Carboxyl-terminal Sequences Critical for Inositol 1,4,5-Trisphosphate Receptor Subunit Assembly*

Daniel L. Galvan and Gregory A. Mignery‡
From the Department of Physiology, Stritch School of Medicine, Loyola University Chicago, Maywood, Illinois 60153

The inositol 1,4,5-trisphosphate receptor (InsP₃R) is a tetrameric assembly of conserved subunits that each contains six transmembrane regions (TMRs) localized near the carboxyl terminus. Receptor subunit assembly into a tetramer appears to be a multideterminant process involving an additive contribution of membrane spanning helices and the short cytosolic carboxyl terminus (residues 2590–2749). Previous studies have shown that of the six membrane-spanning regions in each subunit, the 5th and 6th transmembrane regions, and the carboxyl terminus are strong determinants for assembly. The fifth and sixth TMRs contain numerous β-branched amino acids that may participate in coiled/coil formation via putative leucine zipper motifs. InsP₃R truncation mutants were expressed in COS-1 cells and analyzed by sucrose density gradient sedimentation and gel filtration for their ability to assemble. Chemical cross-linking with the homobifunctional reagents disulfosuccinimidyl tartrate (dSST) or dimethyl suberimidate (DMS) of mammalian and bacterially expressed carboxyl-terminal containing receptor fragments reveals that sequences within the carboxyl terminus confer the formation of subunit dimers. A series of InsP₃ receptor carboxyl-terminal fragments and glutathione S-transferase (GST)/InsP₃R chimeras were expressed in Escherichia coli and used in an in vitro assay to elucidate the minimal sequence responsible for association of the carboxyl terminus into dimers. The results presented here indicate that this minimal sequence is ~30 residues in length and is localized between residues 2629 and 2654. These residues are highly conserved between the three InsP₃R isoforms (~80% identity) as well as the RyR receptor (~40% identity) and suggest that a conserved assembly motif may exist between the two intracellular receptor families. We propose that assembly of the InsP₃ receptor to a tetramer involves intersubunit interactions mediated through both the membrane-spanning regions and residues 2629–2654 of the carboxyl terminus possibly through the formation of a dimer of dimers.

The second messenger inositol 1,4,5-trisphosphate (InsP₃) occupies a crucial role in intracellular calcium signaling. InsP₃ is generated through phosphoinositide turnover via the hydrolysis of phosphatidylinositol 4,5-bisphosphate by phospholipase C in response to G protein or tyrosine kinase-coupled plasma membrane receptor stimulation. InsP₃ rapidly diffuses to the endoplasmic reticulum and binds to the inositol 1,4,5-trisphosphate receptor (InsP₃R) inducing calcium release transients from intracellular stores. InsP₃-mediated calcium signaling is implicated in numerous, diverse cellular processes including cell proliferation/differentiation, transcription, hepatic glycogen metabolism as well as learning and memory (1, 2).

The InsP₃ receptor family is composed of at least three isoforms that share a high degree of sequence identity. The InsP₃R protein is a tetrameric structure resulting from homo- or hetero-oligomerization of the receptor subunits. Each receptor subunit consists of an amino-terminal ligand binding domain of ~600 amino acids containing many positively charged basic amino acids thought to participate in the ligand binding pocket (3, 4, 5). This region is highly conserved among the three isoforms yet the apparent affinity for InsP₃ of each receptor subtype varies considerably (6). The central ~1600 amino acids of the receptor is the least conserved region between the isoforms and has been coined the coupling or modulatory domain. This region has been proposed to couple to or transduce ligand occupancy to channel opening through a conformational shift and contain numerous sequence motifs for potential modulation of the receptors function. These include modulatory phosphorylation sites as well as ATP, calcium, and calmodulin binding sites. The carboxyl-terminal ~500 amino acids encode the calcium channel domain and include the six transmembrane-spanning segments. The ion permeation pathway is localized within the sequences encompassing the fifth and sixth transmembrane-spanning segments (7). The permeation properties of the three InsP₃R isoforms intrinsic calcium channel are very similar. All channels exhibit similar calcium conductances and ionic selectivity. Despite these similarities, InsP₃ and calcium differentially regulate the individual receptor isoforms (8, 9, 10). These properties likely account for the heterogeneity in calcium signaling observed in cells expressing more than one isoform of the receptor.

In addition to forming the calcium channel, the membrane-spanning sequences are key elements that contribute to receptor subunit assembly into tetramers (3, 11, 12). In particular, the fifth and sixth transmembrane regions are critical determinants for oligomerization (12). A plasmid encoding an InsP₃R construction in which the first four membrane-spanning sequences were deleted assembled into tetramers and...
when characterized electrophysiologically, the recombinant protein formed nearly constitutively open calcium channels (7). These had very similar permeation properties to those of the wild type receptor. The fifth and sixth membrane-spanning regions ability to form stable oligomers has been proposed to be a consequence of interactions mediated by β-branched amino acid residues, which resemble a leucine zipper motif (12). Another sequence region identified as critical for oligomerization of the receptor subunits is the cytosolic 160-carboxyl-terminal amino acids. These residues significantly enhance receptor assembly into high apparent molecular weight oligomers (12). This enhanced assembly was observed with protein contexts containing as few as two transmembrane-spanning regions through constructions containing all six (12). In addition to receptor subunit assembly, the carboxyl-terminal region has been implicated in interactions with the amino-terminal ligand binding domain and possibly contributes to channel activity (13–15). A notable feature of this region of the InsP3R protein family is the high degree of homology with the ryanodine receptor.

In this study we investigated the role of the cytosolic carboxyl-terminal amino acids in the assembly and oligomerization of the InsP3R into functional tetramers. Receptor mammalian expression construct series containing exclusively the 5th, 6th as well as sequences encompassed by both membrane-spanning sequences with progressively truncated carboxyl terminus confers the formation of subunit dimers and are essential for receptor assembly.

**EXPERIMENTAL PROCEDURES**

**Materials**

DuBois’s modified Eagle’s medium was obtained from Mediatech. Fetal bovine serum and penicillin-streptomycin were purchased from New England Biolabs, Roche Molecular Biochemicals, and U.S. Biochemical Corp. All other chemicals were of reagent grade and used without further purification.

**Plasmid Constructions**

A series of plasmid intermediates containing the type-1 InsP3R transmembrane regions (TMR) 5 and 6 and sequences encompassing 5–6 were prepared in pGEM3Z. pGem3Z was modified by inserting annealed GM123-AATCTTCGAAAGCGGGAAGATCTGCGM124-GATCACGATCTCCGGCCCTGCGGAGAAGTCCAGGACGCGCC (nt 7622–7645) and GM016-ACTAGCTGGACAGATCTTCTTGCTGTTGCCCTGGAGATATACTCTCTCCTCGGTGGAGGAG (nt 8202–8264). This process resulted in a pGEM-3Z-based vector series encoding TMR 5, 6 with a sequentially greater truncation of the carboxyl terminus (pGEMTRX–40, –60, –80, –100, –120, and –144 amino acids) was achieved by inserting BglII-digested PCR amplification products into similarly digested pGEMTR intermediate. The 5′ oligonucleotide primer GM021-GGATCCCATGACACGTCCCACTTGCGGAGAAGTCCAGGACGCGCC (nt 8581–8597) and the fragment (nt 8140–9471) was then inserted into the pGEM3Z-digested vector, and the resulting four fragments were ligated using T4 DNA ligase. This resulted in the final series of pTMR5–XC, pTMR6–XC, and pTMR5–6XC expression constructs (Fig. 2).

The amino-terminal truncated series of mammalian vectors were prepared using a similar strategy, except that plasmid pETI100 was used as a host vector. This plasmid is missing the 418-amino-terminal amino acid residues, tetramerizes, and does not bind InsP3 (16). Each of the BglII/XbaI fragments of pGEMTR5–6XC series were exchanged for the wild type sequences of pCMV11 producing the amino-terminal truncated pNH2–TMR5–6XC series of clones (see Fig. 2).

**Bacterial Expression Plasmids**

The chimeric glutathione S-transferase (GST)InsP3R carboxyl termini constructs were built by digesting pGEX-2T with BamHI and inserting similarly digested PCR-amplified fragments. The constructs, pGEXI2590–2749 and pGEXII2590–2669 were built with the TMR sequences as follows: pETI2590, GM201-GGATCCGACACCTTTGCTGACCTG (nt 8096–8114). The 3′ antisense primer for pGEXI2590–2749 was GM022-GAATTCCTAGCTGGCGCTGCTGG (nt 8561–8579) and for pGEXII2590–2669 was GM146-CGGAATCTAGATGATTCTTCCT-CTGATC (nt 8324–8336). pGEXII2625–2645 was assembled with the primer pair of GM222-CGGGATCCGCCAGCTGGATGAAGAG (nt 8200–8216) and GM223-CGGGATCCCTACCACTGGAGACGCAG (nt 8250–8264). pGEXII2646–2665 used the pair of GM224-CGGAATCCTGAGCTGGATGAAGAG (nt 8265–8280) and GM225-CGGAATCTAGATGATTCTTCCT-CTGATC (nt 8309–8324). pGEXII2635–2654 was generated using the primer pair of GM226-CGGAATCCGCCAGCTGGATGAAGAG (nt 8265–8280) and GM227-CGGAATCTAGATGATTCTTCCT-CTGATC (nt 8323–8326). The non-chimeric bacterially expressed carboxyl-terminal fragments were generated by PCR amplification following digestion and insertion into BamHI/Ncol digested pET-SC (18). For the carboxyl-terminal deﬁnition series the same ‘5′ primer was used, GM021-GGATCCCATGACACGTCCCACTTGCGGAGAAGTCCAGGACGCGCC (nt 8581–8590). The 3′ antisense primers were as follows: pETI2590–2749, GM022-CGGGATCCCTAGCTGGCGCTGCTGG (nt 8561–8580); pETI2590–2749, GM032-CGGGATCCCTTTGCTAGCTGGCTGCTGG (nt 8383–8396); pETI2590–2669, GM204-CGGAATCCTTACCATCCTGACG (nt 8383–8396); pETI2590–2669, GM205-CGGAATCCCTTTGCTAGCTGGCTGCTGG (nt 8383–8396); pETI2590–2669, GM206-CGGAATCCTTACCATCCTGACG (nt 8383–8396); pETI2590–2669, GM207-CGGAATCCTTACCATCCTGACG (nt 8383–8396); pETI2590–2669, GM208-CGGAATCCTTACCATCCTGACG (nt 8383–8396). The 5′ primers were as follows: pETI2620–2749, GM021-CGGGAATCCCTTACCATCCTGACG (nt 8323–8345); pETI2625–2749, GM021-CGGGAATCCCTTACCATCCTGACG (nt 8323–8345). The 5′ primers were as follows: pETI2620–2749, GM021-CGGGAATCCCTTACCATCCTGACG (nt 8323–8345). The 5′ primers were as follows: pETI2620–2749, GM021-CGGGAATCCCTTACCATCCTGACG (nt 8323–8345). The 5′ primers were as follows: pETI2620–2749, GM021-CGGGAATCCCTTACCATCCTGACG (nt 8323–8345).
**Fig. 1. Carboxyl-terminal effect on sedimentation properties of InsP₄ R constructs containing all six membrane-spanning regions.** Equivalent amounts of microsomal protein from transiently transfected COS-1 cells expressing recombinant wild-type (TMRI–6+C) or a carboxyl-terminal truncation (TMRI–6–C) products were solubilized with 1% CHAPS and applied individually (panel A) or mixed (panel B) to 5–20% linear sucrose gradients (2 ml) to evaluate assembly. Samples were sedimented at 166,000 × gₕ for 5 h, fractionated (n = 24), resolved on 5% SDS/PAGE, and immunoblotted with TIC or proton pump antibodies to reveal the sedimentation profile. Note, the heterologous sedimentation distribution of the TMRI–6–C expression products compared with that of the wild type (TMRI–6+C) containing the carboxyl terminus to the less dense sucrose fractions (lower fraction numbers) independent of whether the two proteins were sedimented as mixed or individual samples.

**Construction of the Type-2 Ryanodine Receptor Carboxyl-terminal Fragment (pETR4870–4969)**

**RNA Isolation—** Frozen rabbit heart ventricle (1 gram) was ground to a powder in liquid nitrogen and solubilized with GTC (4% guanidine thiocyanate, 20 mM sodium acetate, 1% 2-mercaptoethanol, 0.1% dithiothreitol, 0.5% sarkosyl). The sample was clarified by 15 min, 3220 × g, 20 °C centrifugation. The resulting supernatant was layered on top of a 5.7 M cesium chloride cushion and centrifuged for 16 h at 26,700 rpm, 20 °C in a SW41 rotor. The total RNA pellet was allowed to air dry and dissolved in nuclease-free water, precipitated, and quantitated.

**cDNA Synthesis—** 5 μg of the total RNA was used as a template for first strand cDNA synthesis. Each 50-μl reaction contained 500 μM dNTPs (Amersham Biosciences), 20 μg of RNase inhibitor (Promega), 1 μg of pdN₆ random hexamer (Amersham Biosciences) in 50 mM Tris-HCl, pH 8.3, 75 mM KCl, 3 mM MgCl₂, 10 mM dithiothreitol plus 200–400 units of Moloney murine leukemia virus reverse transcriptase (Invitrogen). Reactions were incubated at 37 °C for 60 min and heat-inactivated for 5 min at 95 °C. The RyR carboxyl-terminal clone was built in modified pET-8C. Oligonucleotides GM230-GATCCTGCAG-AGCTTCGAACACATCGGAGGCGGAATTCTCCAGCAGTAG and GM231-GATCTACTCGTCGAACTTGCCCTCGCGGATGTGTTCGA-AGCTTCGAACACATCGGAGGCGGAATTCTCCAGCAGTAG were annealed and inserted into pETR4870–4969. The identity of the pETR4870–4969 was confirmed by DNA sequencing.

**COS Cell Transfection and Purification**

COS-1 cells were transiently transfected with the InsP₄ R plasmid DNA using the DEAE-dextran method (19). Cells were incubated at 37 °C, 5% CO₂ for 48–72 h prior to harvesting for analysis.

**Preparation of Microsomes, CHAPS Solubilization—** Transfected COS-1 cells were harvested 48–72 h post-transfection. Cells were washed twice in phosphate-buffered saline and harvested by scraping into 50 mM Tris (pH 8.3), 1 mM EDTA, 1 mM 2-mercaptoethanol, and 1 mM phenylmethylsulfonyl fluoride. Cells were lysed by 20 passages through a 27.5-gauge needle. Micromosomal fractions were pelleted by centrifugation at 4 °C for 10 min at (106,120 × g). Microsomes were resuspended in buffer and solubilized by adjusting the buffer to 1% CHAPS and mixing on ice for 1–2 h. Prior to oligomerization analysis, the solubilized samples had the insoluble fraction removed by centrifugation at 4 °C for 10 min at (106,120 × g) two times followed by passage through a 0.22-μm cellulose-acetate Spin-X centrifuge filter (Corning; Corning, NY).

**Sucrose Gradient Sedimentation—** Sucrose gradients were run as previously described (12). Essentially, 2 ml of 5–20% continuous sucrose gradients were centrifuged for 4 h and 55 min, 4 °C, at 166,320 × g in a Beckman TLS-55 swinging bucket rotor. Gradients were subsequently fractionated into 25 × 80-μl aliquots, and InsP₄ R protein was detected by immunoblotting.

**Superose 6 FPLC—** FPLC analyses were performed with an Amersham Biosciences AB Superose 6 HR 10/30 column. The mobile phase was 1% CHAPS, 150 mM NaCl, 50 mM Tris (pH 8.3), 1 mM EDTA, and 1 mM 2-mercaptoethanol and run at 0.4 ml/min. The column eluates were fractionated into 0.25-ml aliquots.

**Bacterial Expression**

**Bacterial Transformation—** BL21-CodonPlus Competent Cells (Stratagene) harboring the InsP₄ R plasmids were cultured overnight in Luria Broth + 50 μg/ml chloramphenicol + 100 μg/ml ampicillin. The culture was diluted and then incubated at 37 °C until reaching an optical density (λ, 600 nm) of 0.7. IPTG was added to a final concentration of 1 mM, and cells were incubated for an additional 3–4 h. Cells were pelleted at 4 °C for 20 min at (48,000 × g). Cell pellets were resuspended in liquid nitrogen and stored at −80 °C until further use.

**Bacterial Expression Products—** Frozen bacterial cell pellets were weighed out, and lysis buffer was added STET (8% sucrose, 0.5% Triton X-100, 10 mM Tris-CL, pH 8.0, 50 mM EDTA, 0.25% lysozyme, 1 mM phenylmethylsulfonyl fluoride) and incubated with shaking at ambient temperature for 20 min. Samples were sonicated 2 × 30 s and the DNase and RNase A added to 4 μg/ml and 10 μg/ml, respectively. Samples were incubated at ambient temperature for 30 min after the addition with shaking. Samples centrifuged at 4 °C for 15 min at (15,000 × g), and supernatants were decanted. Pellets were washed three times in Wash Buffer (0.5 mM NaCl, 0.5% Triton X-100, 1 mM phenylmethylsulfonyl fluoride) and incubated with shaking for 15–30 min. Samples were pelleted at 4 °C for 20 min at (48,000 × g). Cell pellets were resuspended in ice-cold water and frozen at −80 °C.

**In Vitro Assembly Assays—** Bacterial expression products were solubilized in 8 M urea, 150 mM NaCl, 50 mM Tris (pH 7.4), 3 mM 2-mercaptoethanol, and 3 mM dithiothreitol. Following solubilization, the samples were centrifuged at 4 °C for 10 min at (106,120 × g). Pellets were discarded, and the solubilized protein was subjected to dialysis against 4 × 1000 volumes of 150 mM NaCl, 50 mM Tris (pH 7.4), 1 mM 2-mercaptoethanol. Recovered dialysates were centrifuged at 4 °C for 10 min at (106,120 × g) to remove the insoluble fraction and ~25% of each sample removed for SDS/PAGE, followed by Coomassie Blue staining to identify the initial starting material. The remainder of              the sample was interacted with glutathione-Sepharose MicroSpin GST Purification Module mini-columns (Amersham Biosciences). Columns were washed 3 × 0.5 ml with the above dialysis buffer. Bound material was eluted with 10 mM reduced glutathione. Eluted material was run on SDS/PAGE and Coomassie-stained to reveal eluted proteins.

**Western Blots**

SDS-PAGE and Immunoblotting—** COS cell expression products were resolved on 5% SDS/PAGE with the exception of the chemically cross-linked material, which was resolved on 4% SDS/PAGE. Bacterially expressed proteins were run on 14% SDS/PAGE and Tris/Tricine
It is well established that the multiple membrane-spanning sequences of the InsP₃R subunits are essential determinants for the targeting, assembly, and formation of the intrinsic calcium release channel (3, 7, 12, 21). The 5th and 6th membrane-spanning sequences, which together with the interceding luminal loop constitute the channel pore, are efficiently assembled into tetramers in the presence of the cytoplasmic carboxyl termini. Previously, Galvan et al. (12) demonstrated that the inclusion of the carboxyl terminus with membrane-spanning region deletions augmented receptor subunit assembly into oligomers. The apparently additive effect of the carboxyl terminus was observed with recombiant protein containing as few as two through those containing all six membrane-spanning regions (12). In addition, yeast two-hybrid experiments using the cytoplasmic carboxyl-terminal fragment indicated a potential interaction between the termini. In support of these observations, Magnino et al. (22) reported similar observations using the type-2 InsP₃R.

**RESULTS**

It is well established that the multiple membrane-spanning sequences of the InsP₃R subunits are essential determinants for the targeting, assembly, and formation of the intrinsic calcium release channel (3, 7, 12, 21). The 5th and 6th membrane-spanning sequences, which together with the interceding luminal loop constitute the channel pore, are efficiently assembled into tetramers in the presence of the cytoplasmic carboxyl termini. Previously, Galvan et al. (12) demonstrated that the inclusion of the carboxyl terminus with membrane-spanning region deletions augmented receptor subunit assembly into oligomers. The apparently additive effect of the carboxyl terminus was observed with recombiant protein containing as few as two through those containing all six membrane-spanning regions (12). In addition, yeast two-hybrid experiments using the cytoplasmic carboxyl-terminal fragment indicated a potential interaction between the termini. In support of these observations, Magnino et al. (22) reported similar observations using the type-2 InsP₃R.
was a substantial amount of material that did not sediment to density gradient positions consistent with a high molecular weight oligomer. It is unlikely that this sedimentation profile is a consequence of proteolytic perturbations since the expressed protein reacted to both amino- and carboxyl-terminal antibodies and had the expected apparent molecular weight on SDS-PAGE. The converse was true for the full-length receptor. In this case the sedimentation pattern for the expressed full-length protein was more homogeneous. The full-length protein sedimented to gradient positions consistent with those of tetramers and exhibited little, if any, protein in the less dense sucrose fractions.

In experiments aimed at further delineating the sequences responsible for the carboxyl-terminal regions contribution to the InsP₃ receptor assembly process, a series of expression plasmids were constructed (Fig. 2A). The initial construct series encode an InsP₃R protein in which the first four membrane-spanning sequences were deleted, and the remaining two membrane-spanning elements (5 and 6) plus the carboxyl terminus were linked to amino acid residue Arg-2225. This construct was previously shown to contain the critical elements necessary for subunit assembly and a functional calcium release channel (7, 11, 12). From this foundational clone, additional expression plasmids were constructed that had progressively truncated carboxyl termini (Fig. 2A). We elected to employ constructs containing exclusively the 5th and/or 6th membrane-spanning segments in this study for several reasons. First, these two segments have been previously shown to compose the strongest membrane-spanning region-derived determinants for the InsP₃R assembly (11, 12). Secondly, previous studies have established that these two membrane-spanning sequences combined with the carboxyl-terminal tail assemble and form an ion permeation pathway that is widely believed to be a consequence of receptor subunit oligomerization. Finally, our hypothesis was that if the effects imparted by the cytoplasmic carboxyl-terminal sequences on oligomerization were a lesser component of assembly, the apparent additive aspects of the other transmembrane-spanning segments might diminish the ability to dissect and identify the sequences responsible. These truncated receptor plasmids express high levels and are targeted to membranous fractions in transiently transfected COS-1 cells (Fig. 3).

The ability of the TMR5–6 carboxyl-terminal truncations to oligomerize was determined using sucrose density gradient sedimentation. Microsomal fractions from transiently transfected COS-1 cells were solubilized with 1% CHAPS and layered onto 5–20% sucrose gradients. Following centrifugation, the gradients were fractionated and resolved on 5% SDS-PAGE, and the receptor sedimentation profiles were determined by immunoblotting using an amino-terminal antipeptide antibody (T1NH, Ref. 17). The protein encoded by pTMR5–6+C sedimented to positions on the sucrose gradient consistent with those of a tetramer (Fig. 4A).

The sedimentation profiles of the carboxyl-terminal deletions were next analyzed. Protein encoded from constructs (pTMR5-6-XC) in which the carboxyl-terminal 40, 60, 80, and 100 amino acids were deleted, formed oligomers which sedimented to gradient positions consistent with a tetramer (Fig. 4A). When the carboxyl-terminal 120 amino acids or greater (-144) were deleted, the receptor proteins sediment as apparent monomers on sucrose gradients (Fig. 4A) suggesting that some sequences between residues -120 and -100 are important in receptor assembly.

To address whether the presence of both the 5th and 6th membrane-spanning regions contribute to the observed carboxyl-terminal effect on assembly, we prepared similar carboxyl-terminal truncations containing only the 5th or 6th membrane-spanning region (Fig. 2, B and C). Previously, a construct encoding TMRI+ was shown not to assemble (12). These constructs expressed to substantial levels in transiently transfected COS-1 cells and were targeted to membranes (Fig. 3). It appears that expression products containing a single mem-
brane-spanning region are not as efficiently localized to membrane structures as those containing both the 5th and 6th transmembrane regions; however, the majority of the protein corresponding to all constructions is targeted to membrane fractions (Fig. 3).

Carboxyl-terminally truncated proteins containing exclusively membrane-spanning region 5 sediment on sucrose density gradients very similar to that observed for constructions containing both transmembrane regions 5 and 6. All of the expressed proteins, with either the full-length or the -40, -60, -80, and -100 carboxyl-terminal truncations, sedimented to gradient positions consistent with tetramers (Fig. 4B). Proteins in which 120 or 144 carboxyl-terminal amino acids are deleted sedimented as apparent monomers.

Analysis of the carboxyl-terminal truncation series containing exclusively membrane-spanning region 6 yielded results consistent with those observed for the TMR5–6 and TMR5 expression product series. Sedimentation of the TMR6 truncation series through sucrose density gradients revealed that receptor assembly is lost when 120 or more carboxyl-terminal amino acids are deleted (Fig. 4C). The distribution of the TMR6 containing protein through the gradient was more diffuse than...
that of the TMR5–6 and TMR5 truncation series. This was especially the case for the carboxy-terminal deletions. The reason for this is unclear; however, it may suggest that the 6th membrane-spanning element, albeit an important determinant of receptor oligomerization, is not as effective as an independent entity as the fifth membrane-spanning sequence. Alternatively, the less defined assembly of TMR6 expression products may be a consequence of the inverted orientation of the membrane-spanning region or perhaps a translocation artifact, since the sedimentation profiles changed when any perturbation was made to the carboxyl terminus. Nevertheless, a shift in sedimentation profile from high apparent molecular weight oligomers to monomeric positions occurred when −120 or more carboxy-terminal amino acids were deleted.

These results suggest that though membrane-spanning regions 5 and 6 are important determinants for receptor assembly and formation of the permeation pathway, the presence of the receptors carboxy-terminal region is crucial for oligomerization into tetramers. From these truncation series, it would appear that perturbation of the carboxyl terminus between residues 2629 (−120 C) and 2649 (−100 C) abolishes the ability of InsP₃R constructs containing the fifth, sixth, or both transmembrane regions to assemble. These experiments also reveal a propensity for constructs containing the fifth (TMR5 and TMR5–6 series) to assemble, as judged by sedimentation, to a greater extent than those containing exclusively the sixth membrane-spanning sequence. These observations may reflect interactions between these two membrane-spanning sequences as postulated by Galvan et al. (12) and are currently under investigation.²

A direct association of the InsP₃ receptor amino-terminal ligand binding domain and the carboxy-terminal region has been reported (13). This interaction results in a head-to-tail arrangement of receptor subunits and it has been proposed that the ligand binding region of one subunit can gate an adjacent subunit (23). In support of these results, an amino-terminal deletion of 418 amino acids, which does not bind InsP₃, was able to oligomerize with wild-type receptor to form tetramers, which exhibited an increase in InsP₃-mediated channel activity (16, 23). It was hypothesized that this enhanced sensitivity was a result of constitutive activation of the amino-terminal deletion mutant subunit(s) in the functional tetramer. Although homotetramers of a similar amino-terminal deletion were inactive when incorporated into planar lipid bilayers (17). In experiments designed to examine whether this “head-to-tail” interaction was involved with the observed effects of the carboxy-terminal region on receptor assembly, we prepared a construct series lacking the amino-terminal 418 amino acids residues of the ligand binding domain fused to the 5th through 6th membrane-spanning element carboxy-terminal truncation series (pNH₅-TMR5–6-X, Fig. 2D). These plasmids were transiently transfected into COS-1 cells and microsomal protein (Fig. 3), was solubilized in 1% CHAPS detergent followed by sedimentation on sucrose density gradients (Fig. 4D). This truncation series did not express to the high levels typified by the other constructions and extended exposures of the immunoblots revealed the endogenous type-1 receptor present in COS-1 cells. Though heterotetramer formation between the transfected and endogenous receptor populations are possible, the vast majority of the overexpressed constructs are homotetrameric assemblies of the transfected template (17, 24). Joseph et al. (25) suggested that formation of the heteromeric complexes was dependent upon relative expression of the two isoforms and that there may be kinetic constraints that favor homo-oligomers over hetero-oligomers.

Consistent with our previous results, the expression products of this truncation series appeared to form high molecular weight assembly complexes until −120 of the carboxy-terminal amino acids were deleted. These results suggest that the assembly phenomena conferred by the carboxyl terminus does not necessitate the amino/carboxyl-terminal interaction. Similar results were observed by Sayers et al. (26) where it was reported a GFP-InsP₃ chimera containing the membrane-spanning regions and carboxyl terminus assembled into apparent tetramers. Examination of the gradient profile for the pNH₅-TM5–6+C expression products reveals a faint, slower migrating immunoreactive band in fractions 16–22 that corresponds to the endogenous type-1 receptor in the COS-1 cells. These receptors likely represent preformed endogenous tetramers and not substantial levels of mutant/full-length heterotetramers.

**FPCL Analysis of Carboxyl-terminal Truncations**—In experiments to further analyze the effect of the carboxyl-terminal truncations on the receptor assembly process, the expression products of the carboxyl-terminal deletion series containing membrane-spanning regions 5–6 and 5 or 6 individually were subjected to size exclusion FPLC over Superose 6 columns. In this series of experiments, the full-length receptor (pInsP₃R-T1), which forms tetramers, and two mutants lacking all membrane-spanning regions with (pTMR0+C) or without (pTMR0-C) the cytoplasmic carboxyl terminus were used as oligomerization indicators. The expression products of pTMR0+C form dimers (shown below) and those of pTMR0-C are monomeric. All TMR5–6 constructs containing carboxy-terminal residues of the InsP₃ receptor (pETI2590–2749) were expressed in E. coli BL21DE3 cells and resolved on SDS-PAGE with increasing concentrations of SDS followed by Western blotting with T1C. Note the diminution of the upper immunoreactive band of apparent dimers of M₅ ≈ 40 kDa as the SDS concentration is increased (panel A). Chemical cross-linking of the bacterially expressed full-length carboxyl terminus, pETI2590–2749 and a fragment encoding residues 2590–2629 (pETI2590–2629) were incubated with the increasing concentrations of sDST (panel B). The products were resolved by SDS/PAGE followed by Western blotting with either an antibody against the carboxyl terminus (T1C) for pETI2590–2749 or with B188 for pETI2590–2629.

² D. Galvan and G. Mignery, manuscript in preparation.

---

**Fig. 7. Bacterially expressed InsP₃R carboxyl-terminal fragments form apparent dimers.** The 160 carboxyl-terminal residues of the InsP₃ receptor (pETI2590–2749) were expressed in E. coli BL21DE3 cells and resolved on SDS-PAGE with increasing concentrations of SDS followed by Western blotting with T1C. Note the diminution of the upper immunoreactive band of apparent dimers of M₅ ≈ 40 kDa as the SDS concentration is increased (panel A). Chemical cross-linking of the bacterially expressed full-length carboxyl terminus, pETI2590–2749 and a fragment encoding residues 2590–2629 (pETI2590–2629) were incubated with the increasing concentrations of sDST (panel B). The products were resolved by SDS/PAGE followed by Western blotting with either an antibody against the carboxyl terminus (T1C) for pETI2590–2749 or with B188 for pETI2590–2629.
terminal sequences ranging from full length to -100 amino acids eluted from the column consistent with that of a high molecular weight oligomer. In contrast the proteins missing the 120 and 144 carboxyl-terminal residues (pTMR5-6–120, pTMR5-6–144) eluted in much later fractions, similar to those of the pTMR0+C control, suggesting that these two truncated proteins are possibly forming dimers (Fig. 5, A and D). The shift in elution profiles from high to low apparent molecular weights for this truncation series occurs in deletions that are identical to those observed using sucrose density gradients to evaluate receptor assembly. Very similar results were observed using constructs containing only a single membrane-spanning region (pTMR5-XC and pTMR5-XC series). In all cases the proteins derived from the full-length, -40, -80, -120, and -100 carboxyl-terminal truncations eluted from the column at fractions consistent with those of an oligomer. The -120 and -144 proteins eluted from the Superose 6 column at significantly later intervals than the proteins thought to be oligomerizing and at positions similar to the monomeric control (Fig. 5, panels B–D).

**Bacterial Expression/in Vitro Assembly Assays**—In experiments aimed at confirming and localizing the elements within the InsP₃ receptors 160 carboxyl-terminal amino acids that confer assembly, a series of bacterial expression plasmids were generated for use in an *in vitro* assembly protocol (Fig. 6). Two GST/InsP₃ chimeras consisting of the complete carboxyl-terminal 160 amino acids (pGEX2590–2749) and one lacking the carboxyl-terminal 80 residues (pGEX2590–2669) were prepared in pGEX-2T vector for use as “bait” in the glutathione-Sepharose-mediated isolation procedure. An additional series of pET-based (18) carboxyl-terminal truncation expression plasmids that correspond to the mammalian series previously described were prepared. These include the complete carboxyl-terminal region (pETI2590–2749) and truncations of -40 (pETI2590–2709), -60 (pETI2590–2689), -80 (pETI2590–2669), -100 (pETI2590–2649), and -120 (pETI2590–2629) carboxyl-terminal amino acid residues. Recombinant proteins were produced in BL21DE3 *Escherichia coli* host upon induction with IPTG.

The bacterial expression product of the full-length carboxyl terminus was examined by SDS-PAGE. Coomassie Blue staining revealed an intensely stained band at the predicted apparent molecular size (120 kDa) consistent with the native carboxyl terminus (pETI2590–2749) and a non-interacting pETI2590–2629 from the GST pull-down experiments (see below) treated with increasing concentrations of sDST. Western blots using the carboxyl-terminal antibody (T1C) revealed an additional immunoreactive band, M, ~40 kDa in the full-length carboxyl-terminal fragment samples and are consistent with the formation of apparent dimers. The pETI2590–2629 formed no additional immunoreactive bands and remained monomeric. Similar results were obtained using DMS over a similar concentration range (not shown).

In addition, these carboxyl-terminal residues appear to be responsible for the formation of dimers in the absence of membrane-spanning regions. A mammalian expression construct missing the entire membrane-spanning complement fused in-frame to the carboxyl terminus (pInsP₃RA2225–2604, called pTMR0+C) was transiently transfected into COS cells. The expression products of both constructs were then subjected to the homo-bifunctional cross-linkers sDST and DMS. The cross-linking reactions were resolved on 4% SDS-PAGE and subjected to Western blotting using T1NH antibody (Fig. 8). An additional immunoreactive band consistent with a dimer is observed in the expression products of pTMR0+C that were exposed to increasing concentrations of cross-linker. However, no additional immunoreactive bands, consistent with the formation of apparent dimers, were observed in the cross-linking reactions with the expression product of pTMR0-C. The only difference between these two expression plasmids is the absence of the cytoplasmic carboxyl terminus. At cross-linker concentrations above 2.5 mM both of these proteins exhibit additional immunoreactive signals. These likely represent nonspecific cross-linking events, due to the high concentration of cross-linker, to other proteins in the transfected COS-1 cell extracts or nonspecific events between cross-linked dimers in the case of TMR0+C. There appears to be little diminution in the apparent dimer band, which would be expected in the case of higher order oligomer formation. In the case of TMR0-C, there appears to be no detectable dimer formation, yet at cross-linker concentrations of 2.5 mM or higher, similar nonspecific events result in an upward smearing effect above the primary expression prod-

![Chemical cross-linking of the carboxyl terminus of the InsP₃ receptor](image352x570_to_528x738)

**Fig. 8.** Chemical cross-linking of the carboxyl terminus of the InsP₃ receptor. COS-1 cells were transiently transfected with InsP₃ receptor constructs lacking the transmembrane regions with or without the native carboxyl terminus (pTMR0+C and pTMR0-C, respectively). Soluble fractions from the transfected cells were subjected to cross-linker (sDST) at the indicated concentrations and resolved on 4% SDS-PAGE followed by Western blotting with an antibody against the carboxyl terminus (T1NH). Note the additional immunoreactive band of apparent dimers observed in the protein containing the carboxyl terminus (pTMR0+C). No significant signal was observed in the sample missing the carboxyl-terminal 160 amino acids (pTMR0-C).
Chimeric GST/InsP₃R in Vitro Assembly Assays—The pETI carboxyl-terminal truncation series expression products were evaluated for the ability to interact with the GST/InsP₃R chimera containing the full-length carboxyl terminus (pGEX2590–2749). Protein samples were treated with 8 M urea in efforts to abolish any intersample assemblages that would likely occur if an oligomerization motif existed. The samples were then mixed with the similarly urea-treated GST/InsP₃R carboxyl-terminal 160-amino acid chimera and subjected to dialysis to remove the urea and facilitate refolding and protein-protein interactions. After dialysis a small (~25%) fraction of each sample was removed for resolution on SDS-PAGE as “starting material” (Fig. 9, top panels A–C). The remainder of the sample was chromatographed over glutathione-Sepharose affinity beads. The bound protein was eluted using reduced glutathione and resolved on 16.5% SDS-polyacrylamide gels as described by Shagger and Von Jagow (20) and stained with Coomassie Blue (G250). As shown in Fig. 8 (panel A), the GST/InsP₃R carboxyl-terminal fragment was purified in all cases using the glutathione-Sepharose beads. All of the non-GST containing carboxyl-terminal fragments, except pETI2590–2629, co-purified with the GST/InsP₃R C-terminal chimera. Identical results were observed using a truncated GST/InsP₃R chimera (pETI2590–2669) missing the carboxyl-terminal 80 amino acid residues (Fig. 9, panel B). No non-GST containing expressed protein (e.g. pETI series) was recovered on the glutathione affinity matrix in the absence of added GST/InsP₃R chimeras. As an additional control, a mixing experiment in which samples were urea-treated and dialyzed separately and then mixed followed by an additional dialysis interval resulted in no interactions. Only the GST/InsP₃R chimeras were recovered upon chromatography over glutathione resin (data not shown). In both
In vitro assembly assays of amino-terminally truncated InsP$_3$R carboxyl termini. The assembly of amino-terminally truncated InsP$_3$R carboxyl-terminal fragments were analyzed using a glutathione affinity purification strategy and resolved on Tris/Tricine SDS-PAGE followed by Coomassie Brilliant Blue G250 staining. The starting material (post-dialysis) is shown (left panel). Samples, from the left to right correspond to pGEXI2590–2749, pETI2602–2749, pETI2625–2749, pETI2645–2749, pETI2666–2749, pETI2602–2669, and pETI2625–2669. Samples in panel B are identical, except that the –80C chimera (pGEXI2590–2669) was used as bait. The right panel (C) represent controls showing starting materials (top) and materials bound and eluted from the glutathione matrix (bottom). Samples, from the left to right correspond to pGEXI2590–2749, pGEXI2590–2669, pETI2602–2749, pETI2625–2749, pETI2645–2749, pETI2666–2749, pETI2602–2669, and pETI2625–2669. Note that only chimeric proteins were recovered in the assay.

In additional experiments designed to localize the sequences responsible for the carboxyl-terminal interaction, a series of amino-terminal deletions of the 160 amino acid C-terminal fragment were prepared. These plasmids encode polypeptides beginning at residues 2602, 2625, 2645, 2666, and extend to 2749. In addition, two constructs beginning at residues 2625 and 2625 that terminate at residue 2669 (carboxyl-terminal –80) were assembled (Fig. 6). These proteins were analyzed for the ability to form stable associations with the GST/InsP$_3$R full-length (pGEXI2590–2749) and –80 carboxyl-terminal fragments (pGEXI2590–2669) as described previously. As shown in Fig. 10 (panels A and B), all protein fragments of both the full-length and truncated GST/InsP$_3$R sequences are capable of interacting with the pETI series, except for pETI2666–2749. This fragment failed to associate with either the complete or truncated carboxyl-terminal GST/InsP$_3$R protein fragment. These results suggest that residues 2629–2666 border the limits of the C-terminal interaction.

This region was further examined by preparing three overlapping GST fusion constructs (pGEXI) containing residues 2625–2645, 2635–2654, and 2646–2665 (Fig. 6). These expression products were used in pull-down experiments with the full-length carboxyl terminus (pETI2590–2749) and select carboxyl-terminal region fragments that overlapped or were adjacent to residues 2629–2666 (pETI2590–2669, pETI2590–2629, pETI2602–2669, pETI2625–2669, and pETI2666–2749). As shown in Fig. 11 (panels A and B), both the GST-InsP$_3$R expression products of pGEXI2625–2645 and 2635–2654 were able to co-purify the full-length, and recombinant proteins encompassing residues 2590–2666, 2602–2669, and 2625–2669. No protein other than the GST-containing bait was recovered using protein fragments that flank residues 2629–2666 (pETI2590–2629 and pETI2666–2749, Fig. 11, panels A and B). In contrast, pGEXI 2646–2666 did not reveal any significant interaction with any of the query fragments (Fig. 11, panel C). It should be noted that very faint signals are detected in lanes representing query samples 2590–2749, 2590–2669, and 2602–2669 (Fig. 11, panel C). Whether these represent interactions between the bait (pGEXI 2646–2666) or chromatography wash artifacts, as a consequence of the large amount of protein present, is not clear. It is possible however that the 10 amino acid overlap between the pGEXI 2635–2654 and 2646–2665 expression products contains a component of the sequence motif responsible for the interaction.

Consistent with these results, bacterial expression products of pGEXI 2625–2645 and 2635–2654 were able to cross-link to
apparent dimers in the presence of sDST whereas no observable dimerization was observed for the protein fragment, pGEXI 2646–2665, that did not substantially interact (Fig. 12). These results, together with the other GST/InsP 3R pull-down experiments summarized in Table I, imply that the sequences responsible for forming carboxyl-terminal dimers in the InsP3R are localized between residues 2629 and 2654. These results are consistent with the shift in oligomerization state observed in our sedimentation and chromatography data using the series of carboxyl-terminal truncations expressed in COS-1 cells. This sequence region is very homologous between the three isoforms of the InsP3R with an average identity of 80%. The InsP3R and ryanodine receptor (RyR) protein families share significant homology across the carboxyl-terminal region (28) and the region defined in this study as a dimerization motif is similarly conserved with a ~40% identity (Fig. 13A). It is interesting that there is not only conservation between the two intracellular calcium release channels across this region but the homologous residues are spaced nearly equidistantly (35 residues) from the last membrane-spanning region in both protein families. This may indicate a conserved structural organization motif between the two similar protein families that are important in assembly and channel function. In support of this notion, we challenged the full-length carboxyl-terminal region of the InsP3 receptor (pGEXI2590–2749), the ~120 carboxyl-terminal deletion (pGEXI2590–2629), as well as the

![Fig. 11. Identification of the carboxyl-terminal sequences conferring dimerization using InsP3R carboxyl-terminal subfragments.](image)

GST/glutathione mediated in vitro assays were performed and resolved on Tris/Tricine SDS-PAGE followed by Coomassie Brilliant Blue G250 staining. The starting materials (post-dialysis) are shown on the top and the glutathione-bound and eluted samples beneath. Panel A shows materials using the InsP3R/GST chimera pGEXI2625–2645 as bait mixed with the full-length (pETI2590–2749), pETI2590–2669, pETI2666–2749, pETI2625–2669, and pETI2590–2629 query sequences. Panels B and C utilized the same query sequences directed against the pGEXI2635–2654 and pGEXI2646–2665 InsP3R/GST chimeras.

![Fig. 12. Minimal interacting InsP3R carboxyl-terminal fragments chemically cross-link to form apparent dimers.](image)
small fragments encompassing the minimal interacting domain (pGEXI2625–2645, pGEXI2635–2654, pGEXI2646–2665) against the carboxyl-terminal region (residues 4870–4969) of the rabbit type 2 ryanodine receptor (RyR2) in a pull-down assay (21). As shown in Fig. 13B, the RyR2 carboxyl-terminal region (pETR4870–4969) interacted with all of the GST-InsP3R carboxyl-terminal fragments with the exception of pGEXI2646–2665, which revealed no apparent interactions. Both the InsP3R and the RyR2 carboxyl termini were recovered when mixed samples were used to challenge the GST-InsP3R carboxyl terminus, pGEXI2590–2749 (Fig. 13B, lower panel; lane 3). These results support the hypothesis that the sequence similarity between the InsP3R and RyR in this region may confer a common assembly motif shared between these two intracellular release channels.

DISCUSSION

The assembly of InsP3 receptor subunits into a homotetrameric structure is a prerequisite for the formation a functional calcium release channel. Previous studies have impli-
cated the fifth and sixth membrane-spanning elements and the carboxyl-terminal region as critical determinants of receptor assembly into tetramers (11, 12). The assembly role of the fifth and sixth membrane-spanning elements has been hypothesized to be a consequence of the preponderance of β-branched amino acids typical of leucine zipper motif promoting protein-protein interactions (12). The role of these sequences in assembly and formation of the intrinsic channel permeation pathway is currently under investigation.² In previous studies, we observed a significant augmentation of receptor assembly in expression constructs containing the 160 amino acids of the carboxyl terminus (12). In this study we have investigated the role of the cytoplasmic carboxyl terminus in assembly of the InsP₃R into tetramers. Using a combination of density gradient sedimentation, size exclusion chromatography and in vitro biochemical methods we have identified a sequence region spanning residues 2629–2654 of the type-1 receptor that confers the formation of subunit dimers.

This region has an ~80% identity between receptor isoforms and is localized to sequences distal to the sixth and final membrane-spanning region. Similar sequences are found in the ryanodine receptor family and may comprise a shared assembly motif in the two channel proteins (Fig. 13). Remarkably, these sequences appear to be nearly equidistant (35 amino acids) from the last membrane-spanning region of the two receptor families. The sequence similarities between the InsP₃R and the RyR are not limited to only the region responsible for forming dimers, but extend through the pore loop and the last membrane-spanning segment into the carboxyl terminus. Previous studies reported that an antibody directed to this region of the InsP₃R resulted in decreased channel activity and increased apparent affinity for InsP₃ (14, 15). Whether the antibody disrupted the carboxyl-terminal interaction or sterically inhibited channel activity is not clear. Taken together these observations implicate this region as not only critical for receptor subunit assembly but important in the ion permeation pathway as well.

Our results demonstrate that residues 2629–2654 mediate the carboxyl-terminal dimerization of the type-1 InsP₃R receptor subunits and in combination with the assembly determinants conferred by the 5th and 6th membrane-spanning regions, result in the tetramerization and formation of the receptor permeation pathway. A likely scenario for the assembly of the receptor is the formation of protein-protein interactions between subunit transmembrane regions 5 and 6 to form dimers upon translocation into the endoplasmic reticulum. A linkage of the “transmembrane dimers” follows via the carboxyl-terminal dimerization event in the cytoplasm to form the functional tetramer.

Acknowledgments—We thank Dr. Thomas C. Sudhof for the generous gift of the type-1 InsP₃R cDNA and Dr. Dan J. Bare and Jessica Solanki for expert technical assistance.

REFERENCES
1. Berridge, M. J., Lipp, P., and Bootman, M. D. (2000) Nat. Rev. Mol. Cell. Biol. 1, 11–21
2. Joseph, S. K., Thomas, A. P., Williams, R. J., Irvine, R. F., and Williamson, J. R. (1984) J. Biol. Chem. 259, 3077–3081
3. Mignery, G. A., and Sudhof, T. C. (1990) EMBO J. 9, 3893–3898
4. Miyawaki, A., Furutani, R., Ryuk, Y., Yushikawa, S., Nakagawa, T., Saitoh, T., and Mikoshiba, K. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 4911–4915
5. Yoshikawa, F., Morita, M., Monka, T., Michikawa, T., Furutani, T., and Mikoshiba, K. (1996) J. Biol. Chem. 271, 18277–18284
6. Newton, C. L., Mignery, G. A., and Sudhof, T. C. (1994) J. Biol. Chem. 269, 26813–26819
7. Ramos-Franco, J., Galvan, D., Mignery, G. A., and Fill, M. (1999) J. Gen. Physiol. 114, 243–250
8. Ramos-Franco, J., Fill, M., and Mignery, G. A. (1998) Biophys. J. 75, 834–839
9. Hagar, R. E., Burgstahler, A. D., Nathanson M. H., and Ehrlich, B. E. (1998) Nature 396, 81–84
10. Mak, D. D., McBride, S., and Foskett, J. K. (2001) J. Gen. Physiol. 117, 445–446
11. Joseph, S. K., Boehning, D., Pierson, S., and Nicchitta, C. V. (1997) J. Biol. Chem. 272, 1579–1588
12. Galvan, D., E. Borrego-Diaz, P. J. Perez, and Mignery, G. A. (1999) J. Biol. Chem. 274, 28483–28492
13. Booneh, D., and Joseph, S. K. (2000) EMBO J. 19, 5450–5459
14. Nakade, S., Maeda, N., and Mikoshiba, K. (1991) Biochem. J. 277, 125–131
15. Miyawaki, S. I., Yuzaki, M., Nakade, S., Shiraoka, H., Nakamichi, S., Nakade, S., and Mikoshiba, K. (1992) Science 257, 251–255
16. Mignery, G. A., Newton, C. L., Archer III, B. T., and Sudhof, T. C. (1990) J. Biol. Chem. 265, 12879–12885
17. Ramos-Franco, J., Caesepool, S., Fill, M., and Mignery, G. A. (1998) Biophys. J. 75, 2783–2793
18. Studier, F. W., Rosenberg, A. H., Dunn, J. J., and Dubendorff, J. W. (1990) Methods Enzymol. 185, 365–383
19. German, C. (1985) in DNA cloning (Glover, D. M., ed) Vol. II, pp. 143–190, IRL Press, Oxford
20. Schagger, H., and Von-Jagow, G. (1987) Anal. Biochem. 166, 368–379
21. Otsu, K., Willard, H. F., Khanna, V. K., Zorzato, F., and Green, N. M., and MacLennan, D. H. (1990) J. Biol. Chem. 265, 13472–13483
22. Magnino, F., Schmidt, K., Mery, L., and Dafnar, J. F. (2001) Eur. J. Biochem. 268, 5881–5888
23. Fischer, G. A., Clementi, E., Radichman, M., Sudhof, T., Ulrich, A., and Meldeol, J. (1994) J. Biol. Chem. 269, 19216–19224
24. Ramos-Franco, J., Bare, D., Caesepool, S., Nani, A., Fill, M., and Mignery, G. (2000) Biophys. J. 79, 1388–1399
25. Joseph, S. K., Bokkala, S., Booneh, D., and Zeigler, S. (2000) J. Biol. Chem. 275, 16084–16090
26. Sayers, L. G., Miyawaki, A., Muto, A., Takeshita, H., Yamamoto, A., Michikawa, T., Furutani, T., and Mikoshiba, K. (1997) Biochem. J. 323, 273–280
27. Maeda, N., Kawasaki, T., Nakade, S., Yokota, N., Taguchi, T., Kasai, M., and Mikoshiba, K. (1991) J. Biol. Chem. 266, 1109–1119
28. Mignery, G. A., Sudhof, T. C., Takei, K., and DeCamillis, P. (1989) Nature 342, 192–195