Subunit Oligomerization, and Topology of the Inositol 1,4,5-Trisphosphate Receptor*

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Daniel G. Galvan, Emma Borrego-Diaz, Pablo J. Perez, 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 (InsP$_3$R) is a tetrameric assembly of highly conserved subunits that contain multiple membrane-spanning sequences in the C-terminal region of the protein. In studies aimed at investigating the oligomerization and transmembrane topology of the type 1 InsP$_3$R, a series of membrane-spanning region truncation and deletion plasmids were constructed. These plasmids were transiently transfected in COS-1 cells, and the resulting expression products were analyzed for the ability to assemble into tetrameric structures. The topology of the membrane-spanning region truncations and the full-length receptor was determined by immunocytochemical analysis of transfected COS-1 cells using complete or selective permeabilization strategies. Our results are the first to experimentally define the presence of six membrane-spanning regions. These results are consistent with the current model for the organization of the InsP$_3$R in the endoplasmic reticulum and show that the truncation mutants are properly targeted and oriented in the endoplasmic reticulum membrane, thus making them amenable reagents to study receptor subunit oligomerization. Fractionation of soluble and membrane protein components revealed that the first two membrane-spanning regions were necessary for membrane targeting of the receptor. Sedimentation and immunoprecipitation experiments show that assembly of the receptor subunits was an additive process as the number of membrane-spanning regions increased. Immunoprecipitations from cells co-expressing the full-length receptor and carboxyl-terminal truncations reveal that constructs expressing the first two or more membrane-spanning domains were capable of co-assembling with the full-length receptor. Inclusion of the fifth membrane-spanning segment significantly enhanced the degree of oligomerization. Furthermore, a deletion construct containing only membrane-spanning regions 5 and 6 oligomerized to a similar extent as that of the wild type protein. Membrane-spanning region deletion constructions that terminate with the receptor’s 145 carboxy-terminal amino acids were found to have enhanced assembly characteristics and implicate the carboxyl terminus as a determinant in oligomerization. Our results reveal a process of receptor assembly involving several distinct yet additive components and define the fifth and sixth membrane spanning regions as the key determinants in receptor oligomerization.

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‡ Supported by National Institutes of Health Grant R29 MH53367 and the Earle M. Bane Charitable Trust. To whom correspondence should be addressed: Dept. of Physiology, Stritch School of Medicine, Loyola University Chicago, 2160 S. First Ave., Maywood, IL 60153. E-mail: gmigner@luc.edu.

Inositol 1,4,5-trisphosphate (InsP$_3$)$_1$ is a well known second messenger that plays a pivotal role in the regulated release of intracellular calcium. Plasma membrane receptor-coupled activation of G-protein or tyrosine kinase-induced hydrolysis of phosphatidylinositol 4,5-bisphosphate results in the production of InsP$_3$ and subsequent efflux of Ca$^{2+}$ from endoplasmic reticulum stores. InsP$_3$-mediated calcium release has been implicated in numerous, very diverse cellular processes including the initiation/propagation of Ca$^{2+}$ waves, cell growth, secretion, fertilization, and development (1, 2).

The InsP$_3$ receptor family consists of three or four highly homologous members with an overall sequence identity of ~69% (3). Most, if not all, cells express one or more of the InsP$_3$R isoforms. The physiological significance of co-expression of multiple isoforms of the InsP$_3$R within a given cell is not clear, but it has been generally thought to confer release channels that were functionally heterogeneous, differentially regulated, and differentially targeted or a combination of all three possibilities. Recent investigations, from this laboratory and others, have defined the intrinsic calcium channel properties for all three isoforms of the receptor (4–8). These studies reveal that the three isoforms of InsP$_3$R have channels with remarkably similar permeation properties. All channels have similar Ca$^{2+}$ conductances (~60 pS) and almost identical reversal potentials using Cs$^+$ or Ca$^{2+}$, indicating similar ionic selectivity. Despite the similarities observed at the single channel level, the individual receptor types appear to be differentially regulated by calcium and InsP$_3$. These studies demonstrated that the type-1 isoform from cerebellum is modulated by calcium in a biphasic manner, whereas the type-2 and type 3 isoforms are not (5, 6, 8, 9). The type 2 receptor Ca$^{2+}$ channel from cardiac myocytes activates at a lower calcium concentration and sustains this activity over a wide range of Ca$^{2+}$ concentrations. Similar results were observed with the type 3 receptor from rat insulinoma cells (RIN-5F) (8). In addition to the differences in calcium sensitivities observed between the type 1 and 2 receptors, the type 2 homologue exhibits approximately a 3-fold increased efficacy to a similar dose of InsP$_3$. Together, these results imply that the intrinsic calcium channels between isoforms are very similar, yet they appear to be regulated quite differently and may help explain the heterogeneity observed in calcium release events in cells and tissue expressing multiple forms of the receptor.

The InsP$_3$R protein is a tetrameric structure resulting from the homo- or hetero-oligomerization of the receptor isoform subunits. Each subunit can be divided into three principle domains consisting of an amino-terminal ligand binding...
region, a carboxyl-terminal channel domain, and a central coupling or regulatory domain (3, 10). Within the carboxyl-terminal region of the receptors there are numerous arrays of hydrophobic amino acids that form membrane-spanning helices that are thought to form the receptor’s intrinsic calcium channel. The role of these multiple membrane-spanning domains in the oligomerization and membrane targeting of the receptor were demonstrated in mutagenesis studies. These studies revealed that the elimination of residues 2205–2225 encompassing the putative membrane-spanning regions resulted in the expression of soluble, monomeric protein that binds InsP3 with similar affinity and specificity as that of full-length native or recombinant receptor (10). Studies using a green fluorescence protein chimera with amino-terminally truncated receptor confirmed these results and proposed that the membrane spanning domains confer reticular targeting and oligomerization. (11). Joseph et al. (12) used cell-free systems to examine the processing of truncated receptor isoforms (types 1 and 3) and concluded that there were two determinants encompassed by this region required for subunit assembly.

The number and topological organization of the transmembrane-spanning regions of the InsP3R subunit has been difficult to determine. A six-transmembrane-helix model has emerged from numerous computer predictions and sequence homology comparisons, which are supported by immunological and N-linked glycosylation studies (13, 14). The details regarding the receptor’s intrinsic calcium channel structure and organization are limited. The calcium channel pore is thought to form as a result of subunit monomer assembly into tetramers. Thus, a detailed analysis of the components involved in tetramerization and topological organization of the membrane-spanning sequences should provide new insights into the formation of the receptors intrinsic calcium release channel.

In this study, we use type 1 InsP3R expression constructions containing differing numbers and combinations of putative membrane-spanning sequences with or without the carboxyl terminus in a mammalian expression system to evaluate the elements involved in subunit oligomerization. In addition, these expression plasmids were used to determine the topological orientation of the membrane-spanning helices through the endoplasmic reticulum using detergent and streptolysin-O permeabilization/immuno-fluorescence analysis. These experiments confirm the six-membrane-spanning hypothesis of the receptors topological orientation. We show that the oligomerization of the receptor is dependent upon several unique, yet additive determinants. We find that InsP3R mutants expressing the first two transmembrane sequences are sufficient to form high molecular weight complexes and that when coupled to the carboxyl-terminal 14 amino acids, the assembly is enhanced. In addition, a construction lacking the first four transmembrane regions, possessing only the fifth and sixth membrane-spanning helices and the intervening luminal loop, is very efficient at forming tetramers.

**EXPERIMENTAL PROCEDURES**

**Materials**—Dulbecco’s modified Eagle’s medium was obtained from Mediatech. Fetal bovine serum and penicillin-streptomycin were purchased from Life Technologies, Inc. Goat serum and streptolysin-O (SLO) were purchased from Sigma. Fluorescein isothiocyanate-conjugated goat anti-rabbit IgG was obtained from Organon Teknika. Restriction enzymes, DNA modifying enzymes and DNA polymerase were purchased from New England Biolabs, Roche Molecular Biochemicals, and U.S. Biochemical Corp. The Fluoromount-G was purchased from Southern Biotechnology Associates, Inc. Protein-A-Sepharose CL4B was obtained from Amersham Pharmacia Biotech. All other chemicals were of reagents grade and used without further purification.

**Plasmid Constructions**—Expression plasmids harboring increasing numbers of membrane-spanning regions that terminate with a common immunological tag were prepared by polymerase chain reaction.

Briefly, a host plasmid was generated by digesting pIP_RSTOP1267 (10) with KpnI/BstBI followed by insertion of the KpnI–BstBI fragment of pCMV-9 (15). This plasmid was then digested with BstBI, and BstBI/ClaI cut polymerase chain reaction products containing defined membrane-spanning regions were inserted. The 5’ cloning linker (ammonium bifluoride, 6945–6968 (GAATCGAATTCTGGAAATATATTAC), was constant, and the 3’ antisense primers were designed to terminate C-terminal to the query sequence. The specific 3’ oligonucleotide primer sequences were as follows: transmembrane region (TMRI) 1 (GAATCGATGTTGCTTCCTCCTCACCTCTTT), TMRI 2 (GAATCGATGTTGCTTCCTCCTCACCTCCTC), TMRI 4 (CGATCGATGTTGCTTCCTCCTCACCTCTC), TMRI 5 (CGCATCGATGTTGCTTCCTCCTCACCTCTT), and TMRI 6 (CGCATCGATGTTGCTTCCTCCTCACCTCTT), and TMRI 7 (CGATCGATGTTGCTTCCTCCTCACCTTTT). In all cases, the full-length receptor pCMV-9/pInsP3R-T1 (6, 15) was used as the amplification template. The construct lacking all membrane-spanning regions (TMRI–0) was prepared by digesting the host plasmid with BstBI, repair with Klenow DNA polymerase, and religation, resulting in the restoration of the reading frame between the InsP3R and the proton pump immunological tag.

Constructions that included the InsP3R carboxyl-terminal 145 amino acids were prepared using similar polymerase chain reaction strategies. In these cases, amplified product was digested with BstBI/BglII and inserted into a similarly digested intermediate plasmid containing nucleotides 6659–9467. These plasmids were digested with BstBI/BamH, and the resulting fragments were inserted into the full-length type 1 receptor (pCMV-9/pInsP3R-T1) at the BstBI/BamHI sites. The 5’ oligonucleotide primer, corresponding to nucleotides 6945–6968 (GAATCGAATTCTGGAAATATATTAC), was constant, and the 3’ antisense primers were as follows: TMRI 1 (ACTAGTCGACGAGATCTTGGTCTTGTCTTGCTTCCTCCTCACCT), TMRI 2 (ACTAGTCGACGAGATCTTGGTCTTGTCTTGCTTCCTCCTCACCT), TMRI 3 (ACTAGTCGACGAGATCTTGGTCTTGTCTTGCTTCCTCCTCACCT), TMRI 4 (ACTAGTCGACGAGATCTTGGTCTTGTCTTGCTTCCTCCTCACCT), TMRI 5 (ACTAGTCGACGAGATCTTGGTCTTGTCTTGCTTCCTCCTCACCT), and TMRI 6 (ACTAGTCGACGAGATCTTGGTCTTGTCTTGCTTCCTCCTCACCT).

**Procedure of Microsomes, CHAPS Solubilization, and Gradient Sedimentation**—Transfected COS-1 cells were harvested 48–72 h post-transfection, and microsomes were prepared as described previously (15). COS cells were washed with PBS; harvested by scraping into 50 mM Tris-HCl, pH 8.3, 1 mM EDTA, 1 mM β-mercaptoethanol, 1 mM PMSF, and lysed by 40 passages through a 27-gauge needle. Membranes were pelleted by a 20-min centrifugation (289,000 g), resuspended in buffer, and either used immediately or frozen at –80 °C. Microsomal fractions were solubilized in 50 mM Tris-HCl pH 8.3, 1 mM EDTA, 1 mM β-mercaptoethanol, 1 mM PMSF, 1% CHAPS on ice for 1 h. Insoluble fractions were eliminated by a 10-min centrifugation (289,000 g), and the supernatant containing solubilized receptor was fractionated through 5–20% sucrose (w/v) gradients. Sucrose gradients (2 ml) were centrifuged for 5 h, 4 °C at 166,320 g (S 289,000 g). The role of these multiple membrane-spanning domains in the oligomerization and membrane targeting of the receptor were demonstrated in mutagenesis studies. These studies revealed that the elimination of residues 2205–2225 encompassing the putative membrane-spanning regions resulted in the expression of soluble, monomeric protein that binds InsP3 with similar affinity and specificity as that of full-length native or recombinant receptor (10). Studies using a green fluorescence protein chimera with amino-terminally truncated receptor confirmed these results and proposed that the membrane spanning domains confer reticular targeting and oligomerization. (11). Joseph et al. (12) used cell-free systems to examine the processing of truncated receptor isoforms (types 1 and 3) and concluded that there were two determinants encompassed by this region required for subunit assembly.

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catalase (250, 11.3 S), and β-galactosidase (540 kDa, 18 S). In addition, CHAPS-solubilized cerebellar microsomes were applied to control gradients as a source of native InsP3R and RyR (30 S) (16, 17) sedimentation controls. Standard proteins were detected using Coomassie staining and immunostaining.

SDS-PAGE and Immunoblotting—COS cell microsomes and sucrose gradient fractions were analyzed by 5% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) as described previously (15), followed by immunoblotting using chemiluminescence reagents (Amersham Pharmacia Biotech).

Immunocytochemical Analysis of InsP3R Topology—Transiently transfected COS cells were harvested by brief trypsinization followed by plating onto poly-d-lysine-coated glass coverslips. Following an attachment interval, the cells were fixed with 2% paraformaldehyde in phosphate-buffered saline (PBS) and permeabilized with 0.3% Triton X-100 and/or streptolysin-O as described below.

Permeabilization by Triton X-100—Transfected COS-1 cells were washed with PBS and fixed in 2% paraformaldehyde-PBS for 15 min at 25 °C. The coverslips were washed with PBS and incubated for 30 min with solution A (20 mM phosphate buffer, pH 7.2, 0.45 mM NaCl, 0.3% Triton X-100, and 1.0% goat serum). The Triton X-100 was removed by washing with PBS, and the cells were blocked in solution B (20 mM phosphate buffer pH 7.2, 0.45 mM NaCl) containing 10% goat serum and stored at 4 °C until use.

Permeabilization by SLO—Selective permeabilization using SLO was performed by the methods described by Otto and Smith (18). Transfected COS-1 cells were washed with PBS and fixed in 2% paraformaldehyde-PBS for 15 min. The coverslips were washed with PBS and incubated for 15 min at 4 °C with streptolysin-O (200 units/ml) that had been preactivated by a 5-min, 0 °C incubation with 10 mM PMSF, and 2% bovine serum albumin). Samples were clarified by centrifugation at 100,000 g for 5 min at 4 °C. The supernatants were removed and mixed with 2 μl (~2 μg) of the secondary antibody used in immunofluorescence staining was a fluorescein isothiocyanate-conjugated goat anti-rabbit antibody (Organon Teknika).

Immunoprecipitations—Cotransfected COS-1 cells were harvested, and microsomes were prepared as described above. A small amount of the microsomes representing total expression products was removed and added to SDS-PAGE buffer. The remaining microsomes were solubilized by stirring on ice for 2 h in Buffer A (1% Triton X-100, 150 mM sodium chloride, 10 mM Tris-HCl, pH 7.4, 1 mM EDTA, 1 mM EGTA, 1 mM PMFS, and 2% bovine serum albumin). Samples were clarified by centrifugation at 100,000 g for 5 min. The supernatants were removed and mixed with 2 μl (~2 μg) of the secondary antibody used in immunofluorescence staining was a fluorescein isothiocyanate-conjugated goat anti-rabbit antibody (Organon Teknika).

Antibodies—The InsP3R-specific amino-terminal (T1NH) and carboxy-terminal (T1C) polyclonal antibodies were generated against residues 308–326 of the type-1 Sib alternatively spliced isoform and the 19 C-terminal amino acids, respectively (6). The luminal loop antipeptide antibody (V753) is directed against residues 2463–2476 of the type-1 receptor (13). The proton pump tag antibody is directed against the 11 carboxy-terminal residues of the 116-kDa subunit of the proton pump (15, 19). All InsP3R peptide antibodies were affinity-purified using immunogenic peptide. The secondary antibody used in immunofluorescence staining was a fluorescein isothiocyanate-conjugated goat anti-rabbit antibody (Organon Teknika).

RESULTS

It is well established that the multiple membrane-spanning sequences of the InsP3R that reside near the carboxy terminus are involved in the targeting and assembly of receptor subunits into functional tetrameric calcium release channels (10, 11). In experiments designed to study the role of these transmembrane sequences as well as other components in the oligomerization and topology of the type-1 InsP3R, a series of expression plasmid mutations were constructed (Fig. 1).

In the initial mutation series, plasmids encoding carboxy-terminally truncated InsP3R proteins containing increasing
numbers of putative membrane-spanning sequences were prepared in a cytomegalovirus promoter-based mammalian expression plasmid. These truncations were transiently expressed in COS cells followed by analysis of oligomerization by sedimentation on 5–20% sucrose gradients. The density gradients were fractionated into soluble (cytosolic) and membranous components. Equivalent samples (2.5 μg) were loaded onto 5% SDS-PAGE and subjected to immunoblotting using TIC affinity pure antipeptide antibody and detected using ECL (Amersham Pharmacia Biotech). Note the large percentage of soluble protein in samples containing only the first membrane-spanning region with or without the 145 C-terminal amino acids and in the construct with membrane-spanning domains 3 and 4 alone. Constructs containing two or more transmembrane regions, with the exception of TMR3–4+C, are almost exclusively detected in the membrane fraction.

The ability of the mutant receptor proteins to oligomerize into tetramers was evaluated by sedimentation through 5–20% sucrose density gradients (Fig. 3). Previous studies (10, 15) have established that the native and recombinant InsP3R sedi-memt as tetramers and when missing all putative transmembrane sequences, as a monomeric protein. In experiments to verify that the 2-ml gradients employed in this study can resolve tetrameric assemblies from monomers, gradients were run that contained protein standards of known molecular weight and sedimentation coefficients. These are indicated in Fig. 3 above gradient sets from each mutation series containing zero and all six transmembrane-spanning regions. The standard sediment in a linear profile ($r^2 = 0.9948$) as a function of sedimentation coefficient versus gradient fraction and are consistent with previous determinations in that constructs lacking all membrane-spanning regions sediment as monomers and those with the entire complement of membrane spanning segments sediment as tetramers (10, 15). Extrapolation from the standard curve suggests that the InsP3R tetramer has an apparent sedimentation coefficient of ~24 S. In these gradients, the native cerebellar and full-length recombinant InsP3R sedimented with very similar profiles. The utility of the sucrose gradients in detecting the shift of receptor subunits from monomeric to tetrameric states is evident; however, the ability to detect potential assembly intermediates (dimers and trimers) is limited. If a stable population of dimers or trimers was encoded by these constructs, we would expect them to sediment to the central region of the gradient.

Sedimentation of monomeric and tetrameric InsP3R proteins on sucrose gradients. Microsomes from transiently transfected COS-1 cells expressing TMR0–C and TMR1–6–C were solubilized with 1% CHAPS (in the case of TMR0–C, cytosolic proteins were used) and applied to 5–20% linear sucrose gradients (2 ml) to evaluate gradient performance. Samples were sedimented at 166,320 $\times g_{av}$ for 5 h, fractionated (n = 25), resolved on 5% SDS-PAGE, and immunoblotted with the TIC or proton pump antibody to reveal the recombinant protein sedimentation profile. Gradient performance and resolution were evaluated by applying standard proteins of known molecular weight and/or sedimentation coefficients. The gradient position of standard proteins are indicated (lettered arrows) and correspond to bovine serum albumin (65 kDa, 4.36 S) (A), alcohol dehydrogenase (150 kDa, 7.4 S) (B), β-amylase (200 kDa) (C), catalase (250, 11.3 S) (D), β-galactosidase (540 kDa, 16 S) (E), and ryanodine receptor (2256 kDa, 30 S) (F). In addition, CHAPS-solubilized cerebellar microsomes were applied to control gradients as a source of native InsP3R and RyR (30 S) sedimentation controls. Standard proteins were detected using Coomassie staining and immunostaining. Regression analysis revealed that the gradients were linear ($r^2 = 0.9948$) with respect to fraction number and known sedimentation coefficient. Both native cerebellar as well as recombinant full-length InsP3R co-sedimented (not shown).

The reasons for the lack of efficient targeting to the ER is not clear, since an amino-terminally truncated protein containing similar transmembrane sequences, when expressed in an in vitro assay, was reported to be targeted to the pancreatic microsomes (12). All of the additional expression products containing two or more putative transmembrane sequences encoded proteins that were efficiently targeted to the ER (Fig. 2).

The inability of the mutant receptor proteins to oligomerize into tetramers was evaluated by sedimentation through 5–20% sucrose density gradients (Fig. 3). Previous studies (10, 15) have established that the native and recombinant InsP3R receptor sediments as tetramers and when missing all putative transmembrane sequences, as a monomeric protein. In experiments to verify that the 2-ml gradients employed in this study can resolve tetrameric assemblies from monomers, gradients were run that contained protein standards of known molecular weight and sedimentation coefficients. These are indicated in Fig. 3 above gradient sets from each mutation series containing zero and all six transmembrane-spanning regions. The standard sediment in a linear profile ($r^2 = 0.9948$) as a function of sedimentation coefficient versus gradient fraction and are consistent with previous determinations in that constructs lacking all membrane-spanning regions sediment as monomers and those with the entire complement of membrane spanning segments sediment as tetramers (10, 15). Extrapolation from the standard curve suggests that the InsP3R tetramer has an apparent sedimentation coefficient of ~24 S. In these gradients, the native cerebellar and full-length recombinant InsP3R sedimented with very similar profiles. The utility of the sucrose gradients in detecting the shift of receptor subunits from monomeric to tetrameric states is evident; however, the ability to detect potential assembly intermediates (dimers and trimers) is limited. If a stable population of dimers or trimers were encoded by these constructs, we would expect them to sediment to the central region of the gradient.

Sedimentation of CHAPS-solubilized recombinant protein containing TMR 1 and the first two membrane-spanning regions (TMR1–C and TMR1–2–C, respectively) on 5–20% sucrose gradients resulted in a diffuse distribution through the
gradient, with receptor protein evident in both the monomeric and tetrameric positions. The gradient fractions (low sucrose percentage) containing the highest levels of protein for TMR1-C corresponded to fractions containing receptor monomers (Fig. 4). In the case of TMR1–2-C, the distribution was nearly equal through the gradient even when nonsaturating exposures of the immunoblots were performed. The sedimentation profile for the construct expressing the first three transmembrane regions (TMR1–3-C) was shifted to more dense gradient fractions, suggesting that the expressed protein was assembling into oligomers. When constructs with transmembrane regions 1–4, 1–5, and all six (TMR1–4-C, TMR1–5-C, and TMR1–6-C, respectively) were analyzed, the majority of the receptor protein was detected in positions consistent with those of tetramers (Fig. 4). These results indicate that the assembly of the receptor subunits is dependent upon the presence of sequences encompassed by the first four putative membrane-spanning regions and possibly as few as three in the absence of additional carboxyl-terminal sequences. The positive identification of multimeric assemblies of the recombinant proteins containing membrane spanning sequences 1 and 2 and 1–3 lacking the carboxyl termini is difficult; however, the sedimentation shift to the more dense sucrose gradient fractions indicates that these proteins are assembling into multimers. The shift of these proteins to more dense gradient positions suggests that self-association occurs as a consequence of the added transmembrane regions, since this is not observed in TMR0-C, which contains zero membrane-spanning sequences. Further investigation was necessary to test whether these expression products were able to interact and assemble with the full-length protein.

In experiments to investigate whether the truncated receptor proteins were multimerizing, we employed an alternative approach using co-expression of full-length receptor and carboxyl-terminally truncated plasmids followed by immunoprecipitation with an antibody directed against the wild-type type-1 receptor carboxyl terminus (T1C). The formation of any co-assembly products of the truncations with the immunoprecipitated full-length receptor could be identified using the unique carboxyl-terminal tag derived from the 116-kDa proton pump subunit. Previous reports have indicated that heteromultimerization of different InsP3R subtypes is a co-translational process and not an artifact of postexpression processing (12). To confirm these observations, we performed co-transfections and mixing experiments of singly transfected cells with full-length and amino-terminally truncated receptor constructs. The co-expressed proteins were readily immunoprecipitable, whereas only the full-length protein could be recovered from mixing experiments using an amino-terminal specific antibody (T1NH) with either CHAPS- or Triton X-100-solubilized material (data not shown).

Cells co-transfected with full-length receptor and the truncations were harvested, and either total protein in the case of TMR0-C or TMR1-C or CHAPS-soluble microsomes from the remaining combinations (TMR1–2-C, TMR1–3-C, TMR1–4-C, TMR1–5-C, and TMR1–6-C) were immunoprecipitated using a type 1 carboxyl-terminal antipeptide antibody. Following extensive washing, the samples were resolved on 5% SDS-polyacrylamide gels and immunoblotted. Total expression products, prior to immunoprecipitation, were detected using an amino-terminal antibody (T1NH) directed against amino acid residues 309–326 of the type 1 receptor (SI splice variant) are shown in Fig. 5A. Immunoprecipitation of the full-length receptor (TMR1–6+C) was verified by Western blotting the immunoprecipitates with the carboxyl-terminal antibody (T1C) (Fig. 5B). Truncated proteins capable of assembling with the full-length receptor were detected with an antibody directed against an 11-amino acid proton pump tag (Fig. 5C). These results demonstrate that the expression plasmids that encode the first two or more membrane-spanning sequences are capable of forming stable and specific assemblies with the full-length receptor protein.

Expression plasmids that encode proteins containing membrane spanning sequences 1–5 and 1–6 result in significantly greater amounts of immunoprecipitable product than the constructs containing four or fewer membrane-spanning sequences. The role for this increase in recovery of hetero-oligomers for these two expression constructions is not entirely clear. This region has been hypothesized to constitute the channel pore and has been shown in vitro to augment receptor subunit assembly (12, 20).

In an additional experiment asking whether the TMR1–2-C proteins observed on the sucrose gradients at the tetrameric positions were aggregates or bona fide assembly products, full-length and TMR1–2-C were co-transfected and sedimented on sucrose gradients (Fig. 6). Gradient fractions containing monomeric TMR1–2-C (fractions 7–10) and multimeric full-length with TMR1–2-C proteins (fractions 17–20) were immunoprecipitated using T1C antibody and immunoblotted with T1NH to detect co-assembled expression products. Monomeric fractions (fractions 7–10) containing exclusively TMR1–2-C protein failed to immunoprecipitate using the carboxyl-terminal antibody, yet tetrameric fractions (fractions 17–20) containing the full-length and truncated receptor co-precipitated (Fig. 6). These results confirm that the TMR1–2–C expression products observed in tetrameric positions on sucrose gradients are probably not nonspecific aggregates.

The role of the carboxyl-terminal 145 amino acids in the formation and/or augmentation of oligomerization of the receptor into multimeric assemblies was next investigated. This was accomplished by preparing an additional set of expression plasmids, analogous to the first series that contain the receptor carboxyl-terminal 145 amino acids fused in frame to the last putative membrane-spanning region (Fig. 1, bottom panel).
Fractionation of expression products from these plasmids into cytosolic and microsomal components reveals an identical pattern to that observed for the initial truncation series (Fig. 2). Transient expression of the plasmid that encodes zero transmembrane-spanning regions (TMR0+C) resulted in the production of soluble, monomeric protein. Expression of a TMR 1-containing fusion (TMR1+C) followed by density gradient centrifugation revealed that, as was the case for the similar construct lacking the carboxyl termini, the protein was distributed over nearly the entire gradient (Fig. 7). Expression products from the plasmid containing the first two membrane-spanning regions fused to the carboxyl-terminal tail (TMR1–2+C) clearly sedimented as high molecular weight multimers (Fig. 7). These results indicate that the addition of the carboxyl-terminal tail to these sequences facilitated the association of subunits as compared with the sedimentation profiles from the identical sequences lacking the C terminus (Fig. 4). All additional constructions containing additional numbers of membrane-spanning sequences oligomerized to an extent in which tetramers were the predominant species detected (Fig. 7).

In efforts designed to investigate the role of the carboxyl terminus in the assembly of receptor subunits, immunoprecipitation experiments were performed. In these experiments, recombinant proteins from COS cells co-expressed an aminoterminally deleted receptor construct (pCMVI-11 (15)) together with individual chimeras from the array of TMR+C mutants. Co-expression products were immunoprecipitated using an amino-terminal antibody (T1NH) that recognized the TMR+C truncations but not the amino-terminal deletion. Co-assembly products between the TMR+C truncations and the aminoterminal deletion containing all putative membrane-spanning sequences were then detected using the carboxyl-terminal antibody (T1C). All truncated proteins containing two or more of the membrane-spanning domains co-assembled with the amino-terminal deletion as was the case for the constructs in which the carboxyl terminus was deleted (data not shown).

Taken together, the immunoprecipitation and sedimentation data suggest that the initiation of receptor subunit assembly into oligomers can occur with only the first two membrane-spanning regions and is enhanced in the presence of the carboxyl-terminal 145 amino acids. In addition, for both arrays of constructs either lacking or containing the cytosolic carboxyl termini, additional membrane-spanning regions enhanced the assembly of the receptor subunits into multimeric structures, implying that several additive determinants are involved in oligomerization.

The observation that the first two membrane-spanning regions are sufficient to initiate subunit assembly, especially in those containing the C termini, suggests that a component of assembly is located within these regions, or possibly it may simply involve the interaction of an amino-terminal domain with a C-terminal domain when oriented on the same face (cytosolic) of the endoplasmic reticulum. To examine these possibilities, two additional constructions were generated that contained sequences spanning the third and fourth as well as the fifth and sixth transmembrane domains (TMR3–4+C and TMR5–6+C). Transient transfection of COS-1 cells with these plasmids resulted in robust expression of recombinant protein that was targeted to the endoplasmic reticulum (Fig. 2). Analysis of these expression products on sucrose density gradients reveals that the TMR3–4+C construct sedimented as a broad peak encompassing both mono- and tetrameric positions exhibiting a profile very similar to that of TMR1–2+C (Figs. 4 and 7). The similarity in the sedimentation profiles for TMR 1 and 2 and TMR 3 and 4 expression products compared with that of the TMR 1–4, which sediment to tetrameric positions, suggest that the assembly phenomena conferred by these domains is additive. In contrast, the majority of the protein encoded by the TMR 5 and 6 construct sedimented to the tetrameric position on the gradient. These results reveal that this region of the receptor is pivotal in the formation of multimeric assemblies and that key sequence specific determinants are involved as well, since the TMR 5 and 6 protein multimerized to a significantly greater extent than any other two membrane-spanning region-containing constructs (e.g., TMR1–2+C or TMR3–4+C).

Analysis of the intervening sequence connecting transmembrane regions 5 and 6 revealed four conserved cysteine residues in all three receptor isoforms. The role of the cysteine residues as possible structural components in the efficient assembly of this construct was examined by sedimentation of TMR5–6+C.
over 5–20% sucrose gradients with or without 10 mM 2-mercaptoethanol. Sedimentation in the presence or absence of reducing agent did not affect the sedimentation or assembly state of this protein and suggests that disulfide bridges are probably not the crucial determinants involved in this assembly phenomenon (data not shown). Similar results were previously observed for the full-length recombinant protein (15).

One feature that the fifth and sixth membrane-spanning sequences possess that is not observed in the other putative spanning domains is a homology to a leucine zipper heptad (see “Discussion”). The role of these sequences in the assembly and function of the receptor intrinsic calcium channel is currently under investigation using site-directed mutagenesis. It is tempting to speculate, however, that a sequence motif such as a leucine zipper may help form a rigid coiled-coil structure, which could augment the formation of the calcium channel and explain the enhanced assembly of receptor subunits. Similar motifs are not uncommon in transmembrane helices and have been identified in phospholamban, M2 of the acetylcholine receptor, and numerous other membrane-spanning domains (21–24).

**Analysis of the Topological Organization of the InsP<sub>3</sub>R**—The InsP<sub>3</sub>R receptor transmembrane topology model currently consists of six membrane-spanning domains. This model has evolved from several computer-derived predictions in conjunction with N-linked glycosylation and immunoelectron microscopy studies (13, 14). In this study, we utilize immunofluorescence assays on COS-1 cells transiently transfected with the full-length receptor and the array of receptor membrane-spanning truncations that have either been permeabilized with Triton X-100 or streptolysin-O. Streptolysin-O is a bacterial pore-forcing toxin that selectively permeabilizes the plasma membrane due to its requirement for cholesterol binding in the bilayer, resulting in the formation of ~30-nm pores without affecting intracellular membranes (25). Thus, the immunoreactivity of a given epitope will be dependent upon the accessibility of the antibody and the orientation (e.g., cytosolic or luminal) of an integral membrane protein. This toxin has been used together with indirect immunofluorescence to determine the transmembrane topology of numerous proteins including presinilin, cytochrome b<sub>5</sub>, and prostaglandin endoperoxide synthases 1 and 2 (18, 26, 27).

COS-1 cells transiently transfected with full-length receptor or the truncation mutants were seeded onto glass coverslips 48 h post-transfection, fixed with 2% paraformaldehyde, permeabilized with either 0.3% Triton X-100 or streptolysin-O, and subjected to indirect immunofluorescence with a repertoire of antibodies. Initial experiments using these permeabilization strategies utilized the full-length receptor with antibodies directed against the amino terminus (T1NH), luminal loop (residues 2463–2476 (13)), and carboxyl terminus (T1C). All antibodies recognized transfected cells that were permeabilized with Triton X-100 and revealed an intense reticular immunoreactivity consistent with the expected targeting of the receptor to the ER (Fig. 8). COS cells permeabilized with streptolysin-O generated similar signals to those observed for the Triton X-100-treated cells with T1NH and T1C but failed to elicit any signal from the luminal loop antibody (Fig. 8). These results confirm the resistance of intracellular membranes to permeabilization by streptolysin-O and are consistent with the data showing that the amino and carboxyl termini are localized to the cytoplasm, where the loop epitope is localized within the lumen of the ER. In control COS-1 cells, transfected with salmon sperm DNA, none of the antibodies elicited immunoreactive signals above background levels observed in nontransfected COS cells.

![Fig. 8. Determination of membrane-spanning topology of the InsP<sub>3</sub>R by immunocytological analysis of transfected COS cells](image-url)
nal tag is localized to the lumen of the ER and is not accessible via streptolysin-O permeabilization.

Expression products encoding receptor proteins with an even number of membrane spanning regions (1 and 2, 1–4, and 1–6) were immunoreactive to both amino-terminal (T1NH) and carboxyl-terminal (TIC) antibodies independent of permeabilization strategies (Fig. 9). These results are consistent with both the amino-and carboxyl termini oriented on the same face (cytosolic) of the endoplasmic reticulum. An additional expression plasmid encompassing amino acid residues 1–2412, which originally was thought to encode five membrane-spanning regions in the eight-membrane-spanning region model proposed by Südhof et al. (3), exhibited immunoreactivity to both amino- and carboxyl-terminal antibodies using either Triton X-100 or streptolysin-O permeabilization. These results demonstrate the presence of only four membrane-spanning regions in this sequence and support the six-membrane-spanning region model. In the case of the TMR1–6-C construct, immunoreactivity to an antibody directed against residues 2463–2476, which has previously been shown by immunogold electron microscopy to localize to the luminal face of the ER, was investigated. In the case of Triton X-100-permeabilized TMR1–6-C-expressing cells, this antiserum bound its cognate sequence and resulted in immunoreactive signals (Fig. 9). However, in streptolysin-O-permeabilized cells, there was no immunoreactivity despite the signals observed for both the NH2- and COOH-terminal antibodies, suggesting that the sequences between membrane-spanning regions 5 and 6 are indeed localized to the luminal side of the ER. These results are consistent with the immunoelectron microscopy and glycosylation data previously reported (13, 14).

COS-1 cells expressing constructs containing an odd number of membrane-spanning sequences (1, 1–3, 1–3X (residues 1–2378), 1–5, and 1–5X (residues 1–2543)) all exhibited immunoreactivity to both antibodies when permeabilized with Triton X-100 but failed to react with the carboxyl-terminal proton pump tag antibody when permeabilized with streptolysin-O (Fig. 8 and data not shown). A construct encompassing residues 1–2378 (1–3X), which would include four putative membrane-spanning regions as predicted from the original eight-membrane-spanning region model, was not immunoreactive to the carboxyl-terminal antibody when cells were permeabilized with streptolysin-O, thus indicating that there are only three potential membrane-spanning sequences present. Construct 1–5X (residues 1–2543), which contains sequences encompassing a hydrophobic region initially proposed to be transmembrane helix 7 in the eight-transmembrane region model, revealed no immunoreactivity in streptolysin-O permeabilized cells (data not shown). These results suggest that this sequence probably does not transverse the ER and does not constitute a membrane-spanning domain. Taken together, these results are consistent with the InsP3 receptor transversing the endoplasmic reticulum six times and confirms that the truncation mutant expression products are correctly targeted and translocated in the ER.

**DISCUSSION**

In this study, we have examined which sequence domains of the InSp3R participate in the assembly of receptor subunits into oligomers, and the topological arrangement of the receptor in the endoplasmic reticulum. The topological organization of the InSp3 receptor in the endoplasmic reticulum was determined by immunocytochemical analysis of transfected COS-1 cells that were either completely permeabilized with Triton X-100 or selectively with the toxin streptolysin-O. The results obtained using these permeabilization strategies provide direct experimental data confirming the six-membrane-spanning region models proposed by several groups and are consistent with the immunogold EM and glycosylation data previously reported (13, 14). We find no evidence that the hydrophobic domain residing between membrane-spanning regions 5 and 6 transverses the ER. This data also establishes that the truncation mutants used in this study are correctly targeted to the endoplasmic reticulum and thus are valid candidates for use in the analysis of determinants involved in the assembly of the receptor into tetrameric calcium release channels.

Analysis of the recombinant proteins ability to assemble into multimeric structures by sucrose density gradient sedimentation and immunoprecipitation reveals that several additive domains are necessary to impart receptor subunit oligomerization. The first requirement appears to be the presence of at least two membrane-spanning regions. Expression products containing the first two transmembrane elements were efficiently targeted to the endoplasmic reticulum and appeared to assemble into high molecular weight complexes on sucrose gradients. This assembly was not extensive, and a large percentage of the recombinant protein sedimented to gradient positions typical for monomers. Immunoprecipitation of full-
length receptor co-expressed with the truncation containing only the first two membrane-spanning regions (TMR1–2-C) revealed that the proteins were indeed co-assembling (Fig. 5). When fractionated on sucrose gradients, these immunoprecipitable co-assembly products sedimented as tetramers (Fig. 6). These results demonstrate that recombinant proteins containing two or more membrane-spanning regions form stable and specific associations with full-length receptor but only reveal that this association can occur between two subunits and do not address the stoichiometry of the interacting subunits. However, the shift of these truncated proteins to more dense positions on the gradients, compared with that of monomeric protein, indicate that they are probably participating in the assembly of the receptor tetramer.

We found that any truncated protein composed of two or more membrane-spanning sequences co-assembled with the wild type receptor, whereas proteins with zero membrane-spanning helices or only the first membrane-spanning helix failed to co-immunoprecipitate (Fig. 5). Constructions containing the first membrane-spanning sequence were not exclusively targeted to the ER as had been expected. In this case, a significant percentage (~50%) of the protein was localized to the cytoplasm, with the remainder associated with the microsomal/ER fractions (Fig. 2). Joseph et al. (12) reported that an in vitro translated construct containing TMR 1 was integrated into pancreatic microsomes. The differences observed between these two studies may reflect that the in vitro translated products were assayed for membrane insertion by sedimentation of the microsomal fraction through sucrose cushions, and thus the ratio of soluble to membrane-associated protein could not be determined. Sucrose gradients did not reveal efficient assembly of InsP$_3$R truncations lacking the carboxyl terminus unless the expressed protein harbored the first three or four membrane-spanning regions. Recombinant proteins with four or more spanning sequences sedimented with the majority of the protein migrating to positions on the gradient, consistent with those of native recombinant or cerebellar receptors. Therefore, we conclude that the first four transmembrane region effects on assembly of the receptor are additive.

The first four membrane-spanning regions of the receptor were reported to be a determinant in assembly based on the heterologomerization of in vitro translated templates (12). Translated protein containing the first four membrane-spanning regions co-precipitated with a type 3 receptor construct containing all six membrane-transversing regions, but failed to co-precipitate with its homologous isoform (type 1) containing all putative membrane-spanning regions. Recombinant proteins with four or more spanning sequences sedimented with the majority of the protein migrating to positions on the gradient, consistent with those of native recombinant or cerebellar receptors. Therefore, we conclude that the first four transmembrane region effects on assembly of the receptor are additive.

The contribution of the carboxyl terminus in receptor oligomerization was revealed by generating a series of transmembrane truncations fused to the carboxyl-terminal 145 amino acids of the native receptor. All expressed proteins from these plasmids containing two or more transmembrane sequences linked to the carboxyl terminus assembled into multimers. These proteins assembled to a significantly greater extent than those lacking the receptor carboxyl terminus. These results indicate that the carboxyl terminus is another important determinant in InsP$_3$ receptor subunit oligomerization. In support of this, preliminary yeast two-hybrid studies in which the carboxy-terminal 160 amino acids were co-expressed on both activating and binding domain plasmids resulted in the activation of reporter gene expression.

Recombinant protein containing the first five (TMR1–5–C), all six (TMR1–6–C), or only the fifth and sixth membrane-spanning regions (TMR5–6–C) sedimented as tetramers. Immunoprecipitations of co-transfected wild type and TMR1–5–C or TMR1–6–C receptors appeared to co-assemble much more efficiently than those with fewer membrane-spanning sequences (Fig. 5). Examination of these sequences indicated potential leucine zipper motifs in both membrane-spanning regions 5 and 6, which may possibly result in coiled-coil interactions.

The coiled-coil has been suggested to be present in numerous proteins with a wide range of proposed functions. It has been implicated in structural proteins, vesicular fusion protein, transcription factors, and, most importantly, ion channels. It has been suggested, based on mutagenesis, circular dichroism, and Fourier transformation IR studies, that the coiled-coil interactions interact to form the pentameric pore of the phospholamban protein (21, 22, 29). Furthermore, modeling studies based on mutagenesis experiments have suggested that the coiled-coil is an important component forming the pentameric M2 helices of the pore of the nAch receptor (23). The increasing number of studies that have implicated this structure in key roles led us to examine the proposed pore region of the InsP$_3$R.

The high degree of oligomerization imparted by transmembrane regions 5 and 6 in our data may be explained by the secondary structure imparted to this region by its primary amino acid sequence. We suggest that these two transmembrane regions display the characteristic heptad consenus se-

\[ \text{D. L. Galvan, E. Borrego-Diaz, and G. A. Mignery, unpublished observations.} \]
sequence of the coiled-coil (leucine zipper) (see Fig. 10). Furthermore, as seen in Fig. 10, the importance of this heptad repeat may be suggested by its strong conservation in all three isoforms of the inositol trisphosphate receptor. This sequence is generally assigned the letters a–g, in which positions a and d are hydrophobic and often β-branched amino acids (30). It is noteworthy that these two regions compose what has been suggested, based on analogy to other ion channels, to be the channel pore of the InsP$_3$ receptor. The presence of a coiled-coil within these regions may participate as a crucial component of oligomerization, as well as in the stabilization and selectivity of the pore of the InsP$_3$ receptor. Interestingly, residues 2431 (Leu) and 2434 (Ile) in the fifth membrane-spanning domain are homologous to those identified in peptide studies in which the residue at regions a and d of the heptad motif and its repetition dictate the oligomerization state of the peptides (31).

In support of this notion, reconstitution of this two membrane spanning region construct (TMR5–6+C) from its tetrameric position on gradients into proteoliposomes and subsequent fusion into planar lipid bilayers reveals that this protein forms ion channels. These channels have nearly identical permeation properties to that of native or recombinant full-length receptor tetramers (32).

These observations, together with the assembly data from the truncation mutants, suggest that the sequences encompassing membrane-spanning helices 5 and 6 are essential components of subunit oligomerization. Since subunit assembly is a co-translational process (12), it is likely that the initial role of transmembrane regions 1–4 is to target and initiate the additive assembly process by sequestering nascent receptor polypeptides in the ER in a conformation such that when transmembrane sequences 5 and 6 are translocated, the receptors complete oligomerization into functional release channels. These interactions ultimately stabilize the structure in conjunction with the additive effects of the carboxyl terminus. These studies reveal that there are several determinants present in the InsP$_3$ receptor subunit that act in synergy to confer oligomerization into functional tetramers. These studies provide the foundation from which additional experiments focusing on the role, if any, the potential coiled-coil plays in the multimerization of the InsP$_3$R and provide insights into the structural organization of the intrinsic calcium release channel.

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