Targeting and Retention of Type 1 Ryanodine Receptors to the Endoplasmic Reticulum*  

Most ryanodine receptors and their relatives, inositol 1,4,5-trisphosphate receptors, are expressed in the sarcoplasmic or endoplasmic reticulum (ER), where they mediate Ca\(^{2+}\) release. We expressed fragments of ryanodine receptor type 1 (RyR1) in COS cells alone or fused to intercellular adhesion molecule-1 (ICAM-1), each tagged with yellow fluorescent protein, and used confocal imaging and glycoprotein analysis to identify the determinants of ER targeting and retention. Single transmembrane domains (TMD) of RyR1 taken from the first (TMD1–TMD2) or last (TMD5–TMD6) pair were expressed in the ER membrane. TMD3–TMD4 was expressed in the outer mitochondrial membrane. The TMD outer pairs (TMD1–TMD2 and TMD5–TMD6) retained ICAM-1, a plasma membrane-targeted protein, within the ER membrane. TMD1 alone provided a strong ER retention signal and TMD6 a weaker signal, but the other single TMD were unable to retain ICAM-1 in the ER. We conclude that TMD1 provides the first and sufficient signal for ER targeting of RyR1. The TMD outer pairs include redundant ER retention signals, with TMD1 providing the strongest signal.

Ryanodine receptors (RyR)\(^{3}\) compose a family of intracellular \(\text{Ca}^{2+}\) channels that mediate release of \(\text{Ca}^{2+}\) from the intracellular stores of excitable and non-excitable cells (1). The three mammalian subtypes of RyR (types 1–3) share \(\sim \)70% amino acid sequence identity, and they are also related to inositol 1,4,5-trisphosphate receptors (IP\(_3\)R) (1, 2). All subtypes of each of the major families of intracellular \(\text{Ca}^{2+}\) channels are expressed predominantly, although not exclusively, in the endoplasmic reticulum (ER) or sarcoplasmic reticulum (3, 4). Both families of channels are regulated by diverse intracellular signals and they also share structural features. All IP\(_3\)R are regulated by cytosolic \(\text{Ca}^{2+}\) and modulated by many of the signals that regulate IP\(_3\)R (1), but RyR subtypes differ in their most important modes of physiological regulation. RyR1, the major isoform of skeletal muscle, is activated by depolarization of the sarcolemma transmitted from dihydropyridine receptors in the plasma membrane to RyR in the junctional sarcoplasmic reticulum (7). In cardiac muscle, the major activator of RyR2 is the local increase in cytosolic \([\text{Ca}^{2+}]\) that follows depolarization-evoked activation of dihydropyridine receptors. In other cells in which each of the three RyR subtypes can be expressed (8), cytosolic \(\text{Ca}^{2+}\) and such signals as cyclic ADP-ribose (9) are probably the major regulators of RyR.

Both IP\(_3\)R and RyR form homo- or heterotetrameric assemblies of subunits (3, 10). Each subunit contains a large cytosolic N-terminal domain, a short C-terminal tail, and a stretch of hydrophobic transmembrane domains (TMD), the last two of which form a cation-selective pore with the intervening luminal loop (11–13). The biophysical properties and probably the structure of the pore are similar for RyR and IP\(_3\)R (14), and a structure similar to the inositol 1,4,5-trisphosphate-binding core is present within a similar part of RyR1 (15).

Considerable evidence suggests that each IP\(_3\)R subunit has six TMD (16, 17). The transmembrane topology of RyR is more contentious (18, 19). It is clear that the N and C termini are cytosolic (20), that the only TMD lie toward the C terminus, and that all models share four TMD, but there have been competing claims for 4–12 TMD (20–23). The position of the final pair of TMD and the role of the intervening loop in forming a selectivity filter are clear (see Fig. 1A) (13, 19, 24). This region is most conserved within RyR and IP\(_3\)R (see Fig. 1B), and point mutations within the loop affect the conductance of the channel (4, 24, 25). Analysis of membrane insertion of RyR1 fragments defined the boundaries of the six TMD shown in Fig. 1A, but suggested also the existence of another membrane-associated region (residues 4329–4381; “M4a–M4b” in Ref. 20) before the TMD1 shown in Fig. 1A. Substantial additional attempts to resolve the topology of this region (26) failed to demonstrate the presence of a pair of TMD before TMD1, suggesting that the region may be associated with the membrane without integrating into it. The latter is consistent also with three-dimensional reconstructions of cryoelectron microscopic images of RyR1. These show that the transmembrane stalk includes at least five \(\alpha\)-helices from each of the four subunits and that it is large enough to accommodate at least six TMD from each (15, 27). The most recent high resolution structure of the pore region of RyR1 suggests also that the M4a–M4b region may lie along the cytosolic membrane surface rather than forming TMD (27).
Collectively, these observations suggest that RyR, like IP₃R, have six TMD (see Fig. 1A). In our analysis, we assumed that RyR1 has six TMD (see Fig. 1A). The boundaries of the shared TMD are the same as those proposed by Bhat and Ma (28) in their four-TMD model, with their TMD1–TMD2 equating with ours, but their TMD3–TMD4 equating with our TMD5–TMD6 (see Fig. 1A and supplemental Fig. S1).

Most cells express IP₃R; many express RyR; and many express both, although tissues differ considerably in their complements of IP₃R and RyR subtypes. The patterns of expression change also during development. Although most IP₃R and RyR in most cells are expressed in the ER or sarcoplasmic reticulum, the two channels may be segregated within those organelles (29), and they may also be expressed in the nucleoplasm (30, 31), secretory vesicles (32), and plasma membrane (4, 33). Such specific subcellular targeting of intracellular Ca²⁺ channels is an important determinant of the spatial organization of intracellular Ca²⁺ signals, but the mechanisms are not understood (34).

ER-resident membrane proteins must first be targeted to the ER, usually by the signal recognition particle, which recognizes a stretch of 7–15 hydrophobic residues as they emerge from the ribosome (35). The signal recognition particle then delivers the nascent protein-ribosome complex to the Sec61 translocon, allowing co-translational insertion of the protein into the ER membrane (36). Once targeted to the ER, resident proteins must be retained by preventing them from leaving or by active retrieval from the Golgi (37). Both cytosolic (di-Arg- or C-terminal di-Lys- or Tyr-based motifs) and TMD sequences can mediate retention or retrieval of ER membrane proteins (discussed in Ref. 34). RyR1 lacks C-terminal di-Lys motifs, and the N-terminal di-Arg motifs are unlikely to be effective. For both IP₃R and RyR, TMD provide the major signals for ER retention (28, 34, 38). Our previous analysis of IP₃R established that ER retention signals are provided by any pair of TMD together with the linking luminal loop or by TMD5 or TMD6 alone, although the single TMD provide weaker retention signals than do the pairs (34). A similar analysis of RyR1 concluded that only TMD6 includes an ER retention signal: TMD1–TMD2 and TMD5 were suggested to be ineffective (28). The signals that target RyR1 or IP₃R to the ER are less clear, although they, too, involve the TMD. A naturally occurring N-terminally truncated RyR1 comprising only its last 656 residues and therefore including TMD1–TMD6 is targeted to and retained within the ER (39). A similar fragment of RyR2 is also targeted to the ER (40). For IP₃R, the first pair of TMD is required for ER targeting, but either TMD when expressed alone is targeted to mitochondria (34).

Expression of fragments of IP₃R or RyR in the ER requires that they are both targeted to the ER and then retained within it: such fragments must therefore include both targeting and retention signals. ER retention signals can be identified in proteins that lack ER-targeting signals by constructing chimeric proteins with intercellular adhesion molecule-1 (ICAM-1), a plasma membrane protein with a single TMD that has its own ER-targeting sequence (28). Here, we used fragments of RyR1 and the same fragments attached to ICAM-1 to address the mechanisms responsible for ER targeting and retention of RyR1.

**MATERIALS AND METHODS**

**Cell Culture and Transfection**—COS-7 cells were cultured in minimal essential medium with 5.6 mM glucose, 10% fetal bovine serum, and 2 mM l-glutamine. They were transfected using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's instructions (34).

**Preparation of Expression Constructs**—Full-length rabbit RyR1 (GenBank™ accession number X15209) (21) N-terminally tagged with enhanced green fluorescent protein (EGFP) was used to examine subcellular targeting of full-length RyR1. This construct was a gift from Dr. David MacLennan (University of Toronto) (20). PCR was used to isolate fragments of RyR1 from the same construct using the primers listed in supplemental Table S1. Fragments of the RyR1 sequence were subcloned in-frame into the pEYFP-C1 vector (Clontech) to allow expression of RyR1 fragments N-terminally tagged with enhanced yellow fluorescent protein (EYFP) (see Fig. 1C). EYFP-ICAM and EYFP-tagged chimeras of ICAM-1 with fragments of RyR1 (Fig. 1C) were produced by PCR amplification of the RyR1 fragment from the pEYFP-C1 clones using the EYFP-ICAM primers (supplemental Table S1) and ligated into the pB1Neo vector containing ICAM-1 (a gift from Dr. Mark Davis, Stanford University) (41). The coding sequences of all constructs were confirmed by sequencing (supplemental Table S2), which also confirmed several minor nucleotide differences from the published RyR1 sequence (20). The expression construct for full-length OIP106 (Q-linked N-acetylglucosamine transferase-interacting protein 106) N-terminally tagged with c-Myc was provided by Dr. Anne Stephenson (School of Pharmacy, London) (42).

**Immunostaining and Confocal Imaging**—Nearly confluent COS-7 cells grown on glass coverslips and transfected with appropriate vectors were used 20 h after transfection. Cells were washed with phosphate-buffered saline, fixed with 4% paraformaldehyde in phosphate-buffered saline for 5 min, and then permeabilized with 0.1% Triton X-100 for 5 min. The following antibodies (in phosphate-buffered saline with 5% bovine serum albumin) were used: rabbit anti-calreticulin antisera (1:100; Calbiochem), anti-GM130 monoclonal antibody (1:100; BD Transduction Laboratories), and anti-Myc monoclonal antibody (1:200; Cell Signaling Technology) with goat anti-rabbit or goat anti-mouse Alexa 633- or Alexa 488-coupled secondary antibodies (1:500; Molecular Probes). Coverslips were mounted in either ProLong (Invitrogen) or 85% glycerol with 2.5% n-propyl gallate and stored at −20 °C. Mitochondria were identified using MitoTracker Red (Molecular Probes) as reported previously (34). Confocal imaging was performed using a Bio-Rad Radiance 2000 or a Leica TCS SP2 AOBS scanning confocal microscope with settings that avoided bleed-through between pairs of indicators. Images were imported into Adobe Photoshop and adjusted to use the full range of pixel intensities. The images shown are typical of many fields from at least three independent transfections (see legend to Fig. 2).

**Western Blotting and Deglycosylation**—Membranes and supernatant fractions were prepared by centrifugation (30,000 × g, 30 min) from transfected cells. The fractions were
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either prepared for Western blotting or incubated with 0.1 unit/ml endoglycosidase H or 10 units/ml N-glycosidase F (both from Roche Applied Science) at 37 °C for 4 h exactly as described previously (34). For Western blotting, EYFP- or EGFP-tagged proteins were identified using rabbit anti-green fluorescent protein (GFP) antibodies (1:1000; AbCam) with horseradish peroxidase-conjugated donkey anti-rabbit second-
ary antiserum (1:5000; AbCam) and SuperSignal West Pico chemiluminescent substrate (Perbio) to visualize antibody binding. Western blotting was performed using the iBlot dry gel transfer system (Invitrogen), and the blots were quantified using GeneTools software (Syngene).

RESULTS AND DISCUSSION

Expression of Full-length RyR1 in the ER—When expressed alone, GFP was diffusively distributed throughout the cytoplasm and nucleoplasm of COS-7 cells and co-localized with neither calreticulin (Fig. 2A) nor cyan fluorescent protein-ER (cyan fluorescent protein tagged with the signal sequence from calreticulin at its N terminus and KDEL at its C terminus) (data not shown). Treatment of GFP-transfected cells with 0.1% Triton X-100 for 5 min at 37 °C released fluorescence from 97% of the cells (Fig. 3E), and after crude subcellular fractionation, most of the GFP was found in the supernatant (S) and membrane (M) fractions. Numbers denote the positions of the M, markers. Subsequent figures are presented in the same format. Here and in all subsequent figures, images are typical (i.e. comparable with >70% of transfected cells) of 30 cells from each of three or more independent transfections. Western blots are representative of more than three independent experiments. Scale bars = 10 μm.

FIGURE 1. Fusion proteins used. A, the proposed positions of TMD within RyR1 are shown. B, the primary sequences of TMDs and TMD6 of rat IP3R1 and rabbit RyR1 are shown. C, the constructs used are shown, with TMD shown by vertical lines, EYFP or GFP by ovals, and ICAM-1 by wavy lines. RyR1 residues are numbered according to Ref. 21. For each construct, the numbers of residues before and after (before/after) the first and last TMD are shown. Supplemental Fig. S1 compares the constructs used previously for analyses of IP3R1 and RyR1 targeting (28, 34).

FIGURE 2. Subcellular distribution of RyR1 in COS-7 cells. A–C, cells were transfected with GFP alone (A) or with full-length RyR1 tagged with GFP (B and C). The distribution of GFP is shown in the left panels, the distribution of calreticulin or calnexin staining in the middle panels, and the merged images in the right panels, with GFP shown in green and ER markers in red. D, Western blots show distribution of GFP and GFP-RyR1 between supernatant (S) and membrane (M) fractions. Numbers denote the positions of the M, markers. Subsequent figures are presented in the same format. Here and in all subsequent figures, images are typical (i.e. comparable with >70% of transfected cells) of 30 cells from each of three or more independent transfections. Western blots are representative of more than three independent experiments. Scale bars = 10 μm.

neither calreticulin (Fig. 2A) nor cyan fluorescent protein-ER (cyan fluorescent protein tagged with the signal sequence from calreticulin at its N terminus and KDEL at its C terminus) (data not shown). Treatment of GFP-transfected cells with 0.1% Triton X-100 for 5 min at 37 °C released fluorescence from 97% of the cells (Fig. 3E), and after crude subcellular fractionation, most of the GFP was found in the supernatant (Fig. 2D). These and previous results (20, 28, 34) confirm that GFP contains no intrinsic ER localization signals.

Full-length RyR1 N-terminally tagged with EGFP was localized to the ER. The receptors were located within fine reticular networks, which were most abundant around the nucleus; co-localized with calreticulin and calnexin (Fig. 2, B and C); and were associated with the membrane fraction (Fig. 2D). We reported previously an indistinguishable distribution for IP3R1 in COS-7 cells (34), and others have reported expression of complete (20, 43) or almost complete (28) N- or C-terminally tagged GFP-RyR1 in the ER of various cell types.

The TMD Region of RyR1 Is Expressed in the ER—COS-7 cells do not express endogenous RyR (44). This allows heterologous expression of RyR1 fragments to be used to identify determin-
nants of ER expression with no risk that fragments might be retained within the ER by forming oligomers with native RyR. A fragment of RyR1 that includes the six TMD but excludes the N-terminal region (RyTMD1–6C) (Fig. 1C) was expressed in the ER, co-localized with calreticulin (Fig. 3A) and calnexin (data not shown), and was retained in cells after permeabilization (Fig. 3E). Identical results were obtained after removal of the C-terminal tail from this fragment (RyTMD1–6) (Fig. 3, B and E). Expression of similar fragments of IP3R1 caused significant changes in cellular morphology that were prevented by introducing an inactivating mutation within the pore (34). We had anticipated similar problems with RyR1, but the morphology of cells expressing RyTMD1–6C, RyTMD1–6, or RyTMD5–6 was normal (Figs. 3 and 4C), although the levels of expression of these fragments were much lower than for similar fragments of IP3R1. Others have also reported low levels of heterologous expression of RyR1 (44).

Previous work showed that removal of either much of the N-terminal (28, 39) or most of the C-terminal tail (28) of RyR1 failed to affect ER targeting and retention. Our results extend these observations and confirm that the region of RyR1 that includes its six TMD is sufficient both to target it to the ER and to retain it there. We have not examined whether additional redundant ER localization signals are present within either of the cytosolic regions, and we cannot exclude expression of some RyR1 in the Golgi (see below), but it is clear that neither RyR1 nor RyTMD1–6 is significantly co-localized with GM130 (Fig. 3, C and D), a marker of the early Golgi (45). We conclude that the TMD region of RyR1 lying between residues 4551 and 4943 is sufficient to mediate both targeting to and retention within the ER.

The First and Last Pairs of TMD from RyR1 Are Expressed in the ER—The first (RyTMD1–2) and final (RyTMD5–6) pairs of TMD were expressed in the ER. They were distributed in a fine reticular network and co-localized with calreticulin (Fig. 4A and C) and cyan fluorescent protein-ER (data not shown); and after subcellular fractionation, both fragments were found in the membrane fraction (Fig. 4D). The subcellular distribution of the middle pair of TMD (RyTMD3–4) was entirely different: it was associated with membranes after cell fractionation (Fig. 4D), but it was not within the ER (Fig. 4B). RyTMD3–4 was instead associated with mitochondrial membranes, forming a ring around each area of staining with MitoTracker Red, a mitochondrial matrix marker (Fig. 5A).

The annular distribution of RyTMD3–4 around the MitoTracker Red staining (Fig. 5A) left open the possibility that this...
fragment might have been targeted to ER associated specifically with mitochondria. To address this possibility, we coexpressed RyTMD3–4 with Myc-tagged OIP106 (42). The latter associates with kinesin and causes mitochondria to aggregate (42). In COS cells, too, expression of OIP106 caused the mitochondria to aggregate (Fig. 5, B, C, and E), and RyTMD3–4 co-localized with these aggregates (Fig. 5B). By contrast, the distribution of RyTMD1–2, which is targeted to the ER (Fig. 4A), was unaffected by coexpression of OIP106 (Fig. 5, D and E). Furthermore, whereas OIP106 and RyTMD3–4 were perfectly co-localized (Fig. 5B, inset), both formed annuli around the Mitotracker Red staining (Fig. 5, A and C, insets). These results confirm that RyTMD3–4 is expressed within the outer mitochondrial membrane and so lacks ER-targeting sequences. Furthermore, expression of RyTMD3–4 within an intracellular membrane (Figs. 4 and 5) is consistent with this region (residues 4770–4830) including two TMD, an even number to allow the N and C termini of the receptor to be cytosolic (20); and the region is too short to accommodate four TMD. Our results are incompatible with a RyR1 topology that includes only four TMD, but consistent with the six-TMD topology (Fig. 1A).

The Single TMD from the First and Last Pairs of RyR1 Are Expressed in the ER—When expressed alone, either of the last pair of TMD (RyTMD5 or RyTMD6) was expressed in the ER. Each co-localized with calreticulin, formed a fine reticular network (Fig. 6, C and D), and was associated with the membrane fraction (Fig. 6E). Similar results were obtained previously for IP3R1, where both TMD5 and TMD6 were expressed in the ER (34). Either of the first pair of TMD from RyR1 (RyTMD1 or RyTMD2) was also expressed in the ER (Fig. 6, A and B; and supplemental Fig. S2), and they, too, were associated with the membrane fraction (Fig. 6E). By contrast, although the first TMD pair of IP3R1 was expressed in the ER, either TMD alone was expressed in mitochondria (34). The difference may result from the shorter TMD1–TMD2 loop in IP3R1 (12 residues versus 62 residues in RyR1). This would allow the sequence recognized by a signal recognition particle within TMD1 of RyR1 to be extruded from the ribosome before translation of TMD2, whereas for IP3R1, it would become accessible only after translation of TMD2.4

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Because RyTMD3–4 was expressed in the outer mitochondrial membrane (Fig. 5), neither TMD alone is likely to mediate ER targeting and retention, although each was expressed in membranes (Fig. 6E). The subcellular localization of TMD3 and TMD4, neither of which was expressed in the ER (supplemental Fig. S3, A and B), confirms this conclusion.

**RyR1 Is Not N-Glycosylated**—Glycosylation can provide another means of examining whether proteins leave the ER. N-Linked glycoproteins remain sensitive to N-glycosidase F throughout their processing, but they become insensitive to endoglycosidase H after processing in the medial Golgi (46). The susceptibility of proteins to endoglycosidase H can thus be used to establish whether proteins have traversed the Golgi (34). Within the luminal loops of RyR1, there is only a single consensus site (Asn4864) for N-linked glycosylation; it lies between TMD5 and TMD6, but only 5 residues from the likely end of TMD5 (Fig. 7C). Under conditions in which we detected glycosylation of IP₃R1, ICAM-1 (34), or ICAM-RyTMD1–6 (Fig. 7, A and B), we detected no glycosylation of RyR1 fragments (Fig. 7). Because N-linked oligosaccharides are added to growing polypeptide chains only when 12–14 residues have emerged from the translocon (46), Asn4864 of RyR1 is unlikely to become accessible to the oligosaccharyltransferase complex. We were thus unable to use glycosylation assays to assess whether RyR1 fragments reach the medial Golgi. More important, we conclude that RyR1 (unlike IP₃R1) (17) is not N-glycosylated.

The analysis so far, using fragments of RyR, required both ER targeting and retention for proteins to be expressed in the ER. Our results thus establish that each of TMD1, TMD2, TMD5, and TMD6 of RyR1 contains signals that allow both ER targeting and retention. Subsequent experiments focused specifically on ER retention by allowing ICAM-1 to direct RyR1 fragments to the ER and then assessing whether those fragments are sufficient to retain the chimeric protein within the ER membrane.

**Pairs of TMD from RyR1 Provide ER Retention Signals**—ICAM-1 was expressed at the plasma membrane (Fig. 8A), associated with the membrane fraction, and its susceptibility to N-glycosidase F and substantial insensitivity to endoglycosidase H (Fig. 8F) is consistent with the fully processed form having progressed through the medial Golgi (34). The first (RyTMD1–2) or last (RyTMD5–6) pair of TMD from RyR1 linked by the luminal loop and then attached via EYFP to the C terminus of ICAM-1 (Fig. 1C) caused retention of the protein within the ER. The chimeric proteins co-localized with calreticulin (Fig. 8B–D), were membrane-associated (Fig. 8E), and were susceptible to both N-glycosidase F and endoglycosidase H (Fig. 8F). The ability of RyTMD5–6 to retain ICAM within the ER is consistent with a previous report (28), but the authors...
concluded that RyTMD1–2 lacked ER retention signals. However, the confocal image from which that conclusion derives (Fig. 4B in Ref. 28) did not show expression of the ICAM-RyTMD1–2 chimera at the plasma membrane and the protein was not fully glycosylated, unlike ICAM itself, which was fully glycosylated and expressed at the plasma membrane (Fig. 3B in Ref. 28). We suggest therefore that our conclusion that RyTMD1–2 includes ER retention signals is consistent with previous observations (28).

Expression of ICAM-RyTMD3–4 often distorted the structure of the ER. In those cells in which the ER remained discernible, ICAM-RyTMD3–4 and calreticulin were co-localized (Fig. 8C), and the chimeric protein associated with the membrane fraction (Fig. 8E), but it was not glycosylated (Fig. 8F). The latter and its low level of expression suggest that ICAM-RyTMD3–4 may have been misfolded and targeted for degradation (47). These results, the observation that RyTMD3–4 alone is expressed in mitochondria (Fig. 5, A and B), our finding that neither TMD3 nor TMD4 is targeted to the ER (supplemental Fig. S3, A and B), and our observation that neither TMD retains ICAM within the ER (supplemental Fig. S3, C and D) suggest that RyTMD3–4 lacks effective ER targeting and retention signals. We conclude that the first and last pairs of TMD from RyR1, together with a linking luminal loop, can each function as an ER retention signal.

**TMD1 Provides the Strongest ER Retention Signal**—Confocal microscopy in combination with analysis of the glycosylation status of the chimeric proteins showed that of the six single TMD of RyR1, only TMD1 reliably retained ICAM-1 within the ER (Fig. 9). The ICAM-RyTMD1 chimera co-localized with calreticulin (Fig. 9A and supplemental Fig. S4) and was entirely sensitive to endoglycosidase H (Fig. 9E). TMD6 provided a less effective retention signal than TMD1. The subcellular distribution of ICAM-RyTMD6 was heterogeneous, with some cells showing substantial co-localization with the ER, others showing expression at the plasma membrane (supplemental Fig. S5), and many showing expression in both membranes (Fig. 9D). In keeping with this subcellular distribution, only a fraction of the fully glycosylated protein was endoglycosidase H-sensitive (Fig. 9E). By contrast, neither TMD2 nor TMD5 prevented ICAM-1 from reaching the plasma membrane (Fig. 9, B and C), and the fully glycosylated ICAM-1 chimeras remained insensitive to endoglycosidase H (Fig. 9E).

Hence, although either of the outer pairs of TMD from RyR1 (RyTMD1–2 and RyTMD5–6) is sufficient to retain ICAM-1 within the ER and to prevent its progress beyond the medial Golgi (Fig. 8), only TMD1 and, to a lesser extent, TMD6 alone are sufficient for ER retention of ICAM-1 (Fig. 9). Our conclusion that TMD1 provides the strongest ER retention signal conflicts with the conclusion of an earlier report (which used ICAM-RyR1 chimeras with a C-terminal GFP tag) in which only TMD6 was suggested to be capable of retaining ICAM-1 within the ER (28). However, our results are consistent with the results shown in Ref. 28 (see above) and are similar to those obtained with IP₃R1, where any pair of luminally linked TMD retained ICAM-1 in the ER, but neither TMD5 nor TMD6 alone was effective (34).

**Conclusion: the First of the Six TMD of RyR1 Allows ER Targeting and Retention**—Our experiments were not designed specifically to address the transmembrane topology of RyR1, but our results support the suggestion that there are six TMD (Fig. 1A). The existence of the first (TMD1–2) and last (TMD5–6) pairs of TMD is not contentious (20–23) and is further supported by our results. Short fragments that include TMD3, TMD4, or TMD3–TMD6 are integrated into membranes (Figs. 4D and 6E), and the region between TMD2 and TMD5 must include a pair of TMD to maintain the cytosolic disposition of the N and C termini (20). We suggest therefore that, like IP₃R, RyR1 probably has six TMD (Fig. 1A).

For both RyR1 and IP₃R (34), the TMD provide ER-targeting and retention signals. The retention signals are redundant because any pair of luminally linked TMD from IP₃R and either of the outer pair for RyR1 provide effective retention signals. For RyR1, TMD1 and, to lesser degree, TMD6 alone are able to mediate ER retention. For IP₃R, the first pair of TMD
must be translated for effective ER targeting (34), whereas for RyR1, translation of TMD1 alone is sufficient (Fig. 6). The difference may reflect the differing lengths of the TMD1–TMD2 loop in the two proteins,4 such that for IP₃R1, the sequence recognized by signal recognition particle is extruded from the ribosome only after translation of TMD2, whereas it requires only translation of the longer TMD1–TMD2 loop for RyR1 to expose the N-terminal end of its TMD1. We conclude that both IP₃R and RyR are targeted to the ER by the first TMD and then retained by redundant signals within the TMD.

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