

Chapter 2

TOPOLOGY AND TRANSMEMBRANE ORGANIZATION OF RYANODINE RECEPTORS

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INTRODUCTION

The transient elevation of Ca^{2+} in the myoplasm is a key element in excitation-contraction (EC) coupling. In muscle, Ca^{2+} is released from a store located in the lumen of the sarcoplasmic reticulum (SR) by a class of Ca^{2+} release channels referred to as ryanodine receptors or RyR. They are located in the junctional terminal cisternae of the sarcoplasmic reticulum, where they form a cluster of tetrameric molecules, each with a transmembrane (TM) component and a much larger cytosolic component. The cytosolic structure is often referred to as the “foot” component, since it appears to be the foot on which the sarcoplasmic reticulum stands as it extends away from the transverse tubular membrane.⁵⁵ The foot component bridges the 140 Å gap that exists between transverse tubular and sarcoplasmic reticulum membranes.

Three-dimensional reconstruction of RyR1 at 25-30 Å has been carried out using averaged electron microscopic images of large numbers of individual molecules.⁵⁶⁻⁵⁸ Isolated RyR1, viewed from above, is roughly 290 x 290 Å with four equal subunits resembling a quatrefoil. These structures are observed in the junctional terminal cisternae of the sarcoplasmic reticulum and in corbular sarcoplasmic reticulum in cardiac muscle, but are absent from *RYR1* null mice.⁵⁹ At 30 Å resolution, the cytoplasmic quatrefoils are seen to be about 120 Å high and to be highly hydrated, with numerous canals lying among at least 10 loosely assembled protein domains (see Chapter 3). The architecture is that of a scaffold that provides a

mechanical linkage between the SR and the transverse tubule, while facilitating flow of Ca^{2+} from a central channel to the periphery. The open channel has a visible, solvent-filled opening on the luminal side, which disappears in the closed configuration. The intramembrane domain, seen from below or from the side, is about 70 Å tall and could accommodate multiple TM amino acid sequences. Estimates have been made of the mass of the protein contained in the TM domains, based on the total volume of the transmembrane domain and the volume occupied by a TM helix. The TM domain could accommodate 24 to 32 TM helices,^{60,61} suggesting that the most likely number of TM helices per monomer is between 6 and 8.

The close apposition between the transverse tubular and junctional terminal cisternae membranes has led to speculation concerning the identity of the proteins in these two membranes that must interact. Block *et al.*⁶² demonstrated that, in skeletal muscle, every other foot protein is directly apposed to a cluster of four L-type Ca^{2+} channel complexes referred to as dihydropyridine receptors or DHPR, located in the transverse tubules. Each of the four DHPR complexes is associated with one subunit of the huge tetrameric RyR molecule. These observations led to the proposal that there is a direct interaction in skeletal muscle between RyR and DHPR molecules. Further support for the interaction between RyR and DHPR comes from physiological evidence which shows that inward Ca^{2+} currents through the DHPR are a late event in skeletal muscle EC-coupling and are not essential.⁶³ An essential component of EC coupling is the “charge movement”, which represents the translocation of a voltage-sensing helix across the membrane, with consequences for the conformation of other physically associated TM helices.^{64,65} These physical movements in the DHPR are believed to be transmitted physically to closely apposed RyR molecules to alter the conformation of RyR1 and bring about the activation of Ca^{2+} release channels that triggers muscle contraction.⁶³

The random apposition of RyR and DHPR molecules that is observed in cardiac muscle shows that cardiac isoforms of RyR and DHPR do not interact directly. Moreover, in cardiac muscle, inward Ca^{2+} through the DHPR is an early and essential event⁶³. These results suggest that Ca^{2+} -induced Ca^{2+} release accounts for virtually all EC coupling in cardiac muscle, but for only 50% of EC coupling in skeletal muscle.

A second protein that helps to bring the transverse tubule into apposition with the terminal cisternae is mitsugumin 29.⁶⁶ This protein has a single TM component and a cytosolic domain which also bridges the gap between the transverse tubule and the terminal cisternae. This protein does not appear to be involved directly with the interaction between RyR and DHPR.

It has been of great interest to understand which parts of RyR and DHPR interact. These segments occur in the part of RyR which is most distal from

the TM segments of the four subunits which make up the RyR molecule. The identity of these segments is covered in Chapter 4 of this book. The focus of this chapter is the question of which parts of the ryanodine receptor form the TM domain. We conclude that there are six essential TM segments which lie between amino acids (aa) 4557 and 4935. They include three TM hairpin loops and a selectivity filter which is located within the final hairpin loop between aa 4854 and 4911. We also conclude that RyR contains an additional upstream TM hairpin loop which is contained within a single contiguous hydrophobic sequence between aa 4323 and 4363 and is preceded by a very basic sequence of about 19 aa which lies in the cytosol. This TM sequence has some regulatory properties, but is not essential to channel function, since recombinant RyR1 molecules in which aa 4274-4535 are deleted retains Ca^{2+} release channel function.⁶⁷ Thus the entire molecule contains 8 TM sequences in 4 TM hairpin loops, but only 3 of these TM hairpins are required for Ca^{2+} release channel function.

IDENTITY OF TM SEQUENCES

RyR Ca^{2+} release channel molecules are homotetramers of 2,350,000 Da, formed from four monomers, each of 565 kDa. TM sequences from each of the four monomers interact to form the ion-conducting pore.⁶⁸ When the sequences of the ~5000 aa that constitute rabbit and human skeletal muscle RyR1^{3,7} were first deduced from cDNAs, they were subjected to analysis using hydropathy plots that were based on criteria defined by Kyte and Doolittle.⁶⁹ The data obtained were interpreted in minimalist fashion by Takeshima *et al.*³ Among the predicted TM sequences, four potential TM sequences are clearly more hydrophobic than others, with hydropathy indices between 2.0 and 2.9. These four sequences: aa **Phe**⁴⁵⁶⁴-**Tyr**⁴⁵⁸⁰; **Pro**⁴⁶⁴¹-**Leu**⁴⁶⁶⁴; **Gln**⁴⁸³⁶-**Phe**⁴⁸⁵⁹; and **Ile**⁴⁹¹⁸-**Ile**⁴⁹³⁷, were designated M1 to M4 and were proposed to form two hairpin loops in the topological model proposed by Takeshima *et al.* (the Takeshima model).³

In the studies of Zorzato *et al.*⁷ a more complex interpretation was attempted, based on previous experience in the prediction of TM sequences in the sarco(end)plasmic reticulum Ca^{2+} ATPase or SERCA.⁷⁰ With the view that TM sequences in pumps and channels might require charged residues in TM sequences, the criteria for prediction of a TM sequence were made less stringent. Nevertheless, the view that each TM sequence would exist in partnership to form a hairpin loop was adhered to. In the topological model proposed by Zorzato *et al.*⁷ (the Zorzato model), eight additional hydrophobic sequences were identified in the C-terminal fifth of the molecule, with hydropathy indices ranging from 0.8 to 1.6, and proposed to

form four additional hairpin loops. The first two sequences in the Zorzato model. M' (Gly³¹²⁴-Phe³¹⁴⁴) and M'' (Pro³¹⁸⁸-Leu³²⁰⁶), were considered to be very tentative; the others were: M1 (Leu³⁹⁸⁵-Ala⁴⁰⁰⁴); M2 (Met⁴⁰²³-Ala⁴⁰⁴¹); M3 (Gly⁴²⁷⁷-Ala⁴³⁰⁰); M4 (Ala⁴³⁴²-Phe⁴³⁶²); M5 (Phe⁴⁵⁵⁹-Tyr⁴⁵⁸⁰); M6 (Leu⁴⁶⁴⁸-Phe⁴⁶⁷¹); M7 (Phe⁴⁷⁸⁹-Val⁴⁸²⁰); M8 (Leu⁴⁸³⁷-Phe⁴⁸⁵⁶); M9 (Met⁴⁸⁷⁹-Gly⁴⁸⁹⁸); and M10 (Val⁴⁹¹⁴-Ile⁴⁹³⁷). M1, M2, M3 and M4 in the Takeshima model correspond to M5, M6, M8 and M10 in the Zorzato model. In further discussion, we will identify the predicted TM sequences through use of the numbering system of Zorzato *et al.*⁷

Subsequent analysis of the aa sequences of RyR2, deduced from cDNAs, confirmed the pattern of hydrophobicity first seen in RyR1.⁴ However, the sequences of M3 and M4 were not well conserved and the hydrophobicity of M4 could clearly be extended to over 40 aa. The sequencing of RyR3,¹² however, led to a different pattern. In RyR3, the sequence corresponding to M3 was not hydrophobic and could not be TM, but the possibility now existed that M4 alone might be long enough to form a hairpin helix.

Brandt *et al.*⁷¹ proposed that as many as four additional TM segments might be located in the first 3000 aa of RyR1: at aa 599-614; 1514-1530; 1838-1855; and 2555-2570. This prediction was based in part on evidence that a 70-kDa fragment, which was deduced to contain the hydrophobic sequences, 1514-1530 and 1838-1855, and a 360 kDa C-terminal fragment containing previously predicted TM sequences, were both labeled with a hydrophobic probe, 3-(trifluoromethyl) 3-(*m*) [¹²⁵I]iodophenyl diazarine.

The first functional evidence for the location of the TM sequences at the C-terminus of the molecule came from investigations by Takeshima *et al.*⁷² which showed that a second RyR1 transcript can be found in brain. This transcript encoded only 656 aa, which would begin just before M⁴³⁸² and extend to the end of the molecule. When a recombinant RyR1 protein with a deletion of aa 183-4006 was expressed in CHO cells, it could be shown to encode a channel, which, when measured in planar bilayers, had a huge conductance, corresponding to that of intact RyR1, and a probability of opening close to 1. Thus, it appeared to contain the channel forming segment, but to be missing key regulatory sequences.⁷³

Studies of a K⁺ channel containing only two TM sequences and a pore⁹ showed that the selectivity filter is located between two C-terminal TM sequences. These findings focused attention on M9 in RyR1 and RyR2 as a candidate for the selectivity filter and M8 and M10 as candidate outer and inner pore helices. Alanine-scanning mutagenesis and analysis of the mutant products in a variety of assay systems^{37,38,74} showed that amino acids in M9 had properties associated with a selectivity filter sequence. These properties included alterations in conductivity, ryanodine binding and channel regulation. If M9 is assigned as the selectivity filter and M8 and M10 as

outer and inner pore helices, then a significant revision is required in the topological predictions of the Zorzato model, but not of the Takeshima model.

The location of other mutation-sensitive aa in TM segments has been investigated in a number of experiment. In a study of the mutation-sensitivity of conserved polar aa in TM sequences,⁷⁵ mutations in **Glu**⁴⁰³² in M2, **Asn**⁴⁰⁸⁶ and **Asp**⁴⁸¹⁵ in M7 and **Asp**⁴⁹¹⁷ and **Gln**⁴⁹³³ in M10 had functional consequences that suggested that they might be involved in channel function and regulation. Indeed, the properties assigned to the mutation **Glu**³⁸⁹⁵ in RyR3, which corresponds to **Glu**⁴⁰³² in RyR1, were those of a **Ca**²⁺ sensor.⁷⁶ These results do not provide direct evidence, however, that the sequences in which these critical residues lie are, in fact, TM sequences.

M10 has been proposed to be the inner pore inner helix of RyR and to play a role in channel activation and gating that is similar to the role of the inner helix in bacterial **K**⁺ channels.^{9,77} When systematic mutagenesis of M10 was carried out, mutants D4847A, F4850A, F4851A, L4858A, L4859A and I4866A had curtailed caffeine-induced **Ca**²⁺ release when expressed in HEK-293 cells and [³H]ryanodine binding was diminished in cell lysates.⁷⁸ Mutants F4846A, T4849A, I4855A, V4856A, and Q4863A lost or had markedly reduced [³H]ryanodine binding, but retained caffeine-induced **Ca**²⁺ release.⁷⁹ These two groups of mutants are largely located on opposite sides of the M10 helix. Single channel measurements showed that mutant Q4863A had altered kinetics and apparent affinity for ryanodine and was insensitive to ryanodol, an analogue of ryanodine. The single channel conductance of the Q4863A mutant and its responses to caffeine, ATP, and **Mg**²⁺, however, were comparable to wild type. The effect of ryanodine on single Q4863A channels was influenced by the transmembrane holding potential. These results suggest that the M10, and **Gln**⁴⁸⁶³ in particular, play an important role in ryanodine binding. Single I4862A mutant channels exhibited considerable channel openings and altered gating at very low **Ca**²⁺ concentrations. All of these data indicate that M10 constitutes an essential determinant of channel activation and gating, in keeping with its proposed role as an inner helix of the pore region of RyR.

In the absence of a high-resolution structure for RyR, attempts have been made to elucidate the structure and topology of RyR using biochemical approaches. Proteolytic digestion of RyR1 yielded several major fragments,⁸⁰⁻⁸² identified by sequencing^{80,82} or by immunoblotting with a series of seven antibodies⁸¹. Five major fragments were estimated to span the molecule. They had masses and deduced aa sequence boundaries as follows: 135 kDa (aa 1-1397 or 1508), 100 kDa (1398 or 1509-2401), 50 kDa (2402-2840), 160 kDa (3119-4475) and 76 kDa (4476-5037). The more N-terminal

150, 50 and 100 kDa tryptic fragments and their subfragments could be extracted by Na_2CO_3 , indicating that they did not contain TM sequences.⁸¹ The more C-terminal 160 kDa fragment reacted with an antibody against an epitope contained within aa 4382-4417 and was resistant to Na_2CO_3 extraction,⁸¹ implying that TM sequences exist in the sequence between aa 3119 and 4476. This sequence would include M', M'', M1, M2, M3 and M4 from the Zorzato model. The 76 kDa C-terminal fragment (4476-5037) could not be extracted into Na_2CO_3 , indicating that it was membrane-associated⁸¹. This fragment would include M5 to M10 from the Zorzato model and M1 to M4 from the Takeshima model.

In a thorough analysis of RyR1,⁸² similar major fragments were generated, which spanned the whole molecule. Sequence analysis clarified which aa were located at the beginning and the end of each of these fragments. The masses and approximate aa sequence of these fragments, beginning at the N-terminus are: 40 kDa (aa 1-426); 110 kDa (aa 426-1508); 100 kDa (aa 1508-2401); 50 kDa (aa 2401-2840); 33 kDa (aa 2840-3119); 150 kDa (aa 3119-4475); and 76 kDa (4475-5037).

According to the hydropathy analysis and evidence obtained from other studies, the N- and C-termini of RyR1 are both located in the cytoplasm,⁸³⁻⁸⁵ indicating that an even number of sequences traverse the membrane. Antibodies against synthetic peptides corresponding to N-terminal aa 2-15⁸⁴ and C-terminal aa 5027-5037⁸⁴ or 4941-5037⁸³ in rabbit skeletal muscle RyR1 bound to intact SR, confirming that both ends of the membrane-embedded RyR1 are exposed to the cytoplasm. Degradation of the C-terminal end of RyR1 in the intact SR, using carboxypeptidase A, caused a loss of reactivity with anti-C-terminus antibodies.⁸⁴ Denaturation of RyR1 in SR vesicles by transient incubation at alkaline pH under condition where few of the vesicles were permeabilized increased its sensitivity to antibodies.⁸³

A useful approach to the topology and localization of specific regions of RyR has involved the insertion of glutathione-S-transferase (GST) or green fluorescent protein (GFP or EGFP) tags into various regions of the molecule. The mutant protein is expressed, purified, and subjected to cryoelectron microscopy and single particle image analysis, and reconstruction of the three-dimensional structure of the tagged protein.⁸⁵ Comparison of the three-dimensional reconstructions of wild type and mutant RyR3 with GST attached to the N-terminus showed that GST was located at the corners of the square-shaped cytoplasmic region of homotetrameric RyR3. This finding proves that the N-terminus of RyR3 lies in the cytoplasm.⁸⁵ Insertion of GFP after Thr¹⁸⁷⁴ in the middle of the highly divergent region 3 (DR3) between aa 1872 and 1923 did not alter the function of the channel.⁸⁶ The tag, representing the DR3 region, was located in domain 9, in the clamp-shaped

structure adjacent to the binding sites for FKBP12 and FKBP12.6. A similar analysis was carried out with RyR2 in which GFP was inserted into divergent region 1 after Asp⁴³⁶⁵.⁸⁷ Again, channel function was not impaired. The inserted GFP, and consequently, DR1, was mapped to RyR domain 3, referred to as the “handle” domain. These studies provide evidence that the N-terminus and divergent regions 1 and 3 are all located in the cytosol.

Confocal microscopy of EGFP fused to a variety of RyR1 sequences indicated that the ER retention signal is present within the C-terminal portion of RyR1 containing the TM sequences⁸⁸. Evidence showed that aa 4918-4943 of RyR1 may be responsible for ER retention of the Ca²⁺ release channel.⁸⁹

Further studies⁸³ have shown that antibodies against aa 2804-2930 of RyR1, lying upstream of M', bound to either intact or permeabilized SR vesicles, confirming the cytoplasmic location of this sequence. Antibodies against aa 4581-4640, between M5 and M6, did not bind to intact SR vesicles but bound well to permeabilized vesicles, supporting a luminal location for this peptide sequence. Antibodies against aa 4860-4886 between M8 and M9 did not bind to intact vesicles, but exhibited weak binding to the permeabilized vesicles, suggesting that this epitope is exposed to the lumen. In a study of human RyR2,¹⁸ an antibody against aa 4594-4718 between M6 and M7 bound to intact or permeabilized SR vesicles, indicating that this epitope is located in the cytoplasm.

These epitope location results are largely consistent with the predictions inherent in both the Takeshima and Zorzato models for membrane topology of RyR1. However, there is a very informative discrepancy for the location of the epitope against aa 4860 and 4886, lying between M7 and M8. In the Zorzato model aa 4860-4886 are predicted to lie in the cytoplasm. In the most recently revised topological model,^{8,90} described in the next section and Fig. 2-1, the value of this antibody is evident: it is consistent with the revised view that M7 (M7a/M7b) forms a hairpin loop, that M8 is oriented cytosol to lumen; that M9 is a selectivity filter located between M9 and M10; and that M10 is oriented lumen to cytosol.

Thus after more than a decade of investigation using a variety of tests, it was still not possible to distinguish clearly between the Takeshima and Zorzato models. Progress had been made, however, since M9 was transferred from TM status to selectivity filter status, allowing M8 and M10 to form a hairpin loop, and M3 was no longer considered to be a TM sequence. Moreover, there was clear recognition that both M4 and M7 are long helical sequences, which might, themselves, form TM hairpin loops. Clearly a systematic investigation of the TM sequences was required and new technologies, such as the fusion of EGFP to various sites in RyR1, was a new technology suited to this type of investigation.

SYSTEMATIC ANALYSIS OF RYR TOPOLOGY

Membrane protein synthesis is initiated at the N-terminus and nascent proteins are translocated into membranes cotranslationally using machinery shared with secretory proteins only to the point where membrane proteins become integrated into the ER membrane.⁹¹ On the basis of these principles, it was possible to design strategies to analyze predicted membrane sequences in RyR1 through the insertion of EGFP, with a stop codon at its C-terminus, into virtually any site in RyR1 where membrane association and orientation was to be interrogated. Nascent proteins could then be truncated sequentially from the C-terminus and tagged with EGFP. Through investigation of the location of EGFP fluorescence in the cell using confocal microscopy, it was possible to determine whether the truncated protein was uniformly distributed in the cell or membrane bound. By examining the fate of the fluorescence after saponin permeabilization of the cell, it was possible to determine solubility – soluble proteins were leached out of the cell; membrane bound proteins were retained within a reticular network.

Other studies could be carried out at the biochemical level following isolation of microsomal fractions from these cells. If EGFP in the fusion protein was soluble, it would be detected in the soluble fraction, but if membrane bound, it would be detected in the microsomal fraction following Western blotting with an EGFP antibody. If loosely bound to membrane fractions, it would be extracted with sodium carbonate, which dissociates peripheral proteins from membranes (but has limited capacity to extract aggregated proteins from membrane fractions). Finally, it was possible to determine whether EGFP was located in the cytoplasm or in the lumen of microsomal vesicles following tryptic digestion. EGFP is intrinsically resistant to digestion by common proteases due to its tight folding. If EGFP were folded appropriately in the cytosol, it would be freed into solution as a trypsin-resistant, 27 kDa product. If it were folded appropriately in the lumen, it would remain in the membrane pellet, but its mass would be increased in proportion to the length of the trypsin-protected TM sequence to which it was attached. Problems that arise using this strategy are that it is difficult to interpret experiments in which EGFP does not fold properly and it is not always possible to insert EGFP into the exact site desired because of technical problems involving the cDNA sequence of RyR1.

The basis for insertional site selection was analysis of the rabbit skeletal muscle RyR1 amino acid sequence using the Argos algorithm^{92,93} for prediction of TM domains (Fig. 2-1 A). Stretches of hydrophobicity, compatible with TM sequences, were found mainly in the last 1000 aa. Those stretches in which the average index for each amino acid is over 1 include: Glu⁴²⁷⁵-Ala⁴³⁰⁰ (average index 1.137); Thr⁴³²³-Gly⁴³⁶³ (1.197);

Arg⁴⁵⁵⁷-Ile⁴⁵⁷⁶ (1.241); Gly⁴⁶³⁷-Asn⁴⁶⁶² (1.170); Gln⁴⁷⁷⁶-Thr⁴⁸²⁵ (1.092); Gly⁴⁸³⁴-Val⁴⁸⁵⁴ (1.201); Leu⁴⁹¹¹ - Leu⁴⁹³⁵ (1.253). These sequences correspond closely to M3, M4, M5, M6, M7, M8 and M10 in the Zorzato model. With the Argos algorithm, M1, M2 and M9 are only weakly hydrophobic, but the hydrophobic region of M4 is over 40 aa long and, of M7, about 50 aa long. Thus both M4 and M7 sequences are long enough to form a TM hairpin. Prediction of TM domains for RyR2 and RyR3 using the same program gave results similar to that for RyR1, except that M3 is not predicted as a hydrophobic TM sequence in RyR3.

In the C-terminal-truncated RyR1-EGFP fusion constructs that were designed to analyze membrane sequences in RyR1, EGFP was attached: PreM1 at aa 3224, which lies after the proposed M'/M'' hairpin loop sequence and just before M1 (designated PreM1); PostM2 (aa 4186); PostM3 (aa 4302); PostM4 (4556); PostMS (aa 4628); PostM6 (aa 4771); PostM7b (aa 4836); PostMS (aa 4888); and PostM10 (aa 5037). The possibility that M7 (aa Gln⁴⁷⁷⁶-Thr⁴⁸²⁵) forms an independent hairpin loop was also examined by placing EGFP after aa 4806 in the middle of M7 (PostM7a).

It was anticipated that any EGFP fusion protein in which EGFP lies before the first TM sequence would be translated in the cytosol, would not enter the secretory protein pathway, would not be translocated into the ER lumen and would not associate with the membrane. As a corollary, any EGFP fusion protein in which EGFP lies after a TM sequence with cytosol to luminal orientation would be translated in the cytosol, would enter the secretory protein pathway, would be translocated into the ER lumen and would associate with the membrane. Finally, any EGFP fusion protein in which EGFP lies after a TM sequence with lumen to cytosol orientation would be translated in the cytosol, where it would remain in association with the membrane. On the basis of these principles, it was possible to determine the boundary between the cytosolic portion of RyR1 and the first membrane-associated sequence. It was expected that EGFP located after TM helices would be translated and translocated to the side of the membrane corresponding to the native orientation of the TM sequence. However, a caveat exists in this reasoning in that a lone signal anchor sequence may not be sufficiently strong to cause translocation into the lumen; in some cases, this occur only in the presence of a combined signal anchor sequence and a stop-transfer sequence.⁹⁴ The fusion-truncation proteins were expressed in mammalian MEF or HEK-293 cells and confocal microscopy was used to visualize the fluorescent proteins in the cell in the absence or presence of saponin, which permeabilizes the cells. Alkali was also used to extract peripheral proteins, leaving behind integral membrane proteins, which

formed a reticular network, but also insoluble recombinant protein aggregates, which were distinguished by their amorphous nature.

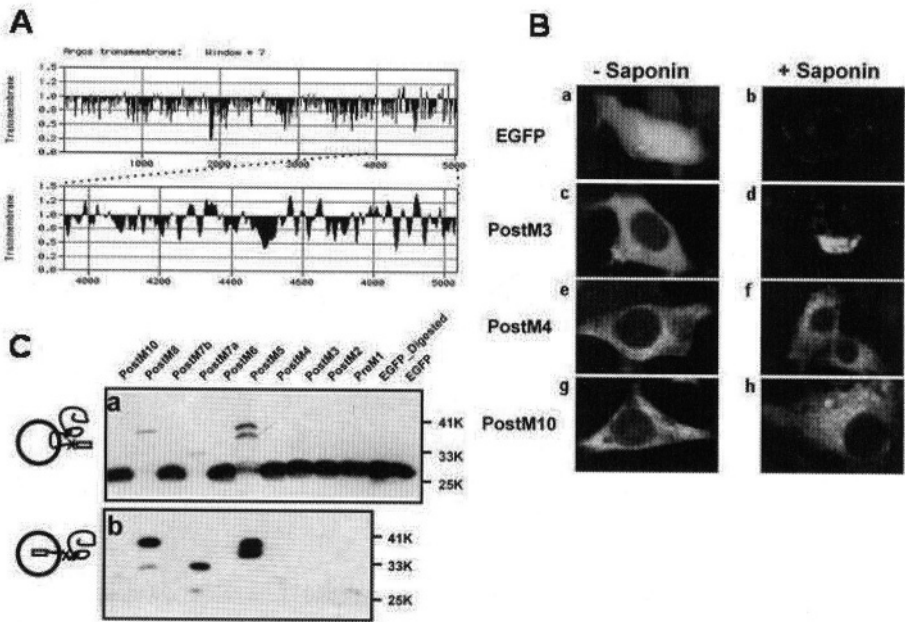


Figure 2-1. **Evidence for a model of RyR1 topology.** **A.** Prediction of transmembrane sequences in rabbit skeletal muscle RyR1 with the Argos transmembrane algorithm.⁹² The top panel shows the analysis of the full length RyR1 sequence and the lower panel shows the expanded C-terminal region. Ten potential TM sequences are apparent, but M4 and M7 are very long. **B.** Confocal microscopy of MEF cells expressing EGFP and RyR-EGFP fusion proteins. The left panel shows transfected MEF cells prior to treatment with saponin and the right panel shows cells after treatment with saponin, which releases soluble proteins (eg. **b** and **d**), leaving aggregated (**d**) and membrane proteins in situ (**f** & **h**). The designation for each construct is on the left side of the image. **C.** Localisation of EGFP in HEK-293 cells transfected with RyR1 fusion protein constructs. Microsomes from transfected HEK-293 cells were digested with trypsin and centrifuged. Proteins in the supernatant (**C, a**) and microsomes (**C, b**) were immunoblotted with anti-EGFP antibody. The cartoon on the left of **C, a** shows that EGFP will be released to the cytosol with a normal mass, following tryptic digestion, if it lies on the cytosolic side. The cartoon on the left of **C, b** indicates that EGFP will remain membrane-bound with an increased mass, following tryptic digestion, if it lies at the luminal side. In PostM8, PostM7a and PostM5, EGFP remained in membranes with an increased mass. EGFP from other fusion proteins was exclusively in the supernatant.

RyR1 fusion proteins truncated PreM1, PostM2 and PostM3 (aa 4302) gave off a uniform fluorescence throughout the cell, indicating that they were located in the cytosol and were not membrane-bound (Fig. 2-1 B). Since they were released from cells treated with saponin and were extracted by Na_2CO_3 it was clear that they were soluble. These studies effectively eliminated M', M'', M1, M2 and M3 as TM helices. Although M2 is not a TM sequence, it contains at least one residue that is critical to channel function and is possibly the Ca^{2+} sensor,^{75,76} indicating that this sequence must form part of an important regulatory domain.

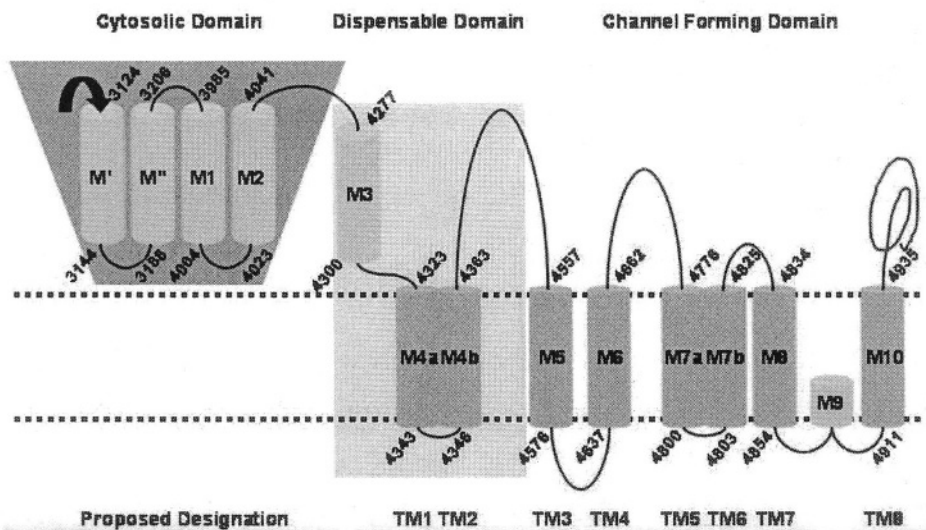


Figure 2-2. Proposal for a model of RyR1 topology. Dark grey cylinders inserted into the membrane represent the eight proposed TM sequences. Numbers inside the cylinders preceded by M are those in the Zorzato model. However, sequences M4 and M7 are now divided into M4a/M4b and M7a/M7b. New designations for these eight TM sequences are proposed at the bottom of the figure, in which the number of the TM sequence is preceded by TM. Light grey cylinders in the cytosol represent those sequences proposed as TM sequences in the Zorzato model, but which are now known to be located in the cytoplasm. They are identified by numbers preceded by M. Cytosolic and luminal boundaries of predicted and confirmed TM segment are indicated by numbers that represent amino acids in RyR1. M9 is now proposed to be the selectivity filter between M8 and M10. The cytosolic domain of RyR is believed to extend to ~aa 4323 at the beginning of the M4 sequence and to include M3. A region which includes aa 4274-4535 is referred to as a dispensable domain, since its removal in the $\Delta 4274-4535$ deletion mutant does not affect structural stability, Ca^{2+} release channel function or ryanodine binding (see Fig. 2-3) and a corresponding sequence is not present in functional IP_3R Ca^{2+} release channels. The last six TM sequences form the Ca^{2+} release channel, but the first two TM sequences may interact with them in a regulatory fashion, thus forming a part of the channel domain.

The RyR1 fusion protein PostM4, truncated at aa 4556 so that it included M4 and the M4/M5 loop, was membrane-associated, as indicated by the reticular nature of its fluorescence, its indifference to saponin permeabilization and its resistance to extraction by Na_2CO_3 . All fusion proteins that were truncated downstream of M4 had similar properties. These results indicate that the region containing the M4 sequence is the first sequence in RyR1 to associate the ~4,300 aa cytosolic portion of the molecule with the membrane.

Further evidence concerning the number of TM sequences and their orientation was derived from proteolytic digestion of microsomal fractions (Fig. 2-1 C). EGFP was recovered after tryptic digestion of SR vesicles as a 28 kDa protein. EGFP from PostM4, PostM5, PostM7a and PostM8 remained largely in the membrane after trypsin digestion and with molecular masses increased in proportion to the mass calculated for each TM domain and/or linker (7-8 kDa for PostM5 and PostM8 and 2-3 kDa for PostM7a). These results show that EGFP in these three proteins was located on the luminal side of the membrane, demonstrating that the orientation for M5, M7a, and M8 is from cytoplasm to lumen. EGFP from all of the other fusion proteins was present in the supernatant and had a molecular mass similar to that of wild type EGFP. These results show that EGFP from PreM1, PostM2 and PostM3 is located in the cytosol because it forms part of a long soluble sequence, while EGFP fused to PostM4, PostM6, PostM7b, and PostM10 lies in the cytosol because it is located at the C-terminus of a hairpin loop. Thus the orientation of M6, M7b and M10 is from lumen to cytoplasm and this must also be true of the second half of M4 (M4b if M4 alone forms a hairpin loop).

These data confirm the view that M5/M6 and M8/M10 form helical hairpin loops and provide new evidence that the long M7 sequence forms a helical hairpin loop. In PostM7a, a construct only 35 aa longer than PostM6, EGFP was translocated to the lumen, preventing its proteolysis by trypsin. In PostM7b, only 30 aa longer than PostM7a, EGFP was dissociated from the membrane after digestion by trypsin, indicating that it was located on the cytoplasmic side. These data provide conclusive evidence that the 50 aa **Gln⁴⁷⁷⁶-Thr⁴⁸²⁵** sequence forms two TM helices with a relatively short luminal loop confirming the prediction of the Argos algorithm (Fig. 2-1 A).

Since M5 is a membrane sequence with an N-cytoplasmic to C-luminal orientation, any TM sequences upstream of M5 must exist as hairpin loops. The PostM3 fusion truncation protein is soluble and PostM4, truncated just before PreM5, is ER associated and located on the cytosolic surface. Thus it is possible that the 40 aa M4 sequence forms a hairpin loop. Indeed, the TMHMM2.0⁹⁵ predicts that M4 in both RyR1 and RyR2 is a TM sequence formed by two helices with a loop in the middle.

In unpublished work, it has been possible to show that fusion truncation proteins immediately PostM4 are insoluble, that fusion truncation proteins immediately PreM4 are soluble and that an RyR2 fusion truncation protein with an epitope in the middle of M4 is soluble. These data are consistent with the view that M4 forms a TM hairpin loop in which the first half (M4a) forms a signal transfer sequence which is not strong enough to anchor the protein, but which can interact with the second half (M4b) to form a signal anchor-stop transfer hairpin sequence, which is the first site of anchorage of RyR1 to the membrane (Fig. 2-2).

Ironically, since so much effort has been spent on investigation of the topology of the M3/M4 region, it is possibly of little functional concern whether M4 does or does not form a hairpin loop in RyR1 (Fig. 2-3). Alignments of the sequence of ryanodine receptors with their homologues, the IP_3 receptors, show that a large gap exists in the IP_3R family, which encompasses the M3/M4 region in RyR1. Thus three C-terminal hairpin loops in IP_3R^{94} are all that are required for Ca^{2+} release channel function. Of more immediate significance is the fact that the RyR1 deletion mutant, $\Delta 4274\text{-}4535$, is expressed in HEK-293 cells at a level that is enhanced up to four-fold over that of wild-type recombinant RyR1 (Fig. 2-3). Thus, the 4274-4535 sequence appears to be inhibitory to expression of RyR1 in HEK-293 cells. The corresponding sequence in RyR2 is not inhibitory, so that expression of RyR2 in HEK-293 cells is also up to four-fold higher than expression of RyR1. These results show that deletion of aa 4274-4535 does not disrupt RyR1 structure - if the protein were misfolded during synthesis, it would have been degraded. The $\Delta 4274\text{-}4535$ deletion mutant also forms a functional Ca^{2+} release channel, which is activated by caffeine (Fig. 2-3 B). Caffeine affinity is intermediate between the high affinity of RyR1 and the lower affinity of RyR2. The deletion mutant also binds ryanodine with the same affinity as RyR1 and RyR2 (Fig. 2-3 C). These results indicate that the aa 4274-4535 region, which encompasses both M3 and M4, is not required for function. In Fig. 2-2, we have delineated the approximate range of this sequence and have labelled it as a dispensable domain. It is probable, however, that the sequence is retained in RyR molecules because it has a unique regulatory function.

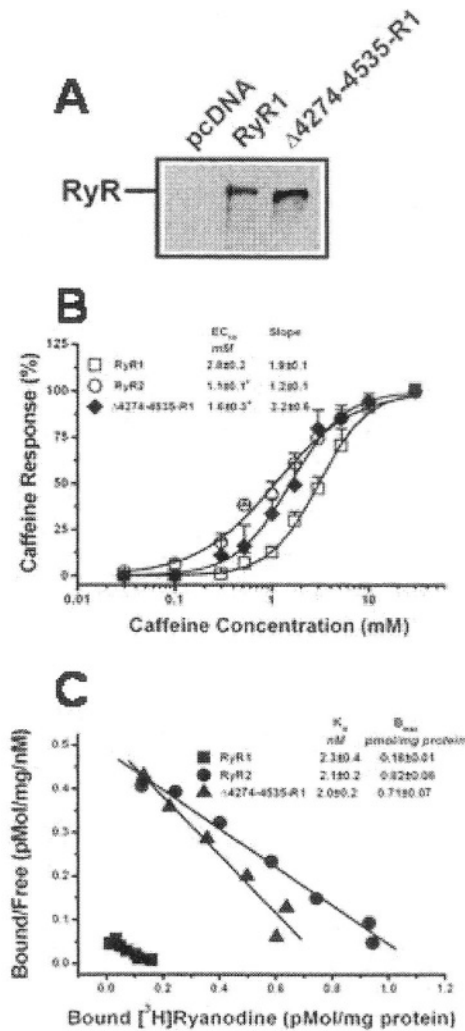


Figure 2-3. Evidence that the region surrounding M3/M4 is dispensable in RyR1. **A.** Expression of RyR1 and the $\Delta 4274-4535$ deletion mutant in HEK-293 cells. CHAPS lysates from HEK-293 cells were harvested 48 h after transfection with cDNAs encoding RyR1 and the $\Delta 4274-4535$ deletion mutant and subjected to Western blotting.⁶⁷ The expression of the $\Delta 4274-4535$ deletion mutant is enhanced over that of RyR1. **B.** Dose-response curves obtained from fluorescence measurements of *in vivo* Ca^{2+} release induced by incremental concentrations of caffeine in HEK-293 cells expressing RyR1, RyR2 and the $\Delta 4274-4535$ deletion mutant. Dose-response curves were used to obtain the EC_{50} values and Hill coefficients for caffeine-induced Ca^{2+} release that are presented as an inset.⁶⁷ **C.** Scatchard analysis of [3H]ryanodine binding to solubilized RyR1 RyR2 and the $\Delta 4274-4535$ deletion mutant expressed in transfected HEK-293 cells. K_d and B_{max} values are inserted.⁶⁷ On the basis of ryanodine binding, presented as an inset, the expression of the $\Delta 4274-4535$ deletion mutant is enhanced four-fold over that of RyR1.

CONCLUDING REMARKS

In summary, it is now clear that M', M'', M1, M2 and M3 do not exist as TM sequences. It is also clear that three hairpin loops do exist: they are formed from M5-M6, M7a-M7b and M8-M10, with M9 inserted between M8 and M10 to form a selectivity filter, as depicted in Fig. 2-2. Results with the M4 sequence show that about 40 aa comprising M4 create the first site of association of RyR1 with the membrane. The precise nature of this association is not yet established, but it is likely to be a TM hairpin loop from M4a/M4b. The cytosolic domain of RyR is believed to extend to ~aa 4323 at the beginning of the M4 sequence and to include M3. A region which includes aa 4274-4535 is referred to as a dispensable domain, since its removal in the $\Delta 4274-4535$ deletion mutant does not affect structural stability, Ca^{2+} release channel function or ryanodine binding, as shown in Fig. 2-3, and a corresponding sequence is not present in functional $\text{IP}_3\text{R Ca}^{2+}$ release channels. The sequence may, however, be retained in RyR1 because it has important regulatory functions. The last six TM sequences are believed to form the Ca^{2+} release channel, but the first two TM sequences may well interact with them in a physical and regulatory fashion, thus forming a part of the channel domain.

ACKNOWLEDGEMENTS

Original work from our laboratory, described in this review, was supported by grant MT-3399 to D. H. M. from the Canadian Institutes of Health Research.

Ryanodine Receptors

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Wehrens, X.H.T.; Marks, A.R. (Eds.)

2005, XXI, 330 p., Hardcover

ISBN: 978-0-387-23187-7