Molecular recognition of the disordered dihydropyridine receptor II-III loop by a conserved SPRY domain of the type 1 ryanodine receptor

Han-Shen Tae, Nicole C. Norris, Yanfang Cui, Yamuna Karunasekara, Philip G. Board, Angela F. Dulhunty and Marco G. Casarotto

Division of Molecular Bioscience, John Curtin School of Medical Research, Australian National University, Canberra, ACT 2601, Australia.

Summary

1. The DHPR II-III loop is an intrinsically unstructured region made up of α-helical and β-turn secondary structure elements with the N & C termini in close spatial proximity.

2. The DHPR II-III loop interacts *in vitro* with a RyR1-SPRY domain through α-helical segments located in the A and B regions. Mutations within the A & B regions in the DHPR II-III loop alter the binding affinity to the SPRY2 domain.

3. The A & C peptides derived from DHPR II-III loop show negative cooperativity in binding to the SPRY2 domain.

4. The SPRY2 domain of RyR1 (1085-1208) forms a β-sheet sandwich structure flanked by variable loop regions. An acidic loop region of SPRY2 (1107-1121) forms part of a negatively charged cleft that is implicated in the binding of the DHPR II-III loop.

5. The mutant E1108A located in the negatively charged loop of SPRY2 reduces the binding affinity to the II-III loop.

Introduction

The cytoplasmic loop between the second and third transmembrane domains (II-III loop) of the skeletal α1-subunit of the dihydropyridine receptor (DHPRα_{1s}) has been identified as an important region for *in vivo* and *in vitro* interactions with the skeletal ryanodine receptor (RyR1).[1-4] *In vivo* studies of skeletal DHPR α_{1s}-null myotubes expressing various DHPR α_{1s} subunit constructs show that the sequence of residues 720-765 (C region) within the II-III loop is essential for skeletal EC coupling.[5] *In vitro*, there is a strong interaction between RyR1 and smaller peptide fragments belonging to the N-terminal A region (residues 671–690, defined by El-Hayek *et al.*, 1995[6]) and weaker interactions with the C region residues (720-765).[3,6-9] The A peptide and its structural/functional analogues, Imperatoxin A and Maurocalcine, have become useful tools in studies of RyR function.[10-13] Much attention has focused on the functional characterization of various II-III loop interacting regions, but few studies thus far have concentrated on structurally mapping microdomains in the RyR that interact with the DHPR *in vitro*. This is chiefly because both DHPR and RyR are large, multidomain membrane protein systems that are notoriously difficult to study *in vitro*. Our group has been responsible for identifying and characterising key structural elements of the DHPR II-III loop involved in the biophysical interaction with the RyR1, and have recently turned our attention to identifying the area of interaction on the RyR1. We have previously suggested that this interaction takes place through a SPRY domain.[14,15] SPRY domains are recognised as protein interacting modules and were so-named because they were identified in a *Dictyostelium discoidueum* splA kinase and in the mammalian RyR.[16] Their specific role in the RyR has not been previously assigned and our work represents one of the first reports to ascribe a functional role (albeit *in vitro*) to any of the three SPRY domains present in all of the RyR isoforms. The region of RyR1 that interacts with the DHPR II-III loop region has been previously reported to involve residues 1085-1112[17,18] and this region overlaps with the second of the three SPRY domains (SPRY2, residues 1085-1208). Our immediate aim is to map the interaction site of DHPR II-III loop and SPRY2 domain of RyR1, thereby allowing this information to ultimately be exploited in future *in vivo* studies. NMR is one of a multitude of biophysical techniques capable of shedding light on the direct molecular interaction between the DHPR and RyR1 molecules, but has the advantage of directly detecting interactions of individual residues in a liquid environment. Here we investigate the molecular interaction of the II-III loop fragments with the aid of NMR complemented by structural homology modeling, fluorescence-binding and site-directed mutagenesis techniques.

Structure and interacting sites on the DHPR II-III loop

The II-III loop is a 126 amino acid fragment of the DHPR and is located in the cytoplasm between the t-tubule and SR membrane of the triad junction. The structure of this fragment has been examined by NMR and found to be composed of a series of α-helical and β-turn secondary structure elements with the N & C termini in close spatial proximity (Figure 1b).[15] Despite the presence of these secondary structure elements, the II-III loop is lacking a stable tertiary conformation and hence has been designated an intrinsically unstructured protein. The II-III loop has been noted to bind *in vitro* to a region of the RyR1 recognised as a SPRY domain with a K_d of 2.3 μM.[15] This is the second of the three SPRY domains located in the RyR1 (SPRY2) and the first time that a binding interaction has been assigned to any of the three SPRY domains in any of the RyR isoforms. The interacting regions on the II-III

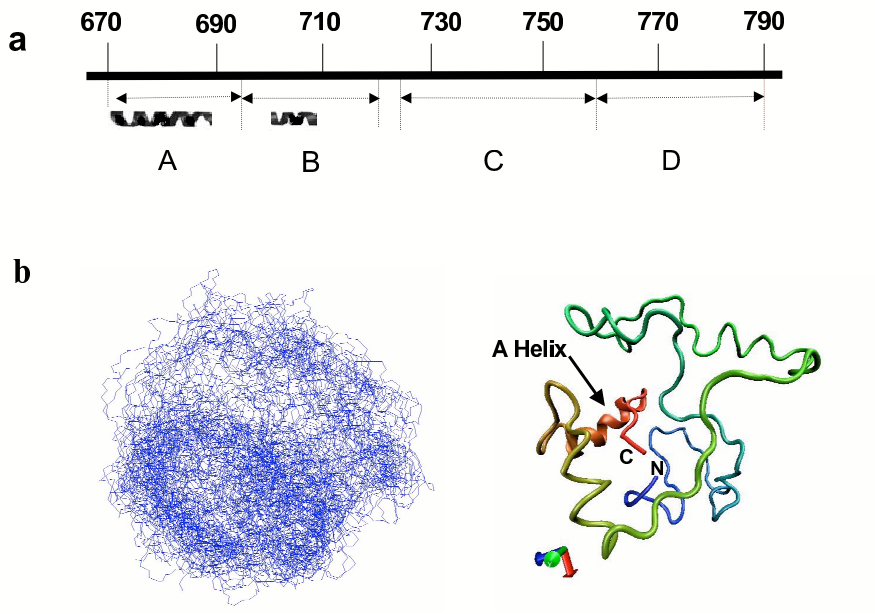

Figure 1. The II-III loop structure of the skeletal DHPR. ***a:*** *The linear structure of the II-III loop (126 amino acids) is subdivided into four regions according to El Hayek* et al.[8] *Regions of helical structure are shown.* ***b:*** *A cluster of 34 NMR derived structures for the II-II loop calculated from NMR constraints (NOEs, dihedral angles, ^{13}C chemical shift values and PRE constraints) (left) and mean structure (right). Prominent structural features include the helical A region (arrow) and the close proximity of the N and C termini.*

loop have been identified by NMR and are located within the A region (670-685), the B region (696-707) and a hydrophobic stretch in the D region (F^{780}-F^{783}) with the principle site of engagement being the basic A region (Figure 1a). As a result of this work, it was of interest to modify these regions and assess the impact of their residues in the molecular recognition of SPRY2. We previously mutated a positively charged sequence within the A region (^{681}RRKRK^{685}) resulting in the reduced *in vitro* binding by ~8 fold. In this current study, we have expanded this mutagenesis analysis by altering residues in the B and D segments of the II-III loop that have been implicated in binding in previous NMR studies (see Cui *et al.*, 2008[15] for methods). These residues include two positively charged residues in the B region (^{703}KK^{704}) and the hydrophobic residues (^{779}FFIF^{782}) in the D region. The mutations in the B region produced a ~1.5 fold reduction in binding, but no change with the hydrophobic mutants (Table 1). Since the A&B regions are involved in binding to SPRY2, the ^{681}RRKRK^{685} and ^{703}KK^{704} mutations were combined and the binding affinity was reduced to 121.1 ± 6.2 μM, a 60-fold reduction relative to the WT SPRY2 (Table 1). These results indicate that the A & B regions of the II-III loop bind in a synergistic manner to the SPRY2 domain. It also suggests that the hydrophobic residues located in the C terminal portion of the II-III loop are unlikely to directly participate in SPRY2 binding; instead, its involvement is most probably a secondary effect resulting from the structural perturbation of the region which result from binding of the A and B helices of the II-III loop.

Table 1. *Binding constants (K_d) of the DHPR II-III loop wild-type/mutants to wild-type RyR1 SPRY2 (see Cui* et al., *2008[15] for methods).*

DHPR II-III loop	K_d (μM)
Wild-type	2.3 ± 0.1
A region mutant ^{681}RKRRK^{685} to AVDAG	18.8 ± 0.4
B region mutant ^{703}KK^{704} to AA	3.2 ± 0.2
D region mutant ^{779}FFIF^{782} to AAAA	2.4 ± 0.1
A & B region mutants	121.1± 6.2

Allosteric effects of A & C II-III loop peptides with the SPRY2 domain

The individual A and C peptides of the II-III loop are known to elicit a functional *in vitro* response upon addition to RyR1[7,9,19-21] and both of these peptide fragments also bind with weak to moderate affinity to the SPRY2 domain of RyR1.[15] These findings however, are not easily reconciled with our NMR results which indicate that the intact DHPR II-III loop interacts with SPRY2 mainly through the A region and not the C region.[15] This raises the possibility that the C region fragment may indeed be capable of binding separately to SPRY2 but, on the whole, II-III loop accessibility of this region to its binding site may be occluded in some manner due to the tertiary structure of

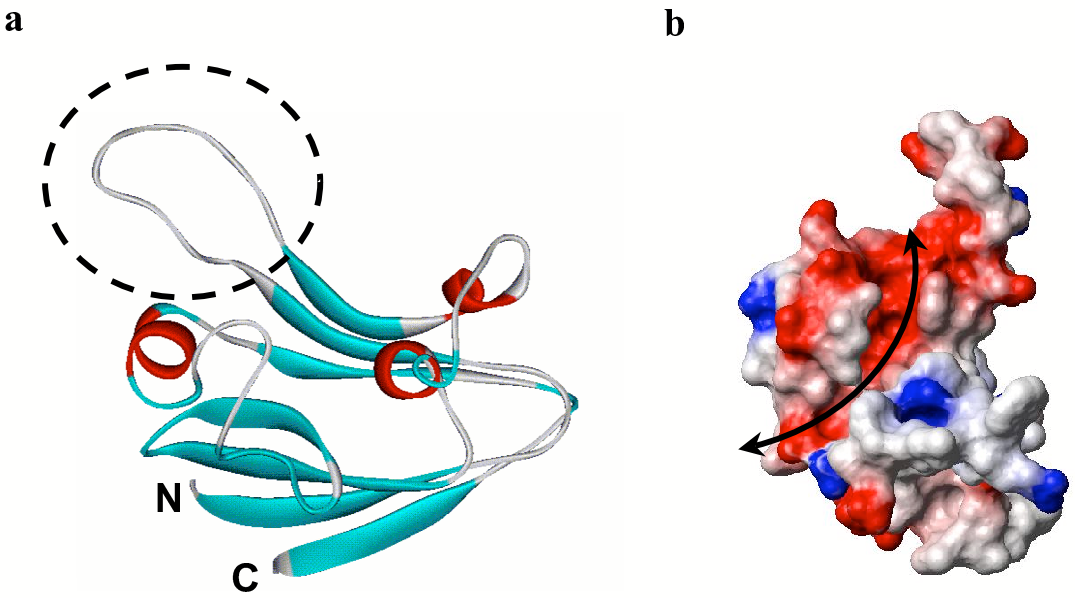

Figure 2. Homology model of the RyR1 SPRY2 domain. ***a:*** *A model of the structure of the SPRY2 domain showing a β-sandwich fold core, flanked by several regions. The major loop in SPRY2 is highlighted in black and contains several acidic residues that contribute to a negatively charged region on the molecule surface.* ***b:*** *Electrostatic surface map of SPRY2 (generated using Pymol – DeLano Scientific) with electrostatic potentials shown in blue and red for the positively and negatively charged regions, respectively. The double arrow curve denotes a potential negatively charged binding site.*

the full II-III loop. In an attempt to determine whether the A and C regions of the II-III loop bind independently, competitive binding studies were set up by monitoring the fluorescence signal upon addition of these two peptide fragments. Table 2 shows that each peptide is capable of binding to SPRY2 in the presence of the other, however, the binding affinity of both peptides is reduced by a factor of ~2 when they are added together, compared to their individual binding affinities. This suggests that each of the binding sites is compromised in some way by the interaction with the other peptide either directly, or by an allosteric effect. These results are consistent with a set of *in vitro* experiments where the addition of peptide C blocks the peptide A interaction by interfering with peptide A binding to the RyR1.[22] These results show that under particular *in vitro* conditions, the C region of the II-III loop may be capable of binding to SPRY2.

Table 2. *Binding constants (K_d) of the DHPR II-III loop peptide fragments to wild-type RyR1 SPRY2 (see Cui* et al., *2008[15] for methods).*

DHPR peptide fragment	K_d (μM)
A (^{671}Thr-^{690}Leu)	8.3 ± 0.3
C (^{724}Glu-^{760}Pro)	20.6 ± 0.4
A + saturated C	17.7 ± 0.5
C + saturated A	41.5 ± 0.6

Homology model of the SPRY2 domain of RyR1

Since the structure of the SPRY2 domain in RyR1 has not been determined, a homology model of SPRY2 was created in order to evaluate potential II-III loop binding sites within this domain. This was achieved through the multiple alignment of the sequences of three published SPRY domain structures with that of SPRY2 from RyR1 using the program HMMER 2.3.2.[23,24] The published SPRY domain structures were of PRY-SPRY-19q13.4.1,[25] B30.2/SPRY domain of GUSTAVUS[26] and SSB-2 [27] (PDB ID codes 2fbe, 2fnj and 2afj, respectively). Models of SPRY2 were created with the program MODELLER 8v1 [28] using the above multiple alignment and the three SPRY structures as the template. When the sequence of known SPRY domain structures (PRY-SPRY-19q13.4.1, B30.2/SPRY domain of GUSTAVUS and SSB-2) were aligned with SPRY2, a sequence conservation for the β-sheet core was observed. From the multiple alignments a homology model of the structure of SPRY2 was constructed and is presented in Figure 2a. The main feature of this model is a β-sheet sandwich structure that is flanked by several loop regions. The secondary structure elements of SPRY2 was confirmed experimentally by using circular dichroism which revealed a minimum at ~215 nm (characteristic of a high β-strand content[29]). A significant feature of this structure is a loop region spanning residues 1107-1121 which is unique amongst the three SPRY domains present in RyR1. We have termed this region the major loop. This major loop contains an abundance of acidic residues that contribute to a negative charge running

from the loop down the molecule (Figure 2b). Because the A region in the II-III loop is very basic, it is feasible that this region may interact with an acidic region in SPRY2. The electrostatic map of the homology model of SPRY2 shows regions of negative surface charge interlaced with smaller positive and hydrophobic patches (Figure 2b). One of these regions shows a high level of negative charge running along the surface of the molecule (see Figure 2b, curved double arrow), which we suggest may form part of a II-III loop binding site.

Specific mutations in a negatively charged loop region of SPRY2

In order to investigate the involvement of the major loop in SPRY2 in the interaction with the basic A region of the DHPR II-III loop, alanine scanning mutagenesis was performed for the acidic residues in the loop region (1107-1121). A set of five mutations was examined (E1108, 1112E, 1114E, 1118D and 1119E) both individually and in combination. When all five residues were mutated a reduction in the binding affinity by ~2-3 fold was observed for the II-III loop (Table 3). When the mutants were probed individually, it was found that only the E1108 mutant significantly affected the binding. The reduction in binding of this mutant was comparable to that of a mutant with 3 and 5 acidic loop residues mutated, suggesting that, within this loop, E1108 is a key residue for binding to the A region residues in the II-III loop. This result is consistent with our hypothesis that the acidic loop in SPRY2 binds to the II-III loop primarily through the A region. To further verify this hypothesis, a binding experiment was performed with the E1108 mutant and the II-III loop A region mutant (^{681}RRKRK^{685} to ^{681}AVDAG^{685}), which lacked the stretch of positive charge deemed important in binding. As expected, no discernable difference in binding of the mutant A region to SPRY2 was observed upon mutation of the E1108, consistent with the ^{681}RRKRK^{685} in the A region binding to E1108 (data not shown). It is noteworthy that a comparison between the RyR1 and RyR2 isoforms reveals that the E1108 amino acid is the only non-conserved acidic residue within this loop region of SPRY2. This suggests that this residue may be involved in an isoform specific interaction with RyR1 and provides encouragement for the future analysis of this residue in a whole cell system.

Conclusions

The skeletal DHPR II-III loop is capable of interacting with the second of three RyR1 SPRY domains in RyR1 *in vitro*. This interaction takes place primarily through basic residues that line the A and B helical elements of the II-III loop. The structure of the SPRY2 has been investigated using homology modeling methods and reveals that this domain exists as a β-sheet sandwich structure flanked by variable loop regions. Preliminary studies have identified that one acidic loop region is involved in the interaction with the basic helical elements of the II-III loop with the E1108 in SPRY2 being an important potential binding site residue.

Table 3. *Binding constants (K_d) of the RyR1 SPRY2 wild-type/mutants to wild-type DHPR II-III loop (see Cui* et al.*, 2008[15] for methods).*

SPRY2 Domain	K_d (μM)
WT-SPRY2	2.3 ± 0.1
× 5 mutant ^{1107}PELRPDVELGADEL^{1120} to ^{1107}PALRPAVALGAAAL^{1120}	6.3 ± 0.3
× 3 mutant ^{1107}PELRPDVELGADEL^{1120} to ^{1107}PALRPAVALGADEL^{1120}	6.3 ± 0.2
E1108A	8.1 ± 0.3

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Author for correspondence:

Dr Marco G Casarotto,
PO Box 334,
Canberra, ACT 2601
Australia

Tel: +61 2 6125 2598
Fax: +61 2 6125 0415
E-mail: Marco.Casarotto@anu.edu.au