in 2 M Urea (Figure 1A) that they used. Therefore, we used higher concentration of urea (8 M), and were able to obtain much more protein from the same amount of inclusion body. The 8 M urea-solubilized R4061-4210 was refolded by

dialysis against a buffer without urea, and the resulting sample was soluble at concentrations as high as 5.5 mg/ml. This dialysis step was very efficient and we obtained almost 100% recovery (Figure 1B). To get purified R4061-4210 without refolding procedure, we tried another vector(His-TRX) (Figure 1C), but it was still found in the inclusion body. Therefore, a refolding procedure was necessary for purification of R 4061-4210.

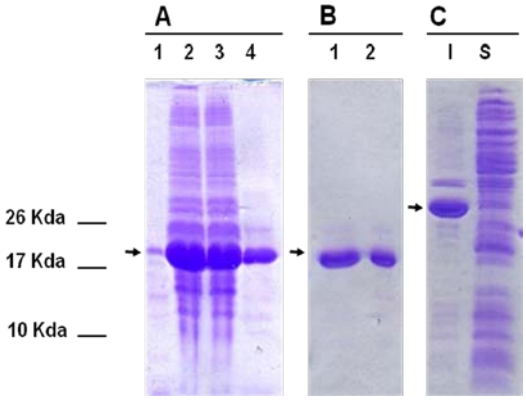


Figure 1. Refolding and expression of R4061-4210. (A) The pET30b-R4061-4210 fusion construct was extracted with Urea. Lane 1: R4061-4210 extracted with 2 M Urea. Lane 2, 3 and 4: R4061-4210 extracted with 8 M Urea. Lane 2 : pellet. Lane 3 : flow-through. Lane 4 : elution (B). Comparison of the protein amount before and after the refolding by dialysis. Lane 1: Extraction with 8 M Urea. Lane 2: After dialysis. (C) Solubility test of TRX-R4061-4210. I represent the insoluble fraction and S represents the soluble fraction

Structural characterization of R4061-4210- As R4061-4210 prepared with 8M urea was quite pure, we obtained the circular dichroic spectrum to check its folding status and secondary structural features. The spectrum showed two minima at 208 and 222 nm (Figure 2A), characteristic of a folded helical protein, consistent with a previous report [15]. This result shows that the preparation with higher concentration of urea (8 M) does not affect the folding of the refolded protein. We then obtained its one-dimensional NMR spectrum to further characterize its structural features. The spectrum exhibited very broad peaks over the aliphatic and amide proton regions, characteristic of an aggregated protein (Figure 2B). Still, up field peaks (<0.5 ppm) that typically appear in a well-folded protein could not be observed. It might be that the portion of the folded region is not large enough, and those relatively small peaks are not visible due to the line broadening of the aggregated protein.

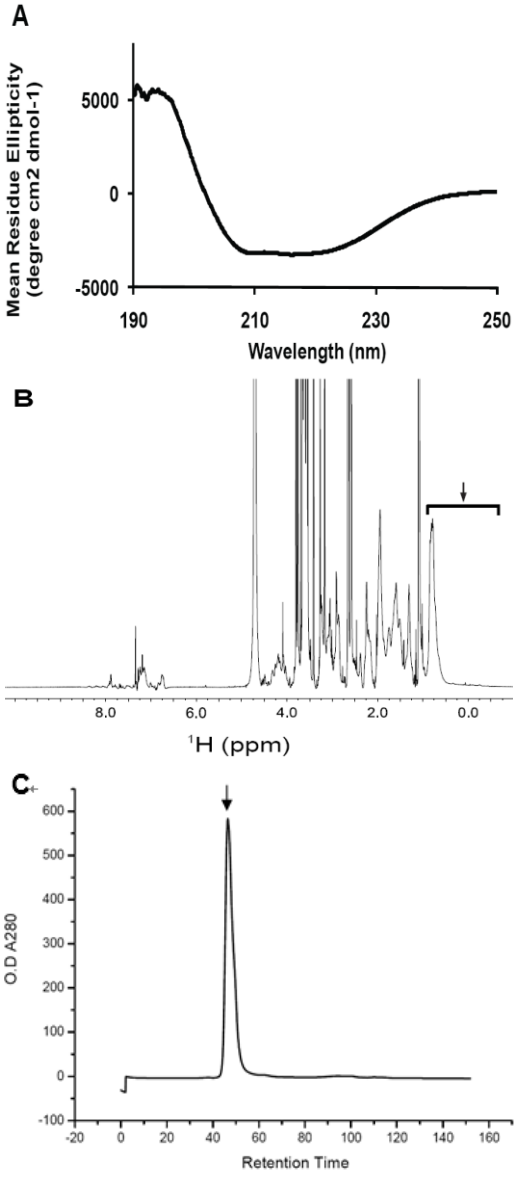


Figure 2. Structural characterization of R4061-4210. (A) Mean residue ellipticity of CD spectrum of R4061-4210. (B) ^{1}H NMR spectrum of R4061-4210. (C) Gel filtration chromatography of R4061-4210. The arrow indicates the target protein peak.

To see if this aggregation is due to the lack of salt in the sample buffer, we added 100 mM NaCl and measured the NMR spectrum again, but there were no significant changes. Further addition of salts led to precipitation of the protein. To confirm the aggregated nature of R4061-4210, we performed gel filtration chromatography. The result showed that the protein elutes at void volume, consistent with large aggregate nature of the protein (Figure 2C).

Characterization of the first half of CaMLD, R4061-4141- Although the whole CaMLD appeared as an aggregate, the sequence analysis suggested that the first half of CaMLD contains two functional EF-hands, while the other half has non-functional EF-hands. Therefore, we split the protein into two halves to see if each can be obtained as non-aggregated form. We constructed the genes for R4061-4141, the first half, and R4141-4210, the rest separately. When R4061-4141 was expressed as a TRX fusion protein and cut from the TRX moiety with TEV protease, it did not form non-specific aggregate, as evidenced by gel filtration chromatography (Figure 3A). Still, R4061-4141 eluted earlier than expected by the molecular weight. By comparison with standard proteins, the peak had apparent molecular weight of approximately 23 kDa (Figure 3B), suggesting that it is dimer with its monomer molecular weight of 9.6 kDa. As R4061-4141 did not form a large non-specific aggregate, which R4061-4210 did, we further characterized its folding and secondary structure with CD and NMR. Similar to R4061-4210, R4061-4141 showed characteristic pattern of a helical protein with two minima at 208 and 222 nm wavelengths (Figure 3C). Contrary to R4061-4210, the NMR spectrum of R4061-4141 showed up field peaks (< 0.5 ppm) characteristic of a well folded protein (Figure 3D). Therefore, we concluded that the first half of R4061-4210 is a natively soluble well-folded helical protein that forms a dimer with two functional EF-hands.

NMR analysis of R4061-4141- To analyze the structure of R4061-4141 in further detail, we carried out ^{1}H-^{15}N HSQC experiment.

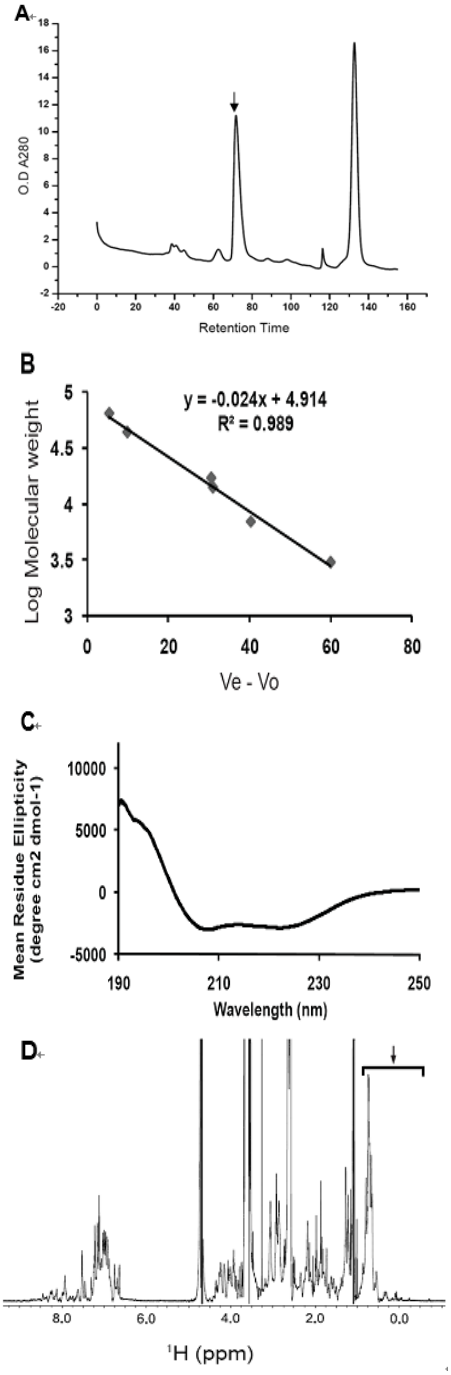


Figure 3. Structural characterization of R4061-4141. (A) Gel filtration chromatography of R4061-4141. The arrow indicates the target protein peak. (B) Regression analysis of gel filtration chromatography retention times of standard proteins (C) Mean residue ellipticity of CD spectrum of R4061-4141. (D) ^{1}H NMR spectrum of R4061-4141.

Unfortunately, the spectrum did not show the expected number of peaks, but showed only a small number of peaks (Figure 4A), which could be due to exchange phenomenon between monomer and dimer. To see if we can get better NMR spectra for structural analysis, we treated the sample salt, calcium in NMR sample, but we did not get noticeable improvement. R4061-4141 has one cysteine in its sequence. Therefore, we treated it with 5 mM DTT and compared it with Non-DTT treated sample with SDS-electrophoresis. The mobility of R4061-4141 was not different between 5 mM DTT treated and Non-treated samples in SDS-electrophoresis (Figure 4B). Therefore the broad peaks were not due to a disulfide bond. To see if attaching another tag could help reduce the dimerization or exchange phenomenon, we constructed GB1-fused R4061-4141, and obtained the NMR spectrum (Figures 4C and 4D). On top of the GB1 peaks, we observed peaks corresponding to the R4061-4141, but the numbers of peaks were still smaller than expected. Based on these results, we concluded that R4061-4141 is natively dimeric through non-covalent interaction, and that it undergoes dynamic exchange between monomeric and dimeric states.

Other constructs for the first half of EF-Hand in CaMLD- To see if the dimeric character of R4061-4141 is due to domain phasing, we built more constructs with different start and end amino acids, such as R4069-4141 and R4061-4137. Then we carried out size exclusion analysis. The results showed that R4069-4141 and R4061-4137 still existed as dimer, just as R4061-4141 (Figure 5).

Expression and characterization of the second half of EF-Hand in CaMLD- To characterize the properties of R4141-4210, we expressed it as recombinant protein. First, we tested the solubility at 37 ℃ and 15 ℃ with the same vector as R4061-4141, TRX. However, TRX fusion protein was expressed entirely as inclusion body (> 99%) (Figure 6A). Therefore, we tried other vectors such as MBP and GB1 fusion vectors (Figure 6A).

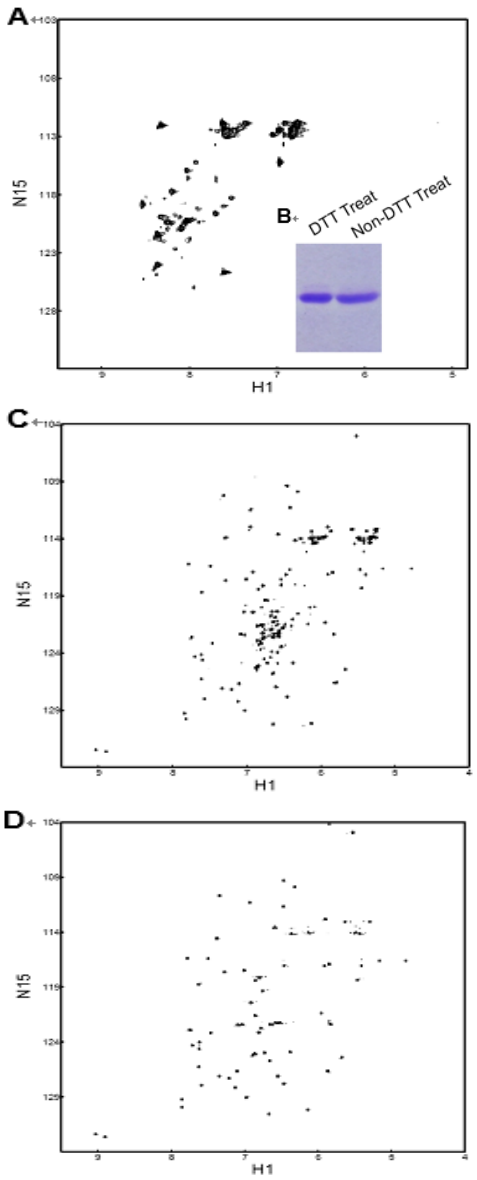


Figure 4. NMR spectra of R4061-4141. (A) HSQC NMR spectrum of R4061-4141. (B) Comparison between 5 mM DTT-treated and Non-DTT treated samples in SDS-electrophoresis, Left : 5mM DTT treated sample. Right : Non-DTT treated sample. (C) HSQC NMR spectrum of GB1-R4061-4141. (D) HSQC NMR spectrum of GB1 only.

MBP fused protein was expressed as soluble protein. However, it could not be cleaved using TEV protease, which is a necessary step to recover R4141-4210 (Figure 6B). The most likely reason is that the TEV cleavage site became inaccessible to the enzyme due

to the aggregative property of the R4141-4210. We, therefore, tried another vector, GB1 fusion vector, a solubility-enhancing vector. We were able to obtain small amount of soluble protein as GB1-fused form. However, the size exclusion result showed that GB1-R4141-4210 eluted in void volume (Figure 6C). Overall, we conclude that R4141-4210 is natively aggregated protein.

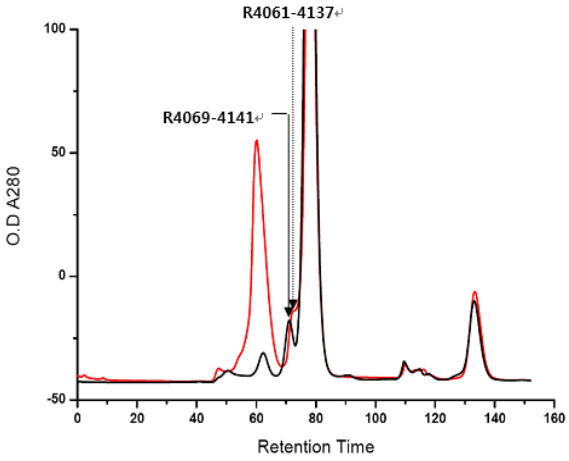
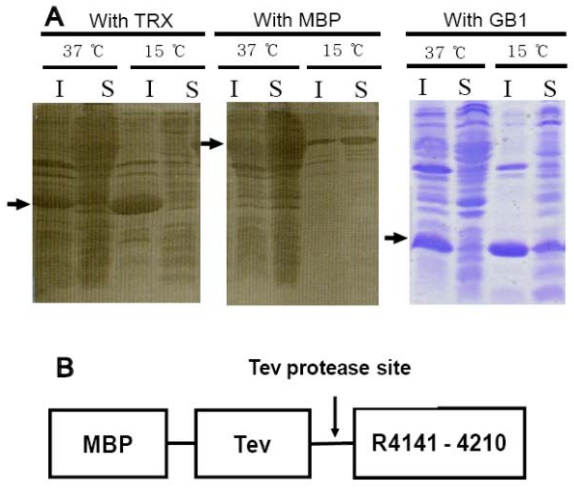


Figure 5. Size exclusion chromatograms of R4069-4141 and R4061-4137. Overlaid Gel filtration chromatograms of R4069-4141 and R4061-4137. Red line: Size exclusion of R4069-4141 (Arrow: Retention time of R4069-4141). Black line: chromatogram of R4061-4137 (Dotted Arrow: Retention time of R4061-4137).



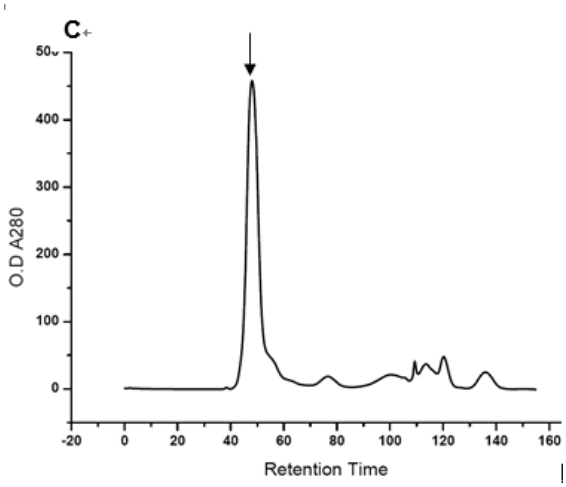


Figure 6. Structural characterization of R4141-4210. (A) To test the solubility, constructs were expressed at 37 ℃ and 15 ℃ with TRX, MBP and GB1 tag, respectively. I represent the insoluble fraction and S represents the soluble fraction (B) TEV protease mechanism diagram by MBP-R4141-4210. (C) Size exclusion of GB1-R4141-4210.

Discussion

RyRs are activated by the binding of Ca^{2+} at mM concentrations, but are inhibited by higher concentrations[18]. According to a previous report, Expressed fragment containing two putative E-F hands (R4064-4210) and a synthetic peptide (R3614-3643) that represents the CaM binding motif of RyR1 interfere with an interaction that regulates the response of RyR1 to Ca^{2+} [15].

Other laboratories have also suggested that CaM might be altering RyR1 activity by interfering with an intramolecular interaction or by inducing a conformational change in the CaM binding site [19]. Demonstrated that a peptide representing the CaM binding site (amino acids R3614-3643) could either activate or inhibit RyR1, depending on the dose and the Ca^{2+} concentration. They proposed that the CaM binding site was an important modulatory site within RyR1. Consistent with this, Rodney et al [20]. According to a previous report, CaMBP (CaM-binding peptide; a peptide corresponding to the

CaMBD) activates the RyR1 channel and This indicates that the CaMBD serves as an intrinsic regulator of the RyR Ca^{2+} channel. There is a segment (residues R4064-4210) in the RyR1 polypeptide chain that resembles the structure of CaM within the RyR1, which is designated as the CaMLD (CaM-like domain). According to recent work by Hamilton and co-workers [15, 19]. Additionally, Ikemoto and co-workers have shown that binding of CaMBP to RyR1 induces protein conformational changes in the RyR1 (or in the expressed peptide containing CaMLD) and induces Ca^{2+} release. On the basis of these pieces of information, they hypothesize that the two in vivo domains of RyR1 corresponding to these peptides, CaMBD and CaMLD, interact with each other, and the mode of interactions between these domains is involved in the tuning of the Ca^{2+} channel function[16]. These results suggest that the R3614-3643 and R4114-4142 regions of RyR1 interact with each other in a Ca^{2+} and agonist-dependent manner, and this serves as a mechanism of Ca^{2+} and agonist-dependent activation of the RyR1 Ca^{2+} channel [17]. To further characterize the CaM like domain of RyR1 structurally, we expressed it recombinant in *E. coli*. In the course of purification of the inclusion-body expressed protein, we improved the refolding procedure and obtained much higher yield. Circular dichroism spectroscopy and NMR data indicated that the refolded protein is helical protein. However, gel filtration chromatography experiment showed that the protein is in nonspecifically aggregated form. To investigate the properties of the individual sub-domains of R4061-4210, we separately expressed the first and second halves of the protein. The first half, R4061-4141, could be obtained in soluble form with TRX vector. It remained soluble after the removal of the TRX fusion tag. The circular dichroism and NMR experiment showed that it is a well folded helical protein, consistent with the presence of functional EF-hands. Gel filtration chromatography results showed that it exists as a dimer in solution. We labeled the protein with ^{15}N- and ^{13}C-isotope and obtained HSQC spectra. However, it exhibited broad peaks with much smaller number of peaks than expected, characteristic of a protein in dynamic equilibrium. The most likely possibility is that the protein in monomer-dimer equilibrium, but it needs to be confirmed. We also recombinant expressed R4141-4210, the second half of CaMLD. It could not be obtained in a soluble form with TRX vector. Although we could obtain a small amount of soluble protein with MBP fusion tag, it could not be cleaved with TEV protease. We were able to obtain relatively large amount of soluble protein with a solubility-enhancing GB1 tag, but it still existed as non-specific aggregate in solution, as determined by gel filtration chromatography. Overall, of the three constructs that we expresses, R4061-4210, R4061-4141 and R4141-4210, only the first half of the CaMLD, only R4061-4141 was expressed in soluble form as TRX fusion protein. In addition, the other two, either refolded or made soluble using GB1 tag, existed as non-specific aggregate. Therefore, we suggested that R4061-4210 does not have typical properties of calmodulin, even though it has been called as “calmodulin-like domain”. Rather, the functional part may be only the first half of the protein, 4061-4141, with two EF hands domain.

References

1. A. F. Dulhunty, and P. Pouliquin, *Clin. Exp. Pharmacol. Physiol*. **30**, 713 (2003)
2. M. Fill, and J. A. Copello, *Physiol. Rev*. **82**, 893 (2002)
3. M. B. Bhat, et al, *Biophys. J*. **73**, 1329 (1997)
4. S. Fleischer, *Biochem. Biophys. Res. Commun*. **369**, 195 (2008)
5. S. G. Priori, et al., *Circulation* **103**,196 (2001)

6. Robinson, R., et al., *Hum. Mutat.* **27**, 977(2006)
7. Hamilton, S. L. and Serysheva, *J. Biol. Chem.* **284**, 4047 (2009)
8. Tripathy, A., et al., *Biophys. J.* **69**, 106 (1995)
9. Meissner, G., et al., *Proteins* **74**, 207 (2009)
10. Yamaguchi, N., et al., *Biochemistry* **44**, 15074 (2005)
11. Moore, C. P., et al., *Biochemistry* **38**, 8532 (1999)
12. Rodney, G. G., et al., *J. Biol. Chem.* **276**, 2069 (2001)
13. Yamaguchi, N., et al., *J. Biol. Chem.* **278**, 23480 (2003)
14. Maximciuc, A. A., et al., *Structure* **14**, 1547 (2006)
15. Xiong, L., et al.,. *Biophys. J.* **90**, 173 (2006)
16. Gangopadhyay, J. P. and N. Ikemoto, *Biophys. J.* **90**, 2015 (2006)
17. Gangopadhyay, J. P. and N. Ikemoto, *Biochem. J.* **411**,415(2008)
18. Meissner, G., et al., *J. Biol. Chem.* **272**,1628(1997)
19. Zhu, X., et al., *Cell Calcium.* **35**,165 (2004)
20. Rodney, G. G., G. M. Wilson, and M. F. *J. Biol. Chem.* **280**, 11713 (2005)