Membrane Insertion, Glycosylation, and Oligomerization of Inositol Trisphosphate Receptors in a Cell-free Translation System*

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In order to study the membrane topology, processing, and oligomerization of inositol trisphosphate receptor (IP₃R) isoforms, we have utilized RNA templates encoding putative transmembrane domains to program a cell-free translation system of rabbit reticulocyte lysates supplemented with canine pancreas microsomes. In the absence of microsomes, translation of the RNA templates encoding all the putative transmembrane domains present in the C-terminal segment of the type I (1TM) and type III (3TM) IP₃R isoforms resulted in a 62- and 59-kDa polypeptide, respectively. In both cases, an additional band approximately 3 kDa larger was observed upon the addition of microsomes. Both bands in the translation doublet were integrated into microsomal membranes and were full-length translation products, as shown by sedimentation through a sucrose cushion and immunoprecipitation with C-terminal isoform-specific antibodies. With both isoforms, N-glycosylase F digestions indicate that the upper band in the doublet corresponds to a glycosylated translation product. A 17-kDa protected fragment was observed after proteinase-K digestion of 1TM translated in the presence of microsomes. The pattern and size of protected fragments was consistent with the current six-transmembrane domain model of IP₃R topology. Cotranslation of both 1TM and 3TM RNA templates in the presence of microsomes followed by immunoprecipitation with isoform specific antibodies revealed coinmunoprecipitation of translation products. This was not observed when the isoforms were translated separately and then mixed, suggesting that heterologimerization occurs cotranslationally. A construct encoding only the first putative transmembrane domain of the type I isoform was found to be sufficient for integration into membranes but was unable to oligomerize with either 1TM or 3TM. Cotranslation experiments using additional constructs indicate that the major structural determinant for homologimerization lies between putative transmembrane domain 5 and the C terminus. A second oligomerization domain involved in stabilization of heteroligomers is present within the first four transmembrane domains.

A family of specific receptors on intracellular membranes mediates the Ca²⁺ mobilization induced by the second messen-

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The abbreviations used are: IP₃R, myo-inositol 1,4,5-trisphosphate receptor; ECL, enhanced chemiluminescence; PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis; TES, 2-[L-hydroxy-1,1-bis(hydroxymethyl)ethylamino]ethanesulfonic acid.

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Assembly of IP$_3$R Isoforms

Pear to assemble in the membrane during biosynthesis, since they are not observed when lysates containing different isoforms are mixed (23). An early step in the biosynthesis of IP$_3$R$_{m}$Rs must involve the insertion of the nascent IP$_3$R$_{p}$ polypeptide into the endoplasmic reticulum membrane and their subsequent folding and assembly into homo- and heteroligomers. However, very little information is available regarding these processes. In order to initiate such studies we have used an in vitro transcription/translation programmed with DNA constructs encoding the transmembrane domains of the IP$_3$R$_{m}$R with canine pancreas microsomes as acceptor membranes. The results indicate that newly synthesized C-terminal domains of type I and type III IP$_3$R isoforms can be cotranslationally inserted into microsomal membranes and N-glycosylated. Protease protection assays have been used to analyze the topology of the inserted protein. Deletion constructs of the type I isoform have been used to show that the first two transmembrane domains are sufficient for membrane insertion but not for oligomerization. In addition, the data show that simultaneous translation of type I and type III IP$_3$R mRNA results in the formation of heteroligomers in vitro. The structural requirements for homo- and heteroligomerization have been investigated.

**EXPERIMENTAL PROCEDURES**

**Materials**

T$_7$, RNA polymerase, Taq polymerase, DNA ligase, RNasin ribonuclease inhibitor, rabbit reticulocyte lysate, and wheat germ lysate were purchased from Promega (Madison, WI). Pfu polymerase, restriction endonucleases were purchased from Stratagene (La Jolla, CA). N-glycosidase F and the long range PCR kit, Amplify, and ECL immunoblotting kits were obtained from Amer sham Corp. Stabilized acrylamide solution (Protogel) for the preparation of SDS gels was obtained from National Diagnostics (Atlanta, GA). Tran$^{35}$S-label was obtained from ICN Radiochemicals (Irvine, CA). Restriction endonucleases were purchased from Promega, Boehringer Mannheim, or New England Biolabs (Beverly, MA). The pcITE T-vector and the sequencing primers U-19, CITE, and T3 were obtained from Novagen (Madison, WI). The cDNA encoding the rat type I IP$_3$R isoforms was kindly provided by Dr. Thomas Sudhof (University of Texas Southwestern Medical Center, Dallas) and Dr. Greg Mignery (Loyola School of Medicine, Chicago). The cDNA encoding the rat type III IP$_3$R isoform was kindly supplied by Dr. Graeme Bell (Univ. Chicago). Bovine preprolactin was transcribed and translated from the plasmid pGEMBP1 (27).

**PCR Primers**

The following primers were synthesized by the Nucleic Acid Facility of the Jefferson Cancer Institute (restriction sites are underlined): ITM-F, 5'-GGCGCATATGAACTGGCAGAAGAAA-3'; ITM-Fb, 5'-GGCGCATATGAACTGGCAGAAGAAA-3'; ITM-R, 5'-TATGATATCGGTACCTTTAGGCTGCTGTGCTG-3'; 1TM-1, 5'-GGCGTGACCTCTAGTGTTTCTCTCT-3'; 1TM-2, 5'-GGCGTGACCTTTAGGCTGCTGTGCTG-3'; 1TM-3, 5'-GGCGTGACCTCTAGTGTTTCTCTCT-3'; 1TM-4-R, 5'-CTGTAAGGTGAGAGGAGGA-3'; 1TM5,6- R, 5'-AAAGGAGTGAGAGGAGGA-3'; 1TM5,6, stop, 1TM-1–4, stop, 5'-AAAGGAGTGAGAGGAGGA-3'; 1TM5,6, stop, 1TM-1–6, stop, 5'-AAAGGAGTGAGAGGAGGA-3'; 1TM5,6, stop, 1TM-1,2, stop, 5'-AAAGGAGTGAGAGGAGGA-3'; 1TM5,6, stop, 1TM-1,2, tag—

**Subcloning the Transmembrane Domain and Constructs into the CITe Vector**

All constructs described below were subjected to automated DNA sequencing to confirm that the products were in frame with respect to the translation initiation site and to verify the sequence of the insert. The amino acid boundaries of the constructs are detailed in Fig. 1.

**Transmembrane Domain of the Type I IP$_3$R and Mutant Constructs**

1TM—The pl7 clone encoding 6 kilobase pairs of the C-terminal portion of rat type I IP$_3$R (2) was used as a template for PCR amplification of the putative transmembrane domains. ITM-F and 1TM-R were used as primers to amplify nucleotides encoding from methionine 2253 to the stop codon. The 1.6-kilobase pair PCR product was ethanol-precipitated and purified from a 1% agarose gel using a Qiagen kit (Qiagen, Chatsworth, CA). The 1TM PCR product was then directly ligated into the pcITE T-vector. Following transformation of Escherichia coli (DH5a), positive clones were identified by colony PCR using a combination of vector (CITE) and insert-specific (1TM-R) primer.

1TM1 and 1TM1,2—The construct encoding the first putative transmembrane domain was amplified using ITM-F and 1TM1-R as primers and cloned into the pcITE T-vector as described above for 1TM. The 1TM1,2 construct, which encodes the first two putative transmembrane domains, was amplified using ITM-F and 1TM2-R as primers and cloned into the pcITE T-vector as described above for 1TM.

1TM1,R and 1TM1,2,R—In these constructs the antibody epitope tag for the type I IP$_3$R was inserted at the C terminus to permit immunoprecipitation. Nucleotides encoding amino acids 2708–2749 were excised from the pcITE-1TM plasmid by digestion with BstEII (cutting at base pair 8450) and NorI (vector site). This “tag” fragment was gel-purified. The 1TM1-R and 1TM1,2-R primers were designed to contain a BstEII restriction site. To make the tagged constructs, the tag fragment was ligated into the BstEII/NorI-digested pcITE-1TM and 1TM1,2 plasmids.

1TM1,4,R—A construct encoding the first four putative transmembrane domains was amplified from the 1TM plasmid using 1TM-F and 1TM4-R. These primers included sites for NdeI and BstEII, respectively. The PCR fragment was digested with these enzymes and ligated into the CITe-1TM plasmid that had been gel-purified after being cut with NdeI and BstEII.

1TM1—Inverse PCR (21) was used to obtain a mutant construct in which the first putative transmembrane domain (corresponding to nucleotides 7140–7218) was deleted from pcITE 1TM. PCR amplification of the entire pcITE 1TM plasmid with the exception of the region of the first transmembrane domain was carried out using 1TM1F and 1TM1R primers and a long range PCR kit obtained from Boehringer Mannheim. The PCR product was blunt-ended with Pfu polymerase (29), gel-purified, and ligated into pcITE 1TM. A forward primer containing an NdeI restriction site (1TM5,6-F) and a reverse primer (1TM-R) that contains an EcoRI restriction site, was used to amplify the segment of the pcITE 1TM plasmid, which encodes the fifth putative transmembrane domain through to the C terminus. The PCR fragment was cut with NdeI and EcoRI, gel-purified, and ligated into NdeI/EcoRI-digested pcITE ITM plasmid.

1TM1,4–stop, 1TM5,6–stop, and 1TM–stop—To study homologimerization with ITM, we designed constructs from which the antibody epitope had been removed and replaced with a stop codon. The alternative approach of using unique restriction sites within the coding sequence to generate truncated transcripts produced results that were difficult to interpret, since multiple translation products were observed. ITM1–4–stop was amplified from ITM1–4–tag DNA template using ITM-Fb as forward primer and 1TM1–4–stop as reverse primer. ITM1–4–stop was amplified from pcITE 1TM DNA template using ITM-F and 1TM1–4–stop as primers. ITM5,6–stop was amplified from ITM1–4–tag DNA template using ITM5,6–F as forward primer and 1TM,stop as reverse primer. 1TM–stop was amplified from ITM DNA template using ITM-F as forward primer and 1TM,stop as reverse primer. All PCR inserts were cut with NdeI and EcoRI, gel-purified, and ligated into NdeI/EcoRI-digested pcITE ITM plasmid.

3TM—The cDNA encoding the rat type III IP$_3$R was used as a template for the PCR amplification of the putative transmembrane domains. 3TM-F and 3TM-R were used to amplify nucleotides encoding from methionine 2132 to the stop codon. The PCR product was made with Pfu polymerase, and overhanging 3’-adenosines were added by incubation of the PCR product with T4 polynucleotide and dATP (30). The product was ethanol-precipitated and directly ligated into the pcITE-T vector. Following transformation of E. coli (DH5a), positive clones were identified by colony PCR using a combination of vector (CITE) and insert-specific (3TM-R) primer.

3TM1—A construct in which amino acids 2519–2645 were deleted from the pcITE-1TM plasmid was amplified using 3TM1-F and 3TM1-R, which contains an EcoRI restriction site. This “tag” fragment was gel-purified. The 3TM1-R and 3TM1,2-R primers were designed to contain an EcoRI restriction site. To make the tagged constructs, the tag fragment was ligated into the BstEII/NorI-digested pcITE-1TM and 1TM1,2 plasmids.

3TM1,3–stop, 3TM5,6–stop, and 3TM–stop—To study homologimerization with 3TM, we designed constructs from which the antibody epitope had been removed and replaced with a stop codon. The alternative approach of using unique restriction sites within the coding sequence to generate truncated transcripts produced results that were difficult to interpret, since multiple translation products were observed. 3TM1–3–stop was amplified from 3TM1–3–tag DNA template using 3TM-Fb as forward primer and 3TM1–3–stop as reverse primer. 3TM5,6–stop was amplified from pcITE 3TM DNA template using 3TM5,6–F as forward primer and 3TM,stop as reverse primer. 3TM–stop was amplified from ITM DNA template using ITM-F as forward primer and 3TM,stop as reverse primer. All PCR inserts were cut with NdeI and EcoRI, gel-purified, and ligated into NdeI/EcoRI-digested pcITE ITM plasmid.
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Cell-free Transcription

Plasmid DNA (5 μg) was linearized with EcoRI. Capped transcripts were synthesized with T7 RNA polymerase using an mRNA synthesis kit (Ambion, Austin, TX). Transcribed templates were purified by phenol/chloroform extraction and ethanol precipitation. The sample was resuspended in 20 μl of diethylpyrocarbonate water and stored at −80°C.

Cell-free Translation and Translocation Assays

Routinely, cell-free translations were carried out for 1 h at 30°C in a final volume of 25 μl and contained 10 μl of rabbit reticulocyte lysate, 0.5 μl of RNasin, 0.5 μl of 1 mM amino acids (minus methionine), 20 μCi of Tran35S-label, 1.5 μl of RNA template (1–3 μg), and 6 μl of buffer A (pH 7.2) containing 110 mM KOAc, 2 mM Mg(OAc)$_2$, and 20 mM KHepes. Where appropriate, the translation reactions contained 3 μl of nuclease-treated canine pancreatic microsomes prepared as described (31). SDS-PAGE gels containing [35S]-labeled translation products were processed for fluorography with 1 M sodium salicylate (32) or Amplify and exposed to Kodak XAR-5 film. In some experiments the translation reactions were carried out using biotinylated lysyl-tRNA using a cell-free translation kit manufactured by Amersham.

For protease protection assays, aliquots of the translation reactions were diluted with 3 volumes of a buffer containing 120 mM KCl, 20 mM Tris-HCl (pH 7.4), 1 mM EDTA, and 100 μg/ml proteinase K. The reactions were incubated on ice for 1 h, and 3 mM phenylmethylsulfonyl fluoride was added for a further 10 min. The sample was then diluted 5-fold into 0.1 mM Na$_2$CO$_3$ buffer (pH 11.0) and spun through a sucrose cushion (1.5 M sucrose, 10 μM TES, pH 7.2) at 60,000 × g for 20 min on a Beckman TLA-100 rotor. The pellet was resuspended in SDS-PAGE sample buffer and analyzed as described above.

For deglycosylation of the translation products, microsomes were spun through a sucrose cushion and resuspended in 0.3% SDS, 100 mM β-mercaptoethanol, 50 mM sodium phosphate (pH 8.6), and 1 mM EDTA. The sample was boiled for 3 min and supplemented with a final concentration of 1.5% octylglucoside. Two aliquots were incubated overnight at 37°C in the presence or absence of 1 unit of N-glycosidase F.

Immunoprecipitation and Immunoblotting

The translation mixture was diluted into 500 μl of a solubilization buffer containing 150 mM NaCl, 50 mM Tris-HCl (pH 7.8), 1% Triton X-100 (w/v), 1 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, and 5 μg/ml each of aprotinin, soybean trypsin inhibitor, and leupeptin. The samples were precleared with 20 μl of Pansorbin (Calbiochem). After removal of Pansorbin by centrifugation (10,000 × g for 10 min), IP$_3$R isoform-specific antibody and 50 μl of a 20% (w/v) slurry of protein A-Sepharose was added, and the sample was incubated for 2 h at 4°C. Immune complexes, precipitated with protein A-Sepharose, were washed three times in solubilization buffer and analyzed by SDS-PAGE. In some experiments the polypeptides in the gel were transferred to nitrocellulose, which was autoradiographed and then immuno-blotted with isoform-specific antibodies to locate the receptor. The polyclonal type I IP$_3$R antibody used in this study has been described previously (33). The type III polyclonal antibody was raised against the C-terminal peptide corresponding to residues 2657–2670. The peptide was synthesized with an additional N-terminal cysteine, which was used for conjugation to keyhole limpet hemocyanin (34). Antibody was raised in rabbits by Cocalico Biologicals (Reamstown, PA). The antibody was affinity-purified using the peptide coupled to Ultralink Iodoacetyl beads as described by the manufacturer (Pierce).

RESULTS

Cell-free Translation of the Transmembrane Domains of the Type I IP$_3$R—PCR was used to amplify the C-terminal segment of the type I and type III IP$_3$R isoforms containing the putative transmembrane domains. Fig. 1 shows the sequence boundaries of the DNA constructs used in the present study. The PCR fragments were cloned into a plasmid behind a modified 5'-untranslated viral sequence (CITE) that is known to enhance translation efficiency in the rabbit reticulocyte system (35, 36). Fig. 2A shows the results of translating the RNA template encoding the 1TM construct using an amino acid mixture labeled with [35S]methionine and [35S]cysteine. In the absence of microsomes, the 1TM construct yielded a single labeled
polypeptide having a molecular mass of 62.3 ± 2.3 kDa (mean ± S.E.; n = 3). The predicted molecular mass of the translation product is 58 kDa, taking into account 13 amino acids derived from the vector and PCR primer (MATTHMDSS-RVE) and the 498 amino acids of the construct itself. Control experiments showed that translation of preprolactin mRNA in the presence of microsomes led to the appearance of a smaller product, corresponding to cleavage of preprolactin by signal peptidase, verifying the efficient processing activity of the microsomal membranes (Fig. 2A, lanes 1 and 2). Translation of 1TM mRNA in the presence of microsomes resulted in the appearance of both the 62-kDa band and an additional translation product that was approximately 3 kDa larger. Both bands in the doublet corresponded to full-length translation products, since they were both immunoprecipitated by an antibody recognizing the 18 amino acids at the C terminus (Fig. 2A, lanes 5 and 6).

In order to assess if one or both translation products are integrated into the microsomal membrane, the translation reactions were diluted with buffer, and the microsomal membranes were recovered by centrifugation through a sucrose cushion (Fig. 2B). Both translation products were recovered in the pellet fraction in these experiments. This was also the case when the translation products were first diluted into a buffer containing Na2CO3 (pH 11), incubation conditions that strip peripherally attached proteins from membranes (37). From these data we conclude that the doublet of translation products was integrated into the microsomal membrane. This occurs cotranslationally, since insertion into membranes was not observed when microsomes were added after translation had been terminated with cycloheximide (data not shown).

Cell-free Translation of the Transmembrane Domains of the Type III IP3R—The type III transmembrane domain construct amplified by PCR corresponds to amino acids 2132–2670 of the rat sequence (5). Fig. 3A shows that the RNA template encoding this construct translated, in the absence of microsomes, as a single labeled polypeptide having a molecular mass of 59.2 ± 0.6 kDa (mean ± S.E.; n = 3). The predicted molecular mass of the translation product is 63 kDa, taking into account 10 amino acids derived from the vector and PCR primer (MATTHMDSPW) and the 539 amino acids of the construct itself. As observed with 1TM mRNA, translation of 3TM mRNA in the presence of microsomes resulted in the appearance of a doublet of translation products consisting of both the 59-kDa band and an additional translation product that was approximately 3 kDa larger. Both bands in the doublet were specifically immunoprecipitated by type III antibody, which did not immunoprecipitate the 1TM translation products (Fig. 3A, lanes 4–6). Almost all of the translation product doublet was recovered in the pellet fraction when the microsomes were stripped with Na2CO3 buffer and then centrifuged through a sucrose cushion (Fig. 3B). These results indicate that the 3TM translation products are also integrated into the microsomal membrane.

Enzymatic Deglycosylation of the Translation Products—It is known that the type I IP3R is N-glycosylated, and two potential sites of glycosylation have been identified in the intraluminal loop between putative transmembrane domains 5 and 6 (19). Although there is no direct experimental evidence that the type III IP3R is a glycoprotein, one of the glycosylation sites in the type I IP3R is conserved in the intraluminal loop of the type III isoform. Therefore, a possible explanation for the appearance of a higher molecular weight band in the presence of microsomes...
is that it represents the glycosylated translation product. Fig. 4 shows the results of experiments in which the 1TM and 3TM translation products were translated in the presence of microsomes were deglycosylated with N-glycopeptidase F. Only a single translation product was observed after deglycosylation of the translation products of both isoforms (lanes 2, 4, and 6). We conclude that the higher molecular weight band seen with both IP₃R isoforms in the presence of microsomes represents a glycosylated translation product.

**Protease Protection Assays**—The current topological model of the IP₃R predicts the presence of three intraluminal loops (6, 16, 17). Two of these loops are relatively small. The third loop, present between putative transmembrane domains 5 and 6, is much larger and encompasses the N-glycosylation sites and the proposed pore-forming domain. Protease cleavage of the translation product inserted into microsomal membranes would be expected to result in the appearance of protected fragments whose size can be predicted from the topological model. In initial trials, we found that labeling the translation product with biotinylated lysine rather than [³⁵S]methionine gave a lower background and a clearer signal in protease protection assays. Fig. 5A shows that proteinase K cleavage of the biotinylated 1TM translation product produces several protected bands (compare lanes 1 and 2). All of the protected polypeptides become accessible to proteinase K in the presence of detergent (Fig. 5A, lane 3). The most prominent of these protease-protected bands (Fig. 5A, lane 2, arrow) has a molecular mass of 16.8 ± 0.5 kDa (n = 3). This molecular mass is in agreement with the value of 16.9 kDa calculated from the primary sequence for putative transmembrane domains 5 and 6 and the intervening large intraluminal loop. A doublet of protected bands above the 19 kDa marker was reproducibly observed in these experiments, although their intensity was variable (e.g. Fig. 5, compare A and B, brackets). Enzymatic deglycosylation of the proteinase K-digested 1TM translation product resulted in a decrease in the doublet of bands above 19 kDa and an increase in the amount of 16-kDa polypeptide (Fig. 5B, lane 3). This suggests that the doublet and the 16-kDa polypeptide correspond, respectively, to the glycosylated and nonglycosylated forms of the large intraluminal loop of the receptor. The presence of a doublet of glycosylated bands may indicate heterogeneity in the occupation of the two available consensus glycosylation sites or heterogeneity in the carbohydrate composition of glycan chains. The two protected bands of the lowest molecular weight seen in Fig. 5A may correspond to the two smaller intraluminal loops expected from the predicted topology, but they were not reproducibly observed in every experiment (e.g. Fig. 5, compare A and B).

**Role of the First Transmembrane Domain**—The IP₃R is an example of a polytopic transmembrane protein that does not contain a cleavable signal sequence. The first transmembrane domains of such proteins are believed to act as topogenic signals for membrane insertion. The sequential arrangement of transmembrane domains that act as “signal anchor” and “stop-transfer” sequences are believed to determine the final topology of the protein (38). We have examined the role of the first transmembrane domain in membrane insertion and receptor oligomerization by making a construct that contains only the first putative transmembrane domain (1TM) or a construct in which only the first putative transmembrane domain has been selectively deleted (1TMΔ1). In order to facilitate immunoprecipitation of the 1TM translation product, the construct was engineered to contain the 42 amino acids present at the C terminus of the type I IP₃R (1TM1,tag), which includes the epitope recognized by the type I-specific antibody. Fig. 6A shows the translation of 1TM1,tag RNA in the presence or absence of microsomes. A translation product with an apparent molecular mass of 13.2 kDa was observed (predicted mass is 12.1 kDa). As expected, the size of the product did not change upon the addition of microsomal membranes, although the translation reaction was more efficient under these conditions. The 1TM1,tag translation product was found in the pellet fraction after treatment of the microsomes with Na₂CO₃ (pH 11.0) and centrifuged through a sucrose cushion (Fig. 6B, lane 1), indicating that the presence of the first transmembrane domain was sufficient to permit insertion into the microsomal membrane. Translation of the 1TMΔ1 construct in which the first putative transmembrane domain has been selectively deleted is shown in Fig. 6C. This product was also integrated into the microsomal membranes, as shown by recovery in the pellet fraction after Na₂CO₃ treatment and centrifugation through a
Membrane insertion of the first transmembrane domain. The PCR was used to make a construct encompassing the first transmembrane domain of the type I IP₃R (ITM₁; see Fig. 1). This construct was additionally engineered to contain the isoform-specific antibody epitope by attaching the 42 amino acids present at the C terminus (ITM₁,tag; see Fig. 1). A shows the in vitro translation of the RNA template encoding ITM₁,tag in the presence and absence of microsomes. The translation products were analyzed on 17.5% SDS-PAGE. In B the ITM₁,tag translation products were diluted in Na₂CO₃ buffer (pH 11.0) and centrifuged through a sucrose cushion, and pellet and supernatant fractions were analyzed as described in the legend to Fig. 2B. C, a construct from which the first transmembrane domain was deleted (ITMΔ₁; see Fig. 1) was translated in the presence of microsomes. An aliquot of the translation reaction was immunoprecipitated with type I-specific antibody (lane 1) or was diluted in Na₂CO₃ buffer (pH 11.0) and centrifuged through a sucrose cushion (lanes 2 and 3) with pellet and supernatant fractions analyzed as described in the legend to Fig. 2B. For comparison, the full-length ITM₁ translation product is shown in lane 4.

Heteroligomerization of the ITM and 3TM Translation Products—Sucrose density gradients were used to investigate the oligomerization status of in vitro translated products. Fig. 7 shows the sedimentation profile of the ³⁵S-labeled ITM₁ translation product after lysis of microsomal membranes in Triton X₁₀₀ or SDS. Analysis of Triton X₁₀₀ lysates revealed a peak of radioactivity at the top of the gradient and a second broader peak located in the middle of the gradient. The broad second peak was absent when the microsomal membranes were lysed in SDS. This suggests that the transmembrane domain of the type I IP₃R can homooligomerize when translated in vitro.

Experiments were then performed to determine if cotranslation of both ITM and 3TM would lead to heteroligomerization. The coimmunoprecipitation of two different translation products possessing heterologous immunological tags has previously been used as an experimental method to study heteroligomerization of other ion channel proteins in a cell-free translation system (36, 39–42). The results using this approach are shown in Fig. 8. When ITM and 3TM RNA templates were translated separately in the presence of microsomes and then immunoprecipitated, it was clear that each of the antibodies was highly specific for its respective isoform (Fig. 8, lanes 1–4). When both templates were translated together, each Ab immunoprecipitated its respective doublet of translation products and also coimmunoprecipitated a proportion of the other isoform. Since ITM and 3TM translation products overlap in their migration, three ³⁵S-labeled bands are observed when both templates are cotranslated and immunoprecipitated with either type I or type III-specific Abs (Fig. 8, lanes 5 and 6). When the templates were translated separately and then mixed before immunoprecipitation, each Ab only immunoprecipitated its cognate antigen (Fig. 8, lanes 7 and 8). From these data we conclude that coimmunoprecipitation of both isoforms reflects the cotranslational assembly of heterooligomers in the microsomal membranes and that this process does not occur when the lysates containing individual isoforms are mixed. This conclusion is consistent with previous studies.
The translation products obtained from 1TM and 3TM are closely spaced on SDS-PAGE gels. In order to accentuate differences in the molecular weight of the two isoforms, we prepared the 3TM “small” (3TMs) construct in which 127 amino acids were deleted from the C-terminal tail, leaving the antibody epitope at the C terminus in place (Fig. 1). Like 3TM, the 3TMs construct was also translated as a doublet but ran on SDS-PAGE at a distinctly smaller molecular weight than the 1TM translation product (Fig. 9). When 1TM and 3TMs RNA templates were translated together and microsomal lysates were immunoprecipitated with type I IP$_3$R Ab, a clear doublet corresponding to coprecipitating 3TMs translation products could be observed (Fig. 9, lane 5). Again, it could be shown that this was not due to cross-reactivity of the Ab (Fig. 9, lane 3), and it was absent when individual templates were translated separately and lysates were mixed after the translation was completed (Fig. 9, lane 7). Attempts to observe 1TM translation product coimmunoprecipitated with type III Ab were unsuccessful (Fig. 9, lane 8), even with more prolonged exposure of the autoradiographs (data not shown). The reason for this lack of reciprocal immunoprecipitation is not clear at present. One possibility is that truncation of the C-terminal tail hinders access of the type III Ab to 3TM when 2TMs is present as a component of heteroligomers with 1TM.

**Structural Features of 1TM Required for Heteroligomerization**

In an attempt to localize the regions of the IP$_3$R that may be important for heteroligomerization, we prepared constructs encoding limited regions of 1TM and tested each for their ability to associate with 3TM in coprecipitation assays. The results obtained with a construct encoding only the region from transmembrane segment 5 to the C terminus (1TM5,6; see Fig.

**Fig. 8. Heteroligomerization of 1TM and 3TM translation products.** Three separate translation reactions containing RNA templates encoding 1TM, 3TM, or both isoforms were carried out in the presence of microsomes and Tran$^{35}$S-label in a final volume of 50 μl. Aliquots of each of the translations were immunoprecipitated with type I- and type III-specific antibodies as described under “Experimental Procedures.” The first four lanes are controls showing the isoform specificity of the Abs used for immunoprecipitation. In lanes 5 and 6, the immunoprecipitates were derived from translation containing both templates. Evidence for coimmunoprecipitating bands is indicated by the arrows. As additional controls, aliquots of the translation in which 1TM and 3TM RNA templates had been separately translated were mixed after translation and then immunoprecipitated with isoform-specific Abs (lanes 7 and 8). After electrophoresis, labeled polypeptides were transferred to nitrocellulose and autoradiographed. Coprecipitating bands are identified by arrows. The data shown are representative of three separate experiments.

**Fig. 9. Heteroligomerization of truncated 1TM constructs.** Three separate translation reactions containing RNA templates encoding 1TM, the truncated 3TMs construct (see Fig. 1), or both constructs were carried out in the presence of microsomes and Tran$^{35}$S-label in a final volume of 50 μl. Samples from each translation were solubilized and immunoprecipitated with isoform-specific antibodies as described for Fig. 8. The first four lanes are controls showing the isoform specificity of the Abs used for immunoprecipitation. In lanes 5 and 6, the immunoprecipitates were derived from translation containing both templates. Evidence for coimmunoprecipitating bands are indicated by the arrows. As additional controls, aliquots of the translation in which 1TM and 3TMs RNA templates had been separately translated were mixed after translation and then immunoprecipitated with isoform-specific Abs (lanes 7 and 8). Coprecipitating bands are identified by arrows.

**Fig. 10. Heteroligomerization of truncated 1TM constructs with 3TM.** A, RNA templates encoding 1TM5,6 and 3TM were translated together (lanes 1 and 2) or were translated separately and then mixed (lanes 3 and 4). Both sets of samples were immunoprecipitated as described under “Experimental Procedures” with type I- (lanes 1 and 3) or type III-specific antibody. Translation products were analyzed on an 8% SDS-PAGE gel. B, the procedures were the same as described for A except that the combination of RNA templates used was 1TM1–4, tag and 3TM. Translation products were analyzed on a 15% SDS-PAGE gel. C and D, 3TM (lane 1), 1TM, tag (lane 2), and 1TM1,2, tag (lane 3) RNA templates were translated separately or in the indicated combination with 3TM (lanes 4 and 5). Translation of 3TM alone is shown in lane 1. The translations were divided into two aliquots, which were solubilized and immunoprecipitated with type I Ab (C) or type III Ab (D). Translation products were analyzed on a 17.5% SDS-PAGE gel. Coprecipitating bands are identified by arrows.
independently interact with 3TM. However, the first two transmembrane and the last two transmembrane domains can each of the receptor containing the first four transmembrane domains attached to the type I epitope tag (1TM1–4,tag; see Fig. 1). This construct also proved directly quenched (lanes 1, 3, and 5). The translation products present in 1 μl of the combined 1TM and 1TM1–4,stop reaction are shown in lane 7. Coprecipitating bands are identified by arrows.

In this case both templates when translated are expected to yield products of similar molecular weights. However, it is clear that the slightly smaller doublet of bands corresponding to 1TM,stop coprecipitates with 1TM when the two templates are cotranslated (Fig. 11, lane 2) but not when the templates are translated separately and then mixed (Fig. 11, lane 1). Similar evidence for coprecipitation was observed when 1TM5,6,stop was combined with 1TM (Fig. 11, lane 4). However, in contrast to the results observed with heterologimerization assays, there was no indication of coprecipitation of 1TM1–4,stop (Fig. 11, lane 6). This was not due to impaired translation of template, since both translation products were present in samples derived directly from the translation mixture (Fig. 11, lane 7). Similar experiments combining 1TM1 and 1TM are shown in Fig. 12. Immunoprecipitation of lysates containing both 1TM and 1TM1 templates with type I IP3R-specific antibody (lanes 4–6).

Structural Features Required for Homoligomerization—In order to determine if the same structural requirements apply for homoligimerization of receptors we modified the coprecipitation assay so that 1TM template was combined with truncated 1TM constructs from which the antibody tag had been deleted and replaced with a stop codon (1TM1-stop, 1TM5,6-stop, and 1TM1–4,stop; see Fig. 1). In initial experiments we first verified that homoligomerization could be demonstrated by this procedure by combining 1TM with 1TM,stop.

1) is shown in Fig. 10. 1TM5,6 translates in the presence of microsomes as a lower band of approximately 36 kDa, above which lies a series of closely spaced bands that merge together at the autoradiograph exposures shown (Fig. 4, lane 5, and Fig. 10A, lane 3) but resolve into three distinct bands at lower exposures (data not shown). All of the upper bands are removed upon digestion with N-glycopeptidase F (Fig. 4, lane 6), and they must therefore correspond to differentially glycosylated 1TM5,6 translation products. When 1TM5,6 is cotranslated with 3TM and microsomal lysates are immunoprecipitated with type I specific Ab, an additional band is seen that corresponds to the glycosylated 3TM translation product (Fig. 10A, lane 1).

Similarly, immunoprecipitation of the lysates with type III-specific Ab shows the presence of coprecipitating 1TM5,6 (Fig. 10A, lane 2). The control lanes, in which 1TM5,6 and 3TM were translated separately and then mixed, show no evidence of coprecipitation (Fig. 10A, lanes 3 and 4). The same experiment was also carried out with a 1TM construct encoding the first four putative transmembrane domains attached to the type I epitope tag (1TM1–4,tag; see Fig. 1). This construct also showed evidence of coprecipitation with 3TM (Fig. 10B, compare lanes 1 and 3). The results of cotranslation of 3TM with smaller segments of the receptor encoded by 1TM1,tag and 1TM1,2,tag are shown in Fig. 10C. Although both of these constructs were effectively translated when combined with 3TM, neither product had the ability to associate with 3TM as judged by the absence of coprecipitation when tested with either type I- (Fig. 10C, lanes 4 and 5) or type III-specific Abs (Fig. 10D, lanes 4 and 5). These results suggest that the region of the receptor containing the first four transmembrane domains and the last two transmembrane domains can each independently interact with 3TM. However, the first two transmembrane domains do not have the ability to heteroligomerize.

**FIG. 11. Homoligomerization of 1TM with truncated 1TM constructs.** Translation reactions were carried out in the presence of microsomes using individual or combined templates. The individual translations contained 1TM, 1TM,stop, 1TM5,6,stop, and 1TM1–4,stop. The combined reactions contained 1TM combined with 1TMstop, 1TM5,6,stop, or 1TM1–4,stop. 15 μl of the combined translation reactions (lanes 2, 4, and 6) were solubilized and immunoprecipitated with type I IP3R Ab. As controls, 15 μl of each of the individual translations were mixed after translation, solubilized, and also immunoprecipitated with type I IP3R Ab (lanes 1, 3, and 5). The translation products present in 1 μl of the combined 1TM and 1TM1–4,stop reaction are shown in lane 7. Coprecipitating bands are identified by arrows.

**FIG. 12. Homoligomerization of 1TM with 1TM1.** RNA templates encoding 1TM and 1TM1 were translated separately or together in the presence of microsomes. Aliquots of the translation mixture were either directly quenched (lanes 1–3) or immunoprecipitated with type I-specific antibody (lanes 4–6).

**DISCUSSION**

In the present study we have utilized a rabbit reticulocyte lysate system to study membrane integration, processing, and assembly of IP3-Rs. Only RNA templates encoding C-terminal segments were used in these experiments, since the putative transmembrane domains of the receptor are confined to this...
Our data show that the first transmembrane span of the IP₃R segments can have signal sequence activity, stop-transfer activity, or both, depending on their sequence context (43, 44). Mutagenesis studies have suggested that the transmembrane segments play a primary role in the assembly of IP₃R tetramers (11). In common with what is known regarding the architecture of voltage-gated ion channels (45, 46), it is believed that a functional inositol trisphosphate-gated Ca²⁺ channel is tetrameric and that transmembrane domains from each monomer contribute to the formation of a central ion-conducting pore. The precise domains that line the pore of IP₃R and the types of interactions holding the tetramer together are unknown. In the case of the Shaker K⁺ channel, ionic interactions have been proposed to occur between negatively charged residues in transmembrane segments 2 and 3 and positively charged residues in transmembrane segment 4 (47, 48). Analysis of the six putative transmembrane segments of all three of the IP₃R isoforms indicates the presence of only one highly conserved positively charged residue in putative transmembrane segment 3 (lysine). By this criterion, electrostatic interactions between the membrane embedded segments of the receptor are unlikely to play a major role in maintenance of the tetrameric structure. Hydrophobic interactions, interactions between charged residues located on cytosolic or luminal loops, and intersubunit disulfide bridges (49) may all contribute to stabilization of the oligomer.

Heteroligomerization between closely related subunits has been described for many ion channel proteins, including the amiloride-sensitive Na⁺ channel (50), cyclic nucleotide-gated cation channel (51, 52), G-protein-gated inward rectifying K⁺ channel (53, 54), and voltage-gated K⁺ channels (36, 55, 56). Several studies have documented the presence of heteroligomers of IP₃R isoforms in cell lysates (23–26). In the present series of experiments, we have provided evidence for heteroligomerization between 1TM and 3TM constructs in an in vitro translation system. The ability of a single specific antibody to coimmunoprecipitate both translation products was observed only when both isoforms were cotranslated and not when they were translated separately and then mixed. This indicates that association between isoforms is not an artifact of the detergent solubilization and immunoprecipitation procedures. In general, it was easier to observe 3TM coimmunoprecipitated by type I-specific Ab than to observe 1TM coimmunoprecipitated by type III-specific Ab. The lack of reciprocal coimmunoprecipitation is particularly evident when using truncated 1TM constructs. The results were not dependent on the particular type of antibody used, since different type I- and type III-specific antibodies gave similar results in these experiments (data not shown). Other factors that may contribute to this result are the relative translation efficiency of each RNA template when added in combination, differential occlusion of C-terminal antibody epitopes in homo- and heteroligomers, and the possibility that there is a preferred stoichiometry for heteroligomers favoring type I subunits. Because of these factors, the results obtained from coprecipitation assays are necessarily qualitative, and it is difficult to estimate the exact fraction of translation products that exist as heteroligomers. However, the data indicate that heteroligomers are a small fraction of the total translation product in the in vitro system. Analyses of cell lysates have produced estimates of heteroligomerization that range from involvement of only a minor fraction in WB rat liver

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**TABLE I**

<table>
<thead>
<tr>
<th>Homologomerization</th>
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<th>Template 2</th>
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</table>

* The boundaries of the constructs used are shown in Fig. 1. NS, data not shown.

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2 S. K. Joseph, unpublished observations.
epithelial cells (24) to the entire fraction of type I IP$_3$Rs being heteroligomerized in RINm5F cells (26). The regulatory mechanisms that favor (or prevent) the formation of heteroligomers during receptor biosynthesis in the endoplasmic reticulum of different cell types remains to be defined.

An important advantage of the cell-free translation system is that it allows the structural requirements for oligomerization to be investigated using truncated RNA templates. Table I shows a summary of the data obtained from coprecipitation experiments using truncated 1TM constructs to study homo- and heteroligomerization. Constructs containing 1TM1 and 1TM1.2 proved incapable of homoligomerization or heteroligomerization. Thus, the first transmembrane domain, while necessary for membrane insertion, does not play a role in oligomerization. Our experiments indicate that 1TM5,6 is the smallest truncated 1TM construct that associates with 1TM or 3TM. We therefore conclude that this region contains the primary oligomerization domain of IP$_3$Rs. The 1TM5,6 construct encompasses the proposed pore domain of the channel and the glycosylation sites. The putative transmembrane segments 5 and 6 are highly conserved among the different isoforms and also share a high degree of homology to corresponding domains in the ryosynode receptor (9). Inward rectifying voltage-dependent K$^+$ channels have a similar topology consisting of two transmembrane segments with an intervening pore domain. These channels have also been shown to form homo- and heterotetramers (53, 54).

A surprising observation in these experiments was the finding that the 1TM construct containing the first four transmembrane segments could associate with 3TM but was inactive in homoligomerization assays. Since both 1TM5,6 and 1TM1-4,tag can independently associate with 3TM, this suggests that a secondary oligomerization domain is located in the TM1-4,tag construct. This domain would function to stabilize heteroligomeric IP$_3$R subunit interactions. Multiple sites of interaction have also been proposed to be involved in the self-assembly of tetrameric voltage-dependent K$^+$ channels (36, 39, 48, 56, 57). Further mapping of the oligomerization domains and analysis of transmembrane topology should provide valuable information on the structure and assembly of this important class of ion channel proteins.

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REFERENCES


