The Transmembrane Segment of Ryanodine Receptor Contains an Intracellular Membrane Retention Signal for Ca\textsuperscript{2+} Release Channel*

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The ryanodine receptor (RyR) is a large homotetrameric protein with a hydrophobic domain at the C-terminal end that resides in the endoplasmic reticulum (ER) or sarcoplasmic reticulum membrane and forms the conduction pore of a Ca\textsuperscript{2+} release channel. Our previous studies showed that RyR expressed in heterologous cells localized to the ER membrane. Confocal microscopic imaging indicated that the ER retention signal is likely present within the C-terminal portion of RyR, a region that contains four putative transmembrane segments. To identify the amino acid sequence responsible for ER retention of RyR, we expressed fusion proteins containing intercellular adhesion molecule (ICAM), various fragments of RyR, and green fluorescent protein (GFP) in Chinese hamster ovary and COS-7 cells. ICAM is a plasma membrane-resident glycoprotein and serves as a reporter for protein trafficking to the cell surface membrane. Imaging analyses indicated that ICAM-GFP fusion proteins with RyR sequence preceding the four transmembrane segments, ICAM-RyR-(3661–3993)-GFP, and with RyR sequence corresponding to transmembrane segments 1, 2, and 3, ICAM-RyR-(4558–4671)-GFP and ICAM-RyR-(4830–4919)-GFP, were localized to the plasma membrane; fusion proteins containing the fourth transmembrane segment of RyR, ICAM-RyR-(4913–4943)-GFP, were retained in the ER. Biochemical assay showed that ICAM-RyR-GFP fusion proteins that target to the plasma membrane are fully glycosylated, and those retained in the intracellular membrane are core-glycosylated. Together our data indicate that amino acids 4918–4943 of RyR contain the signal sequence for ER retention of the Ca\textsuperscript{2+} release channel.

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HindIII-BamHI sites of pcDNA3 expression vector followed by cloning of the cDNA for GFP in frame to the 3′-terminal end of ICAM using the restriction sites EcoRV and XbaI to generate pcDNA3 (ICAM-GFP). The various fragments of RyR (generated by PCR) were then inserted in frame between ICAM and GFP using the restriction sites for BamHI and EcoRV.

**Cells and Expression System**—Cells were grown at 37 °C in a humidified atmosphere with 5% CO₂ in Ham’s F-12 medium (CHO cells) or RPMI 1640 medium (COS-7 cells) supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. The expression plasmids were introduced into cells that were 60–70% confluent using LipofectAMINE PLUS reagent (Invitrogen). The expression of chimeric RyR proteins was analyzed using Western blot assays.

**Immunoblot and Glycosylation Assay**—Cells were lysed with ice-cold modified radioimmune precipitation buffer in the presence of protease inhibitors as described previously (3). For the deglycosylation reaction, 5–25 μg of protein in the cell lysate was incubated at 37 °C in the presence of 2.5 milliliters of recombinant peptide N-glycosidase F (Glyko, Inc., Novato, CA). Control and glycosidase-treated protein samples were separated using 3–15% linear gradient SDS-PAGE and transferred to a polyvinylidene difluoride membrane. Western blotting was performed with a polyclonal antibody against ICAM (Santa Cruz Biotechnology, Santa Cruz, CA) or against GFP (CLONTECH, Palo Alto, CA) using a horseradish peroxidase-linked ECL detection system.

**Confluent Microscopic Imaging**—CHO and COS-7 cells transfected with GFP-RyR or ICAM-GFP-RyR-GFP were grown in glass-bottomed microwells (Biotech Inc., Butler, PA). The GFP signals were visualized with a Zeiss laser scanning confocal microscope (LSM410) using a 63× oil immersion objective. A fluorescein isothiocyanate dichroic filter with excitation at 488 nm and emission at 515 nm was used. Immunostaining of CHO cells stably expressing RyR was performed with an anti-RyR monoclonal antibody (34C) and a secondary antibody conjugated with Texas Red (Molecular Probes, Eugene, OR). For visualization of endoplasmic reticulum, cells were labeled with an ER marker carbocyanine dye, 3,3′-dihexyloxacarbocyanine iodide (Molecular Probes).

**RESULTS AND DISCUSSION**

**RyR Expressed in CHO Cells Is Localized to Intracellular Membranes**—Using GFP as a reporter protein attached to the N-terminal end of RyR, we observed that the expressed GFP-RyR proteins were localized to the perinuclear area of CHO cells (Fig. 1A). The subcellular distribution of GFP-RyR-(290–5037) was similar to that of the nontagged RyR stably expressed in CHO cells determined by immunostaining with an anti-RyR monoclonal antibody (Fig. 1B). The pattern of distribution of both nontagged and GFP-tagged RyR proteins resembled that of the ER membrane labeled with 3,3′-dihexyloxacarbocyanine iodide dye (Fig. 1C). The C-terminal hydrophobic domain of RyR amounting to about 20% of the entire protein was found to contain structures sufficient to form a functional Ca²⁺ release channel (4). The distribution pattern of GFP-RyR-(3661–5037) also resembled that of the ER membrane (Fig. 1D). These results indicate that the ER retention signal for RyR likely resides within the C-terminal portion of RyR. We did not examine the functional properties of the GFP fusion constructs of RyR in this study. However, a recent study demonstrated that RyR with GFP fused to its C-terminal end was fully functional when expressed in RyR-null (dyspedic) myotubes (13).

To further identify the putative ER retention signal(s) for RyR, we constructed a series of deletion mutants of GFP-RyR as illustrated in Fig. 2A. These mutants contained deletions in the N-terminal portion of RyR proximal to the putative transmembrane segments as well as deletions in the C-terminal cytoplasmic tail region of the protein. Since some of the ER retention signals have been shown to be located in the C-terminal end of polypeptides, we generated constructs with deletion of 15, 40, and 97 amino acids from the C-terminal tail of RyR. A previous study from Gao et al. (11) showed that deletion of 15 residues from the C-terminal end of skeletal muscle RyR resulted in an inactive channel when expressed in HEK 293 cells. However, the subcellular localization of this mutant RyR was not examined. Representative confocal images of CHO cells expressing some of the GFP-RyR mutants are shown in Fig. 2B. All mutant GFP-RyR fusion proteins listed in Fig. 2A exhibited fluorescence distribution similar to that of GFP-RyR-(290–5037) in that the fluorescence signal was concentrated in the perinuclear region, suggesting their localization to the intracellular membranes.
These results suggested two possible mechanisms for ER localization of the GFP-RyR fusion proteins. First, the actual retention signal is localized within a domain that is common in all the mutant proteins, namely the transmembrane segments and their connecting cytoplasmic and luminal loops (e.g. amino acids 4297–4943). Second, the addition of the GFP tag or large deletions introduced in the RyR sequence may cause improper folding of the fusion polypeptide, leading to retention in the ER and subsequent degradation of the misfolded proteins (14).

ICAM as a Reporter Protein for Plasma Membrane—To test the latter possibility and to further characterize the ER retention signal of RyR, we used ICAM, also known as CD54, as a reporter protein (12). ICAM is a plasma membrane glycoprotein containing one transmembrane segment with eight N-glycosylation sites on its extracellular domain (Fig. 3A). Other related glycoproteins such as CD4 and CD8, which are also expressed on the plasma membrane, are commonly used to study protein trafficking and to identify the ER retention signal (15, 16). To ascertain that tagging of GFP onto a protein does not interfere with proper folding of the fusion protein, an ICAM-GFP fusion construct was made by attaching the sequence for GFP to the C-terminal end of ICAM (Fig. 3A). The ICAM-GFP construct expressed in COS-7 cells was targeted to the cell surface membrane as shown in Fig. 3B, whereas GFP expressed alone in these cells exhibited a diffuse fluorescence throughout the cell with the exclusion of what appeared to be mitochondria (Fig. 3C). COS-7 cells are larger than CHO cells and have distinct patterns of ER network, which is convenient for subcellular localization of expressed membrane proteins. COS cells have also been used previously for functional expression of RyR (17).

To test whether the C-terminal hydrophobic domain of RyR contains the ER retention signal, a tripolar fusion construct was generated by inserting the sequence encoding the C-terminal region of RyR (amino acids 3661–5037) in frame between ICAM and GFP to obtain ICAM-RyR-(3661–5037)-GFP (Fig. 3A). We speculated that if the ER retention signal is present within the RyR sequence and is dominant, then the fusion protein would reside in the ER membrane. Otherwise the signal within ICAM would shuttle the entire polypeptide to the surface membrane. As shown in Fig. 3D, ICAM-RyR-(3661–5037)-GFP exhibits a fluorescence pattern resembling that of GFP-RyR-(3661–5037) indicating that this protein is localized to the ER. As a control, a fragment of RyR that resides proximal to the hydrophobic domain was inserted into ICAM-GFP to generate ICAM-RyR-(4558–4671)-GFP. As shown in Fig. 3E, COS-7 cells expressing this construct exhibited green fluorescence predominantly on their surface membrane. The dense fluorescence spots present in the central region of the cell likely represent misfolded proteins in the form of aggresomes (see also Figs. 4B and 5B) (26). The result showed that the specific sequence of RyR is responsible for the targeting of ICAM-GFP.

Transmembrane Segments and ER Retention of RyR—Since the experiments described thus far indicated that the transmembrane segments might play a role in the retention of RyR in the ER, we decided to further test this by examining the minimal sequence involved in the ER retention using the
ICAM-GFP approach. Two additional ICAM-RyR-GFP fusion constructs were generated by introducing sequences for (i) transmembrane segments 1 and 2 and their connecting intraluminal loop (amino acids 4558–4671) or (ii) transmembrane segments 3 and 4 and their connecting intraluminal loop (amino acids 4830–4943). These fusion proteins were transiently expressed in COS-7 cells, and their subcellular localization was examined using confocal microscopy (Fig. 4). ICAM-RyR-(4830–4943)-GFP exhibited a fluorescence pattern identical to that of the reticular structure of ER in COS-7 cells (Fig. 4A), whereas cells expressing ICAM-RyR-(4558–4671)-GFP exhibited fluorescence at the surface membrane similar to what was observed with the ICAM-RyR-(3661–3993)-GFP construct. The data indicate that transmembrane segments 3 and 4 of RyR contain sequences sufficient to retain the entire fusion polypeptide in the ER membrane. The ICAM sequence appears to exert a dominant effect on the ICAM-RyR-(4558–4671)-GFP fusion protein, and hence a fraction of it is transported to the surface membrane (Fig. 4B). Similar incomplete redistribution to the surface membrane has been observed with voltage-dependent Ca\(^{2+}\) channels when co-expressed with auxiliary \(\beta 3\) subunit (18).

Furthermore, incubation at a lower temperature (24–26 °C) resulted in an increase in the surface membrane fluorescence of cells expressing ICAM-RyR-(4558–4671)-GFP but had no effect on cells expressing ICAM-RyR-(4830–4943)-GFP (not shown). Lower temperature is thought to help proper folding of proteins that are otherwise misfolded and promote maturation and transportation of surface membrane proteins (19).

**Glycosylation as a Biochemical Marker for the Surface Expression of ICAM—**ICAM is a glycoprotein containing eight putative N-glycosylation sites on its extracellular domain (12, 20). ICAM-GFP is expressed on the surface membrane of transfected COS-7 cells (Fig. 3B). When probed with an anti-ICAM-1 antibody, ICAM-GFP exhibits two bands on SDS-PAGE (Fig. 4C). The majority of the protein runs as a higher molecular mass band representing the fully glycosylated state and a lower core glycosylated band (Fig. 4C, lane 1). Incubation of total cell lysate from these cells with \(\beta\)-N-glycosidase F removes all sugars from the protein resulting in higher mobility and the appearance of a single low molecular mass band (Fig. 4C, lane 2). ICAM-RyR-(4830–4943)-GFP exhibited one single band that is mostly core-glycosylated (Fig. 4C, lane 7), whereas the ICAM-RyR-(4558–4671)-GFP construct showed two bands on Western blot (Fig. 4C, lane 5): a lighter higher band and a lower thicker band. Both proteins are sensitive to digestion with \(\beta\)-N-glycosidase with apparent increase in molecular mobility (Fig. 4C, lanes 6 and 8). While the lower thicker band in lane 5 represents a core glycosylated protein that is retained in the intracellular membranes, the upper minor band likely represents a fully glycosylated fusion protein that is targeted to the cell surface. The small proportion of the fully glycosylated band of ICAM-RyR-(4558–4671)-GFP reflects the low percentage of green fluorescence observed at the cell surface membrane measured under confocal microscopy (see Fig. 4B). Similar results were obtained with Western blot analysis using anti-GFP antibody (not shown). Together our data indicate that the sequences within the transmembrane segments 3 and 4 of RyR and/or their connecting intraluminal loop contain the ER retention signal for the Ca\(^{2+}\) release channel.

**Amino Acids 4913–4943 of RyR, Conserved Sequence among \(\text{IP}_3\) Receptors, Contain ER Retention Signal for Ca\(^{2+}\) Release Channel—**To further dissect the ER retention sequence within the transmembrane segments 3 and 4 of RyR, three additional ICAM-RyR-GFP constructs were generated: ICAM-RyR-(4830–4865)-GFP, containing the sequence corresponding to the third transmembrane segment of RyR, ICAM-RyR-(4913–4943)-GFP, containing the fourth transmembrane segment of RyR, and ICAM-RyR-(4860–4919)-GFP, containing the intraluminal loop connecting the third and the fourth transmembrane segments of RyR. These constructs were transiently transfected into COS-7 cells, and the subcellular localization of expressed fusion proteins was examined (Fig. 5). ICAM-RyR-(4913–4943)-GFP exhibited a fluorescence signal in intracellular membranes resembling that of the ER network (Fig. 5A), whereas the fusion protein containing RyR-(4860–4919) and RyR-(4830–4865) were expressed only on the surface membrane (Fig. 5, B and C). Similar observation was also made in CHO cells transfected with these constructs (not shown). These results indicate that the ER retention signal of the RyR is localized specifically within the 24-amino acid region (amino acids 4920–4943) that forms the fourth transmembrane segment of the Ca\(^{2+}\) release channel.

Several ER retention/retrieval signals have been proposed for membrane proteins that involve both the transmembrane domains as well as cytoplasmic sequences (21). Examples of these include dilsyline-, diarginine-, and tyrosine-based motifs in the cytoplasmic domains of proteins (22) and sequences in the transmembrane domains. While the mechanism(s) by which transmembrane segments participate in protein sorting is not clearly understood, the length of the transmembrane segment and its hydrophobicity and the physical interaction between the transmembrane segments and their surrounding lipid environment are thought to be involved (23). Our findings in this study indicate that a sequence of 24 amino acids within the fourth transmembrane segment is important in the retention of RyR in the ER membrane. It is also interesting to note that this sequence is highly conserved in different subtypes of RyR (skeletal, cardiac, brain, and Drosophila) (24) and members of the \(\text{IP}_3\) receptor family (4, 25). And the C-terminal region of \(\text{IP}_3\)R also contains structures sufficient for its ER retention (3, 11). This suggests that these two proteins likely contain common ER retention signal sequences within their C-terminal region. When the amino acid sequences within the C-terminal region of RyR and \(\text{IP}_3\)R are compared, there is a high degree of sequence identity (11 of 18 residues) in the region of transmembrane segment 4 of RyR and the corresponding region of \(\text{IP}_3\)R (Fig. 6). A lesser degree of identity is evident in the regions of transmembrane segments 1, 2, and 3 of RyR and the corresponding regions of \(\text{IP}_3\) receptor (25).

In summary, our results demonstrate that the transmembrane segments of RyR (and \(\text{IP}_3\) receptor) not only contribute to
the formation of the Ca\(^{2+}\) channel pore but also participate in the sorting of these proteins to proper subcellular compartments. While the exact mechanism by which ER retention of RyR occurs is not known, we cannot rule out the possibility of interaction with other common cellular proteins in this protein sorting process. Further experiments involving mutations of amino acids in the transmembrane segments that are common to RyR and IP\(_3\) receptors will help us better understand the structural basis for ER targeting of RyR.

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