The Role of the S4-S5 linker and C-terminal Tail in Inositol 1,4,5-trisphosphate Receptor Function
Zachary T. Schug and Suresh K. Joseph
From the Department of Pathology, Cell Biology and Anatomy, Thomas Jefferson University School of Medicine, Philadelphia, PA 19107
Running title: Gating mechanism of the IP₃R
Address correspondence to: Suresh K. Joseph, Department of Pathology, Anatomy and Cell Biology, Thomas Jefferson University, 1020 Locust Street JAH 230A, Philadelphia, PA 19107, Tel: 215 503-1222; fax. 215 923-6813; E-mail: suresh.joseph@mail.tju.edu

In previous studies we have suggested that spatial proximity of the C- and N-terminal domains of inositol 1,4,5-trisphosphate receptors (IP₃Rs) may be critical for the channel gating mechanism. In the present study we have examined the sites of C-N interaction in more detail. We report that deletion mutations within the S4-S5 linker (aa2418-2437) prevent co-immunoprecipitation of the C- and N-terminal domains, inhibit channel activity and enhance IP₃ binding. We also show that a region of the C-terminal tail (aa2694-2721), predicted to be a coiled coil, is also required for channel activity. Circular dichroism spectroscopy and gel filtration studies confirm that this region has a helical structure with the ability to form tetramers. We propose a model in which IP₃-induced conformational changes in the N-terminal domain are mechanically transmitted to the opening of the pore through an attachment to the S4-S5 linker. The coiled coil domain in the C-terminal tail may play a critical role in maintaining the structural integrity of the channel.

Inositol 1,4,5-trisphosphate receptors (IP₃R) are tetrameric ligand-gated cation channels located in the membrane of the endoplasmic reticulum that serve to mobilize Ca²⁺ into the cytosol in response to cell stimulation (1, 2). There are three mammalian IP₃R isoforms (types I, II and III) encoded by three distinct genes (3). Each subunit of the IP₃R can be functionally divided into a ligand-binding domain (LBD), a regulatory domain and a channel domain (4). Based on crystal structure and mutagenesis studies the LBD can be subdivided into three regions: the suppressor domain (aa1-223), the β-domain (aa224-436) and the α-domain (aa437-604) (5, 6). Two models have been put forward to explain how the binding of IP₃ at the N-terminus gates the opening of the channel pore some 2000 amino acids away at the C-terminus. The first envisions that conformational changes in the LBD may be transmitted through the regulatory domain and suppressor domain to the channel domain (7). The second model is based on the observation that the LBD is directly coupled to the channel domain (8, 9). This latter model predicts that conformational changes in the LBD can gate the opening of the channel through a direct mechanical interaction between the domains. Overall, very little is known about the exact gating mechanism of IP₃R channels.

The coupling of the LBD to the channel domain was proposed to be intermolecular and to involve interaction sites located between aa1-340 in the N-terminus of one subunit and aa2418-2749 in the C-terminus of the adjacent subunit (9). In the present study we have utilized mutations in the C-terminal channel domain to further define the sites of C-N interaction. We show that one C-terminal site of interaction is the cytosol-exposed loop between transmembrane segments 4 and 5 (S4-S5 linker). The 159 amino acids of the cytosol-exposed C-terminal tail were not involved in the C-N interaction, but deletion of 60 amino acids from the tail, which completely removes a predicted coiled coil, was sufficient to impair channel function. A model of IP₃R channel gating is proposed that involves the S4-S5 linker and a coiled coil in the C-terminal tail as key components of the gating mechanism.

EXPERIMENTAL PROCEDURES
Expression cloning - The cDNA encoding the IP₃R type I in pCMV3 was the kind gift of Dr. Thomas Südhof (University of Texas Southwestern Medical Center). All amino acid numbering is with reference to the rat Type I IP₃R (10). The splice variant used in this study was SI (-), SII (+), SIII (-). All point mutants were made using the QuikChange or the QuikChange Multi Site-Directed Mutagenesis Kit (Stratagene, CA) utilizing a cassette encompassing the BstBI/XbaI fragment of the type I IP₃R in pBluescript (Invitrogen Corp., CA). Mutants were confirmed by sequencing and the BstBI/XbaI digested inserts were subcloned into the full length IP₃R or HA-tagged full length IP₃R cDNA in pCMV3. For the GST-fusion protein an insert of nucleotides 8050-8205 was amplified using Pfu DNA Polymerase (Stratagene, CA) and primers encoding BamHI and EcoRI sites. The BamHI/EcoRI PCR product was ligated into similarly digested pGEX-2T (Amersham Biosciences, NJ). This construct expresses aa2684-2735 of type I IP₃R with GST fused to its amino terminus. All primer sequences are available upon request.

Cell culture and transfection - COS-7 cells were grown on 100mm or 150mm plates (Sarstedt, NC) in DMEM (Sigma, MO) supplemented with 10% FBS (Gibco, NY), 0.1mg/ml streptomycin (Gibco, NY), 100 I.U./ml penicillin (Gibco, NY) and 0.04mg/ml Gentamicin (Gibco, NY) until 70-80% confluent. Transfections typically were done overnight in DMEM without serum. LT-1 (Mirus, WI) and NovaFECTOR (VennNova, Inc, FL) were used together during transfections and each was added at a cationic lipid to DNA ratio of 1:1. Transfections typically were done overnight in DMEM without serum. LT-1 (Mirus, WI) and NovaFECTOR (VennNova, Inc, FL) were used together during transfections and each was added at a cationic lipid to DNA ratio of 1:1. Transfections typically involved 5µg and 20µg of DNA for 100mm and 150mm plates, respectively. Co-transfections were done on 100mm plates with 5µg of IP₃R and 5µg of SERCA2b. After 24 hours, serum containing DMEM was added and cells were allowed to grow for 48-72 hours.

Expression, purification and concentration of GST-fusion proteins - GST-aa2684-2735 was propagated in BL21-Gold (DE3) Escherichia coli (Stratagene, CA) and induced with 1mM isopropyl β-D-thiogalactopyranoside (IPTG) at 37°C for 3 h. After induction, bacteria were pelleted and resuspended in a lysis buffer (PBS pH 7.8, 10mM EDTA, 0.25% lysozyme, 5mM DTT, 1% Triton X-100 and a broad spectrum protease inhibitor cocktail (Roche, IN). Bacteria were rotated at room temperature for 30min then sonicated. Lysed bacteria were spun down at 20,000 x g for 30 min at 4°C. The supernatant was rotated overnight at 4°C with 5ml of a 50% slurry of glutathione sepharose 4B (Amersham Biosciences, NJ). Beads were spun down, washed 3x with PBS and resuspended in elution buffer (50mM Tris-HCl pH 8.0, 10mM glutathione, 150mM KCl). After overnight rotation at 4°C the supernatant was removed and concentrated 10x using an Amicon-10 Ultra centrifugal device (Millipore, MA). Cleaved GST fusion protein was concentrated using Vivaspin 2 concentrators with a 3kDa molecular weight cut-off (ISC BioExpress, UT).

Preparation of microsomal vesicles - COS-7 cells were harvested in isolation buffer (320mM sucrose, 20mM Tris-HCl pH 7.4, 0.5mM EGTA). The cell suspension was then lysed by 6-8 passes through a 26½ gauge needle. Lysates were spun at 750 x g for 5 minutes. Supernatants were spun at 115,000 x g for 50 minutes. The pellets were resuspended in resuspension buffer A for flux assays (500mM sucrose, 1mM DTT, 20mM Tris-HCl pH 7.8, 300mM KCl) or in resuspension buffer B for trypsin digestion (250mM sucrose, 20mM MOPS-KOH pH 7.6, 20µM EGTA).

Sephacryl S-200 column - 2-5mg of cleaved and purified GST fusion protein was loaded onto the column. Fractions were eluted with a column buffer (10mM sodium phosphate pH 7.4, 150mM NaCl) and 130 fractions were collected (1ml/tube). 50-200µl was removed from each fraction and assayed for protein. Fractions containing the eluted coiled coil were pooled and concentrated for use in circular dichroism spectroscopy.

Circular dichroism - Far UV CD spectra from 190-250nm (in steps of 1nm) were obtained by averaging 2-5 scans on a CD spectrometer (Jasco J-810). The recordings were done with 0.45mg/ml peptide in column elution buffer using a cuvette with a 1mm path length. The helical content of the peptide was predicted by deconvolution of the spectra using Selcon3 (11).

Trypsin digestion and co-immunoprecipitation - Microsomal vesicles containing recombinant IP₃Rs were incubated (0.25µg/µl) in 200µl trypsin digestion buffer (20mM Tris-HCl pH 7.8, 120mM KCl, 1mM EDTA). Trypsin was added to the vesicles at 4µg/ml and incubated at 37°C for 10 minutes. The reaction was stopped by the addition of 40µg/ml soybean trypsin inhibitor (Sigma, MO).
complete protease inhibitor cocktail (Roche, IN), and 1mM PMSF (Sigma, MO). Microsomes were solubilized in 600µl of solubilization buffer (50mM Tris-HCl pH 7.8, 1mM EDTA, 150mM NaCl, 1% Triton X-100) and 10µl of *Staphylococcus aureus* cell wall (Pansorbin cells; Calbiochem, CA). The supernatants were collected after centrifugation (12,000 x g; 10 min) and 70µl of PrA sepharose (20% slurry) and immunoprecipitating antibody were added for 4-16 hours at 4°C on a rocker. PrA-sepharose beads were spun down and washed twice in solubilization buffer. Samples were quenched, boiled for 5 minutes, and loaded on 10% SDS-PAGE gels (unless stated otherwise).

**Trypsin digestion in the presence of a cross-linking reagent** - Microsomal vesicles containing recombinant IP$_3$Rs were incubated (0.5µg/µl) in 100µl of PBS with 0.7mM dithiobis(sulfosuccinimidylpropionate) (DTSSP) for 2 hours. After the incubation, 1.5M Tris-HCl pH 8.8 was added to a concentration of 35mM to stop the crosslinking reaction. An equal volume of trypsin digestion buffer was added and microsomes were processed for trypsin digestion as described above. After immunoprecipitation, the Protein A Sepharose beads (Sigma, MO) were incubated an additional 15 minutes at 4°C in 1% Zwittergent 3-14 to disrupt any non-covalent interactions. Samples were then washed in PBS and quenched in sample buffer supplemented with 10mM DTT to ensure cleavage of the cross-linker.

**$^{3}H$-IP$_3$ binding assays** - COS-7 cells from a 150mm plate were harvested by trypsinization, washed once in 10ml of HRB-HEDTA (20mM Tris-HEPES pH 7.5, 120mM KCl, 2mM HEDTA, 1X protease inhibitor cocktail and 1mM PMSF) and resuspended in 800µl of HRB-HEDTA. The suspension was permeabilized by the addition of 40µg saponin/mg protein. Saponin-treated cells (0.8ml) were incubated with a medium containing 120mM KCl, 20mM Tris-HEPES (pH 7.2) and 10mM $[^{3}H]$-IP$_3$. Non-specific binding was estimated by inclusion of 10µM cold IP$_3$. After 5 minutes on ice, the incubations were vacuum filtered on type A/E glass fiber filters (Pall Corporation, MI) and washed with 15mls of buffer (50mM Tris-HCl pH 8.3, 1mM EDTA, 1mg/mL BSA). Filters were counted in Budget-Solve complete counting cocktail (Research Products International, Corp., IL).

**$^{45}$Ca$^{2+}$ flux assays** - Assays were performed as previously described (12). Briefly, microsomal vesicles prepared from COS-7 expressing SERCA2b and IP$_3$R were incubated for 25 minutes at 30°C in a 200mM Ca$^{2+}$ buffer supplemented with ATP and an ATP regenerating system, $^{45}$Ca$^{2+}$, and 20µM ruthenium red. $^{45}$Ca$^{2+}$ uptake was estimated in the absence of any addition, 10µM IP$_3$ or 1µM A23187. After incubation, microsomes were vacuum filtered over a 0.3µm filter (Millipore Corp. MA), washed (150mM KCl) and filters were counted in Budget-Solve complete counting cocktail (Research Products International, Corp., IL). Depending on the exact level of SERCA2b expression the $^{45}$Ca$^{2+}$ counts accumulated in the vesicles ranged from 16,000 to 112,000 cpm in the absence of IP$_3$ for all the constructs used in this study with the exception of TL-6. For the latter construct the accumulated counts were significantly lower (2342 ± 30.5 cpm, n=4) which may indicate that TL-6 encodes a constitutively open channel.

**Membrane Attachment Assays** - Microsomal vesicles were prepared from COS-7 cells transiently transfected with various IP$_3$R constructs. 40µg of vesicles were incubated in a buffer (120mM KCl, 20mM Tris, 1mM EDTA, 1mM DTT) supplemented with 10µg/ml of trypsin for 0-60 minutes. The reactions were quenched by the addition of soybean trypsin inhibitor (100µg/ml) and 1mM PMSF. After digestion the vesicles were spun down at 62,000 x g and then pellets and supernatant fractions were quenched in SDS sample buffer and processed by 10% SDS-PAGE. Alternatively, a single time point of 7 minutes of trypsin digestion was used for the quantitative analysis of point mutations. All mutants were run in parallel with LoopA so that the loss of fragment I could be compared for each mutant. After SDS-PAGE both the pellets and the supernatants were immunoblotted with NT-1, an N-terminal specific IP$_3$R antibody. Densitometric analysis of bands was carried out using the program ImageJ (NIH, Bethesda, Maryland, [http://rsb.info.nih.gov/ij/](http://rsb.info.nih.gov/ij/)). The loss of fragment I into the supernatant for each loop mutant was expressed as a percentage of the loss of fragment I for the LoopA mutant. For analysis of the tail-less mutants the TL-2 mutant was used as a normalization control.

**RESULTS**
Assembly status of IP₃R mutants  Fig. 1A depicts a linear cartoon of the IP₃R showing the three main functional domains. IP₃Rs are cleaved by limited trypsin digestion into five distinct fragments (I-V) whose boundaries and molecular weights are labeled in Fig. 1A. We and Yoshikawa et al. (13) have shown that these five fragments remain bound to one another through non-covalent interactions after trypsin cleavage, such that immunoprecipitation of the 95kDa tryptic fragment V with an antibody against the C-terminus will co-immunoprecipitate the 40kD fragment I of an adjacent subunit in the tetramer (8, 9, 13). Further studies showed that fragment I and V could be directly cross-linked indicating that they were within the cross-linking distance of ~12 Å. GST pull-down assays using a GST-tagged LBD and various in vitro translated transmembrane segments from the channel domain, showed that the interacting sites within the channel domain resided downstream of S4 (i.e. between aa2418-2749, see also Fig. 1B) (9). Within these residues the only exposed cytosolic regions which could be involved in the C-N interaction are the S4-S5 linker and C-terminal tail (Fig. 1B). To further narrow down the interaction site(s) we deleted 10 amino acids from the proximal or distal portion of the S4-S5 linker and also made progressive deletions of the C-terminal tail. All mutant constructs were tagged with an HA epitope for detection and immunoprecipitation of the C-terminal tail (Fig. 1B). Dithio-bis(sulfosuccinimidylpropionate) (DTSSP) is a cross-linking reagent reactive towards lysines that have previously been shown to cross-link fragments V & I in a manner that survives immunoprecipitation in the presence of Zwittergent 3-14, a detergent that disrupts all intra- and intermolecular non-covalent interactions (9, 10, 15). If the loop mutants caused gross structural changes in the receptor, then DTSSP cross-linking of fragments V & I would be expected to be altered. Fig. 2A (lanes 6 & 9) shows this was not the case, indicating that the cross-linked lysine side-chains in the two tryptic fragments remain within ~12Å in both loop mutants. These data suggest that the S4-S5 linker may be one major site of interaction between the ligand-binding and channel domains. In the absence of cross-linking reagents, this interaction may be destabilized in the loop mutants during the

Limited tryptic digestion and co-immunoprecipitation  We analyzed the C-N interaction of ΔLoopA, ΔLoopB and TL-6 mutants employing a co-immunoprecipitation assay we have used previously (8, 9). All the mutants formed a 40kDa N-terminal fragment I (Fig. 2A, lanes 4, 7 & 10) and a ~90-95kDa HA-tagged C-terminal fragment V (Fig. 2A, lanes 13-20). As observed previously, the immunoprecipitation of wild type (FL-HA) fragment V with HA antibody resulted in the co-immunoprecipitation of fragment I (Fig. 2A, lane 2). This was also observed for TL-6, a mutant IP₃R lacking 159 amino acids of the C-terminal tail (Fig. 2A, lane 11). From this result we conclude that the C-terminal tail region is not necessary for C-N interaction. The same procedure, however, showed that fragment I of the ΔLoopA and ΔLoopB mutants was not co-precipitated by HA-tagged fragment V (Fig. 2A, lanes 5 & 8). Dithio-bis(sulfosuccinimidylpropionate) (DTSSP) is a cross-linking reagent reactive towards lysines that has previously been shown to cross-link fragments V & I in a manner that survives immunoprecipitation in the presence of Zwittergent 3-14, a detergent which disrupts all intra- and intermolecular non-covalent interactions (9, 10, 15). If the loop mutants caused gross structural changes in the receptor, then DTSSP cross-linking of fragments V & I would be expected to be altered. Fig. 2A (lanes 6 & 9) shows this was not the case, indicating that the cross-linked lysine side-chains in the two tryptic fragments remain within ~12Å in both loop mutants. These data suggest that the S4-S5 linker may be one major site of interaction between the ligand-binding and channel domains. In the absence of cross-linking reagents, this interaction may be destabilized in the loop mutants during the
prolonged period required for immunoprecipitation.

Fragment I (aa1-340) encompasses the entire suppressor domain (aa1-223) as well as some of the IP₃ binding core (aa224-576) (5, 13). We considered the possibility that a loss of C-N interaction could affect the IP₃ binding characteristics of the IP₃R. We performed ³H-IP₃ binding assays on each of our constructs as previously described (16). To correct for different expression levels the data was normalized to the binding and expression levels of wild type Type I IP₃R measured in parallel. Both loop deletions displayed statistically significant increases in the binding of IP₃ relative to the wild type IP₃R (Fig. 2B). The finding that deletions made to the S4-S5 linker have effects on the binding of IP₃ to the receptor is consistent with an interaction between these two domains and suggests that the C-N interaction may indirectly affect the structure of the N-terminal LBD. None of the tail-less mutants tested showed any significant changes in ³H-IP₃ binding (data not shown; for TL-5 see also (7, 17)).

Structural and functional characterization of loop mutants Previous studies have shown that a major proportion of the cleaved fragment I remains associated with the membrane fraction after trypsin digestion (8, 13). If the interaction between fragment I and fragment V is weakened in the loop mutants we would predict that a greater proportion of fragment I would be released into the supernatant. To test this, the supernatants of trypsin digested FL-HA, ΔLoopA or ΔLoopB were assayed for fragment I. FL-HA had almost no loss of fragment I from the membrane after 60 minutes of trypsin digestion (Fig. 3A). However, fragment I dissociated from the membrane in a time dependent manner in both ΔLoopA and ΔLoopB mutants (Fig. 3A). The results observed with the loop mutants in membrane attachment assays are consistent with the findings obtained in co-immunoprecipitation assays and suggest that C-N interactions are weakened by deletions made within the S4-S5 linker.

In order to further narrow down which amino acids might be directly involved in the C-N interaction we created a series of alanine point mutations within the ΔLoopA and ΔLoopB regions. The mutations were designed to remove charged or hydrophobic residues and also to determine the relative importance of residues within the ΔLoopA and ΔLoopB regions (Fig. 3B). All the point mutants were expressed at similar levels in microsomal vesicles (upper panel, Fig. 3C). The amount of fragment I lost from the membrane by each point mutant was expressed as a percentage of the amount lost from ΔLoopA membranes which were run in parallel and assigned as 100% (Fig. 3C, mutant 1). ΔLoopB behaved identically to ΔLoopA in membrane attachment assays (Fig. 3C, mutant 2). Mutations made to the charged residues within the ΔLoopA region led to a substantial loss of fragment I from the membrane (Fig. 3B, mutants 3, 4 & 5). Mutation of charged residues in ΔLoopB (mutant 6) or two hydrophobic residues in ΔLoopA (mutant 7) had minimal effects. The partial effect on stability of removing a positive charge in ΔLoopA (mutant 4) was not augmented by the additional removal of positive charges in ΔLoopB (mutant 10). The data suggest that C-N interactions may involve electrostatic associations primarily with charged residues in the ΔLoopA portion of the S4-S5 linker.

To test if mutations in the S4-S5 linker affected function, we employed a ⁴⁵Ca²⁺ flux assay using microsomal vesicles prepared from COS-7 cells co-transfected with various mutant IP₃Rs constructs along with the Ca²⁺ pump, SERCA2b. The assay measures the activity of transfected, rather than endogenous IP₃Rs, and has been described in detail elsewhere (9, 12). The last lane in Fig. 3D (labeled SERCA2b) shows the IP₃-mediated inhibition of ⁴⁵Ca²⁺ flux in microsomes prepared from COS-7 cells transfected with SERCA2b alone (no recombinant IP₃R) and represents the background contribution from endogenous IP₃Rs. Both ΔLoopA and ΔLoopB were inactive in IP₃-mediated ⁴⁵Ca²⁺ flux assays and behaved like the control cells transfected with SERCA2b alone (mutants 1 & 2, respectively). Functional characterization of selected loop point mutants by ⁴⁵Ca²⁺ flux assays showed qualitatively similar results to the membrane attachment assays. Mutation of either acidic residues (D2418, E2423, E2424) or a single basic residue (R2422) in the ΔLoopA region had partial inhibitory effects on channel function (Fig. 3D, mutants 3 & 4), which was further increased when the mutations were combined (Fig. 3D, mutant 5). However, mutation of the charged residues...
Properties of the IP$_3$R C-terminal tail region

The C-terminal tail region encompasses aa2590-2749 and has been shown to be involved in the assembly and function of the IP$_3$R (7, 14, 18). Previous studies have shown that deletion of 13aa from the C-terminus does not affect channel function (7, 19) but removal of 139aa inactivates the channel (7). Two cysteine residues just downstream of S6 were also shown to be crucial for activation gating (white circles, Fig. 4A) (7). Other studies located a “minimal interaction domain” (MID) between aa2629-2654, which plays a role in the dimerization of C-terminal tails as well as in the assembly and stability of tetramers (Fig. 4A) (14). Membrane attachment assays were carried out on the series of tail-less mutant constructs with progressive deletions of the C-terminal tail (shown in Fig. 1B). All the tail-less deletions were expressed at similar levels in microsomal vesicles (upper panel, Fig. 4B). Wild type, TL-1, TL-1A and TL-1B did not show a substantial loss of fragment I from the membrane. However, the deletion of more than 43 amino acids (i.e. TL-2 through TL-6) caused a loss of fragment I from the membrane compared to wild type (Fig. 4B). 45Ca$^{2+}$ flux studies on the tail-less deletions are shown in Fig. 4C. Interestingly, function was completely lost when more than 43 amino acids were deleted from the C-terminus (TL-2 through TL-6). Since the membrane attachment and the functional assays both show major effects beginning at TL-2 we conclude that the amino acids between 43 and 60 residues from the C-terminus are also crucial to maintaining a functional channel.

Structural characterization of the C-terminal tail region

Analysis of the C-terminal tail using the program MultiCoil (20) indicates that the residues between TL-1 and TL-2 (aa2690-2725) have a high probability of being part of a coiled coil (Fig. 5A). The analysis indicates that there are four heptad repeats which make up the coiled coil beginning at L2694 and continuing through Q2721. The inset in Fig. 5A is an end-on view of C-terminal tail residues 2694-2721 shown as a single helix of the coiled coil. The core hydrophobic residues at positions a and d of the heptad are indicated (black circles). Removal of the last two heptads displayed a statistically significant inhibition of function (TL-1B, Fig. 4C) but the removal of all 4 heptads from the C-terminal tail correlated with a complete loss of channel function (TL-2, Fig. 4C).

In order to examine the structural and biophysical properties of this region of the C-terminal tail, a GST fusion protein of aa2684-2734 was expressed and purified (Fig. 5B, lane 1). The fusion protein was cut with thrombin to separate the GST (24kDa) from the predicted coiled coil region (6kDa) (Fig. 5B, lane 2) and then further purified by incubation with glutathione sepharose 4B beads (lane 3) followed by size exclusion FPLC (lane 4). A portion of the linker region remained attached to the coiled coil after thrombin digestion and created a peptide with the predicted molecular weight of 10.5kDa. Analysis of the molecular weight of the purified protein on a sephacryl S-200 column indicated a size of ~45kDa (fraction 48) with no evidence for the significant formation of a monomer (fraction 82) (Fig. 5C). We conclude that aa2684-2734 from the C-terminal tail has the ability to assemble into tetramers and that this higher order structure may be necessary for channel function. The secondary structure of aa2684-2734 was assessed by circular dichroism (CD) spectroscopy. CD wavelength scans showed intense minima near 208nm and 222nm which are characteristic of highly helical proteins (Fig. 5D). When the spectra was analyzed by a secondary structure prediction program Selcon3, (11) this region of the C-terminal tail was predicted to be 42% helical (data not shown).

**DISCUSSION**

A hypothetical model of IP$_3$R gating based on our studies is shown in Fig. 6. The main feature of the model is that the gating mechanism couples the conformational change in the LBD, resulting from IP$_3$ binding, to the mechanical movement of the S4-S5 linker through a direct interaction between these regions of the IP$_3$R. In our mutagenesis experiments, shortening the S4-S5 linker (ΔLoopA or ΔLoop B) prevented interactions between the C- and N-terminal domains and yielded a non-functional channel. Point mutations suggested that charged residues in the proximal region of the linker...
LoopA) may have a dominant role. The importance of the S4-S5 linker has also been emphasized in studies of the gating mechanism of several voltage-gated channels. In the Kv1.2 channel the S4-S5 linker is an amphipathic helix which runs parallel to the plane of the membrane and is arranged in a manner that constricts the S6 pore-lining helix bundle at the cytosolic aspect of the membrane, thereby maintaining the channel in a closed conformation (21, 22). The voltage induced movement of the charged S4 helix is proposed to displace the S4-S5 linker and thereby permit the S6 helix bundle to separate which allows ion conduction (22). Similarly, in KirBac1.1 channels a homologous S4-S5 linker structure (referred to as a “sliding helix”) has been suggested to play a critical role in its gating mechanism (23). In both these K+ channels, interactions between the S4-S5 linker and the distal segments of the S6 helix have been documented (24, 25).

Secondary structure programs predict that the S4-S5 linker would also form an amphipathic helix in IP3Rs (data not shown). Whereas the voltage-dependent shift of the S4 helix drives the movement of the S4-S5 linker in K+ channels, we suggest that the direct coupling of the LBD to the S4-S5 linker causes the opening of the channel upon IP3 binding. Previous studies have shown that the presence of IP3 does not interfere with the co-immunoprecipitation of C- and N-terminal domains in the absence (8) or presence of a cross-linking reagent (data not shown). This suggests that any changes in the interactions between these domains involved in the gating mechanism may cause a subtle mechanical movement of the S4-S5 linker rather than a gross formation/disruption of molecular interactions. The movement of the linker would allow the S6 helix bundle to separate and open an ion conduction pathway for Ca2+ (Fig. 6B). Ca2+ itself has profound effects on IP3R channel gating (26). Although not specifically considered in Fig. 6, it is clear that the multiple binding sites for Ca2+ and calmodulin present in the receptor could potentially influence the binding of IP3 and the conformational movements involved in the gating mechanism at many steps.

What is the evidence that C-N interactions are direct and involve the suppressor domain (aa1-223) as indicated in Fig. 6? GST-LBD (aa1-605) has been shown to specifically pull down an in vitro translated segment of the IP3R containing the S4-S5 linker (aa2418-2749), which together with cross-linking data (9) suggest that the interaction is direct and lies within aa1-340. The suppressor domain makes up the majority of this region and we have therefore speculated that it contains the site of interaction. Interestingly, deletion of the suppressor domain does inactivate IP3Rs despite increasing the affinity of the receptor for IP3 by over 10-fold (7). Others have proposed that segments from the regulatory domain maintain the channel in the closed state and that binding of IP3 relieves this inhibition (27). Varnai et al. have shown that expression of an ER-tethered form of the α-domain of the LBD (aa427-605) can gate endogenous IP3Rs (28). Further work is required to identify the exact N-terminal segments interacting with the S4-S5 linker.

A second significant observation of this study is the identification of a coiled coil domain in the distal portion of the C-terminal tail (Fig. 4A and Fig. 5). Removal of this region eliminated IP3R activation and destabilized C-N interactions as measured in membrane attachment assays (Fig. 4B-C), but did not increase IP3 binding or affect the co-immunoprecipitation of tryptic fragments as observed for the loop mutants (Fig. 2). Therefore, we conclude that deletion of the C-terminal tail must destabilize the IP3R in a manner distinct from the loop deletions. Co-immunoprecipitation assays (Fig. 2A) or pull-down assays using a GST fusion protein of the C-terminal tail (aa2590-2749) and recombinant LBD (aa1-605) from transiently transfected COS-7 cells provide no evidence that the tail is directly involved in the interaction with the N-terminal domain (Supplemental Data, Fig. S2). This conclusion may appear to be inconsistent with the finding that an enhanced release of fragment I was noted for both the loop and tail-less mutants in the membrane attachment assays. However, it should be pointed out that the co-immunoprecipitation and membrane attachment measurements are very different assays and that many additional factors (other than interaction between the S4-S5 linker and fragment I) may be involved in maintaining the adherence of trypsin-digested receptor segments to membranes. For example, the void created by deletion of the C-terminal tail and the absence of the stabilization contributed by the coiled coil domain could potentially interfere with the packing of the tetrameric
subunits and could indirectly impair interactions between the five tryptic fragments.

Coiled coils have been identified in the C-terminal tails of a diverse set of ion channels such as voltage gated potassium channels (Eag1, Eag2, Elk, Erg) (29, 30), cyclic nucleotide channels (CNQ and KCNQ) (31-34), calcium-dependent potassium channels (SK) (35), non-selective cation channels (PKD) (36) and calcium channels (TRP) (37), where they typically aid in the assembly and stability of tetramers (38). However, the primary determinants for tetramer assembly in IP$_3$Rs are contained within the S5-S6 transmembrane domains (18). Therefore the coiled coil may contribute to the stability of the IP$_3$R tetramer but is unlikely to have a primary role in tetramer formation. The C-terminal tails of both ryanodine receptors (RyR) and IP$_3$Rs have been shown to self associate (14) and the recombinant IP$_3$R coiled coil domain (aa2684-2734) forms tetramers as determined by FPLC chromatography (Fig. 5C). At present we can only speculate as to why the deletion of 60aa from the C-terminal tail activates the IP$_3$R channel. Clearly the loss of the coiled coil domain correlates with the loss of structural stability of the receptor as indicated from the membrane attachment assays. The lack of the proper associations between the C-terminal tails in the tail-less mutants could affect a number of channel characteristics such as the packing of subunits around the channel, the structural integrity of the pore lining S6 helix or the occlusion of the normal exit pathway for the Ca$^{2+}$ ion. Interestingly, a coiled coil domain is absent from the shorter C-terminal tail of RyRs, although removal of 15 amino acids from the C-terminal tail of RyRs is sufficient to disrupt RyR activation (39, 40).

Ramos-Franco et al. have shown that deletion of the first four transmembrane domains (Δ2211-2416) produced a constitutively open channel (41). Presumably, the absence of a membrane anchor on the N-terminal end of the S4-S5 linker allows the linker to adopt a position where it can no longer compress the S6 pore lining helix bundle. This mutant was also found not to be gated by IP$_3$, as would be expected if the channel lacked the C-N interaction necessary to relay the signal to the channel domain. In our studies, none of the loop mutants or TL-1 through TL-5 resulted in a constitutive activation of the channel, as indicated by a robust $^{45}$Ca$^{2+}$ uptake into microsomal vesicles in the absence of IP$_3$ (data not shown). However, preliminary studies suggest that the TL-6 mutant, which includes deletion of the cytoplasmic end of the helix bundle, may be constitutively active (data not shown).

A number of predictions and future directions arise from the model in Fig. 6. The S6 helix is shown as extending beyond the membrane but its exact boundaries remain to be established. Many ion channels contain a glycine which is proposed to act as a hinge to allow the S6 helix to bend during channel opening (24, 25, 42). Mutagenesis of a putative hinge residue G2586 in the S6 helix of IP$_3$Rs should test this proposed mechanism. Interestingly, mutation of the analogous residue in RyR2 (G4864A) did not affect caffeine-mediated Ca$^{2+}$ release (43). The section of the S6 helix, proposed to extend into the cytoplasm and interact with the S4-S5 linker, is presumably critical for channel gating. C2610 and C2613 in this region have been identified as being crucial for activation gating of IP$_3$Rs (7). Salt bridges between residues in the S4-S5 linker and the region adjacent to S6 have been suggested to stabilize the closed state of HCN channels (44). Further studies are needed to identify interaction sites between the S4-S5 linker and S6 helix in IP$_3$Rs and also to document the movement of these regions predicted from the proposed gating mechanism.

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FIGURE LEGENDS

Fig. 1. Schematic representation of the rat Type I IP\(_3\)R deletion mutants.  
A, Linear cartoon depicting the location and molecular weights of the five fragments (I-V) generated by tryptic digestion of the IP\(_3\)R. The three functional domains of the receptor are indicated.  
B, Schematic representation of the 11 deletion mutants used in defining the region of C-N interaction. Deletions were made in either the S4-S5 linker (\(\Delta}\text{LoopA} \& \Delta}\text{LoopB}) or in the C-terminal tail region (TL-1 through TL-6). The amino acid numbers for the loop mutants and tail-less mutants were as follows: \(\Delta}\text{LoopA} (\Delta2418-2427), \Delta}\text{LoopB} (\Delta2428-2437), \text{FL-HA} (\text{aa}1-2749), \text{TL}-1 (\text{aa}1-2725), \text{TL}-1A (\text{aa}1-2716), \text{TL}-1B (\text{aa}1-2706), \text{TL}-2 (\text{aa}1-2689), \text{TL}-3 (\text{aa}1-2656), \text{TL}-4 (\text{aa}1-2626), \text{TL}-5 (\text{aa}1-2609), \text{TL}-6 (\text{aa}1-2590).

Fig. 2. Co-immunoprecipitation and cross-linking of tryptic fragment I by HA-tagged fragment V in Type I IP\(_3\)R deletion mutants.  
A, COS cells were transfected with FL-HA (wild type), \(\Delta}\text{LoopA}, \Delta}\text{LoopB} or TL-6. Microsomal vesicles were prepared and digested with trypsin as described in ‘Methods’ in a final volume of 400\(\mu\)l (0.125\(\mu\)g protein/\(\mu\)l). An aliquot (5\(\mu\)g protein) of each digestion was saved for immunoblot analysis (lanes 1, 4, 7 and 10). The remaining digested IP\(_3\)R was subjected to immunoprecipitation with HA mAb and protein A sepharose for 4-16hrs at 4°C (lanes 2, 5, 8 and 11). Alternatively, vesicles were cross-linked with 0.7mM DTSSP for 2hrs on ice prior to trypsin digestion and immunoprecipitation (lanes 3, 6, 9 and 12). Lanes 13-20 show analysis of lysates by immunoblotting with HA mAb and indicate that fragment V is formed normally in the loop and tail-less mutants.  
B, WT and loop mutant cDNA were transiently transfected into COS-7 cells. The cells were permeabilized with saponin and binding was measured with a sub-saturating concentration of \(^3\text{H}\text{-IP}_3\) (10nM) as described in ‘Methods’. The expression of the loop mutants were quantified densitometrically from immunoblots and expressed as a ratio (R) relative to the WT receptor. The binding values of the loop mutants were divided by R to correct for small variations in expression (16). WT binding values were between 0.8-1pmol/mg protein. Data are the means ± S.E.M. of 6-8 independent measurements.

Fig. 3. Assessment of the stability of the C-N interaction and functional analysis of S4-S5 linker mutations.  
A, Microsomal vesicles expressing wild type, \(\Delta}\text{LoopA} or \(\Delta}\text{LoopB} IP\(_3\)Rs were treated with trypsin for 0-60min. The reaction was quenched and pellets and supernatants were separated and subjected to SDS-PAGE. The trypsin digested IP\(_3\)Rs were probed with N-terminal antibody (NT-1) to recognize tryptic fragment I.  
B, Schematic diagram of the S4-S5 linker mutants. All mutations were made to alanine. \(\text{WT–full length HA-tagged IP}_3\text{R, 1}\)–\(\Delta}\text{LoopA} (\Delta2418-2427), \(\text{2}\)–\(\Delta}\text{LoopB} (\Delta2428-2437), \(\text{3}\)–(D2418, E2423, E2424), \(\text{4}\)–(R2422A), \(\text{5}\)–(D2418, R2422, E2423, E2424), \(\text{6}\)–(K2431, R2435), \(\text{7}\)–(L2419, V2420), \(\text{8}\)–(D2418, E2423, E2424, R2435), \(\text{9}\)–(D2418, E2423, E2424, K2431, R2435), \(\text{10}\)–(R2422, K2431, R2435), \(\text{11}\)–(D2418, R2422, E2423, E2424, K2431, R2435).  
C, Membrane attachment assay of S4-S5 linker mutants. upper panel, Western blot of microsomal lysate (pellet + supernatant) after trypsin digestion using NT-1. Molecular weight marker is shown on the left and lanes are aligned with the re-
spective bar for each mutant construct. lower panel, supernatants and pellets were collected after 7 min of trypsin digestion and the levels of fragment I were determined by immunoblotting with NT-1 antibody. The amount of fragment I released for each mutant was expressed relative to ΔLoopA (run in parallel) and defined as the 100% value. *, significance of $p \leq 0.05$ versus wild type (WT) and is representative of at least 3 independent measurements. The absolute level of release for ΔLoopA expressed as a supernatant/pellet ratio was $35.2 \pm 2.6\%$ ($n = 10$). D, The effect of IP$_3$ on $^{45}$Ca$^{2+}$ flux was measured in microsomes prepared from COS-7 cells co-transfected with SERCA2b and IP$_3$R cDNA as described in ‘Methods’. The cells transfected with SERCA2b alone was taken as the control (12). *, significance of $p \leq 0.05$ versus wild type (WT) and is representative of at least 4 independent measurements.

Fig. 4. Assessment of the stability of the C-N interaction and functional analysis of tail-less deletion mutants. A, Linear cartoon representation of the C-terminal tail of IP$_3$Rs showing the S6 helix, C2610, C2613 (white circles), the minimal interaction domain (MID) and a predicted coiled coil domain. The C-terminal end of each tail-less deletion (italics) is marked by an arrow. B, Membrane attachment assay of tail-less deletion mutants. upper panel, Western blot of microsomal lysate (pellet + supernatant) after trypsin digestion using NT-1. Molecular weight marker is shown on the left and lanes are aligned with the respective bar for each mutant construct. lower panel, supernatants and pellets were collected after 7 min of trypsin digestion and the levels of fragment I were determined by immunoblotting with NT-1 antibody. The amount of fragment I released for each mutant was expressed relative to TL-2 (run in parallel) and defined as the 100% value. *, significance of $p \leq 0.05$ versus wild type (WT) and is representative of at least 3 independent measurements. The absolute release for TL-2 expressed as a supernatant/pellet ratio was $30.3 \pm 4.7\%$ ($n = 4$). C, The channel function of the tail-less deletion mutants was assessed using a $^{45}$Ca$^{2+}$ flux assay as given in Fig. 3D. *, significance of $p \leq 0.05$ versus wild type (WT) and is representative of at least 4 independent measurements.

Fig. 5. Biochemical and biophysical analysis of a coiled coil in the C-terminal tail of the IP$_3$R. A, Line graph of the probability of the amino acids in the C-terminal tail forming a coiled coil as predicted by the program MultiCoil (http://multicoil.lcs.mit.edu/cgi-bin/multicoil). The inset is a typical helical wheel projection for the four heptad repeats of the coiled coil (aa2694-2721) (45, 46) showing the core hydrophobic residues of the heptad as filled circles. B, A GST fusion protein of aa2684-2734 was expressed and purified (lane 1). The GST-portion was then cleaved from the coiled coil by thrombin digestion (lane 2). The purified coiled coil was further purified by incubation with glutathione sepharose 4B (lane 3). The purified coiled coil was then subjected to size exclusion FPLC and concentrated (lane 4). 5µg of protein was loaded per lane and run on a 4-20% SDS-PAGE gel and coomassie stained for bands. A black arrowhead marks the position of the coiled coil after cleavage of the GST. C, Size exclusion FPLC profile for the cleaved GST fusion protein of aa2684-2734. Peak elution profiles for GST alone (50kDa), soybean trypsin inhibitor (20.5kDa) and aprotinin (6.5kDa) are marked by dotted lines. Size exclusion FPLC of aa2684-2734 (10.5kDa) peaked at fraction 48 and had a predicted molecular weight of 45kDa. The elution profile of the coiled coil region corresponds with the formation of tetramers. D, Secondary structure was measured by CD spectroscopy as given in ‘Methods’. The spectrum is compatible with a predominantly helical structure.

Fig. 6. Model for the gating mechanism of the IP$_3$R. For simplicity only the S4, S5 and S6 helices of two subunits of the IP$_3$R tetramer are shown. The S4-S5 linker is shown as a helix running parallel to the membrane and arranged in a manner that constricts the pore-lining S6 helix into a closed configuration (panel A). Based on the present study we propose that the S4-S5 linker is connected via multiple interactions with residues in the N-terminal suppressor domain (SD) and that binding of IP$_3$ to the ligand-binding domain (LBD) causes a conformational change that induces a displacement of the S4-S5 linker that allows the constriction of the S6 helix at the bundle crossing to be relieved and thereby provide a conduction pathway for Ca$^{2+}$ (panel B). The maintenance of the tertiary structure of the gating/conduction localis.
pathway is also dependent on the integrity of two key cysteine residues (filled circles) and a coiled coil domain (CC) in the C-terminal tail. For additional details see text.
Figure 1
Figure 2

Panel A: Western blot analysis showing the protein expression levels of FL-HA, ΔLoopA, ΔLoopB, and TL-6. 

- **IP: HA**
  - FL-HA: -
  - ΔLoopA: +
  - ΔLoopB: +
  - TL-6: +

- **DTSSP**
  - FL-HA: -
  - ΔLoopA: -
  - ΔLoopB: +
  - TL-6: +

- **Zwt 3-14**
  - FL-HA: -
  - ΔLoopA: -
  - ΔLoopB: +
  - TL-6: +

Blot: NT-1 Ab

(38 kDa bands)

Blot: HA Ab

(90 kDa bands)

Panel B: Quantitative analysis of 

\[ ^3H \text{-IP}_3 \text{ Binding (\% of Control)} \]

- **FL-HA**
  - 100 (control)

- **ΔLoopA**
  - 200

- **ΔLoopB**
  - 500

* indicates statistically significant differences compared to control.
Figure 4

(A) Schematic representation of the protein domain structure. The diagram shows the domains S6, MID, and Coiled Coil with corresponding amino acid positions.

(B) Bar graph showing the percentage of Fragment I in the supernatant for different constructs. The x-axis represents the constructs (WT, TL-1, TL-1A, TL-1B, TL-2, TL-3, TL-4, TL-5, and TL-6), and the y-axis represents the percentage of Fragment I (% of TL#2).

(C) Graph depicting the uptake of $^{45}Ca^{2+}$ for different constructs. The x-axis represents the constructs (SERCA2b, WT, TL-1, TL-1A, TL-1B, TL-2, TL-3, TL-4, TL-5, and TL-6), and the y-axis represents the uptake as a percentage of control.

Blot: NT-1 Ab

* indicates statistically significant differences.
Figure 6

A. CLOSED

B. OPEN

Key:
- **SD**: Scent Detection Domain
- **LBD**: Ligand Binding Domain
- **CC**: Cys-Cellulose Complex
- **IP3**: Inositol 1,4,5-Trisphosphate
- **C**: Channel
- **lumen**: Lumen of the cell
- **cytoplasm**: Cytoplasm of the cell

The figure illustrates the transition from a closed to an open state of the channel, highlighting the interactions of the SD and LBD domains with IP3 in the act of opening.
The role of the S4-S5 linker and C-terminal tail in inositol 1,4,5-trisphosphate receptor function
Zachary T. Schug and Suresh K. Joseph

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