Cryo-EM structure of human type-3 inositol triphosphate receptor reveals the presence of a self-binding peptide that acts as an antagonist

Received for publication, October 22, 2019, and in revised form, January 6, 2020. Published, Papers in Press, January 8, 2020, DOI 10.1074/jbc.RA119.011570

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Edited by Karen G. Fleming

Calcium-mediated signaling through inositol 1,4,5-triphosphate receptors (IP3Rs) is essential for the regulation of numerous physiological processes, including fertilization, muscle contraction, apoptosis, secretion, and synaptic plasticity. Disregulation of IP3Rs leads to pathological calcium signaling and is implicated in many common diseases, including cancer and neurodegenerative, autoimmune, and metabolic diseases. Revealing the mechanism of activation and inhibition of this ion channel will be critical to an improved understanding of the biological processes that are controlled by IP3Rs. Here, we report structural findings of the human type-3 IP3R (IP3R-3) obtained by cryo-EM (at an overall resolution of 3.8 Å), revealing an unanticipated regulatory mechanism where a loop distantly located in the primary sequence occupies the IP3-binding site and competitively inhibits IP3 binding. We propose that this inhibitory mechanism must differ qualitatively among IP3R subtypes because of their diverse loop sequences, potentially serving as a key molecular determinant of subtype-specific calcium signaling in IP3Rs. In summary, our structural characterization of human IP3R-3 provides critical insights into the mechanistic function of IP3Rs and into subtype-specific regulation of these important calcium-regulatory channels.

Inositol 1,4,5-triphosphate receptors (IP3Rs)3 are ligand-gated calcium (Ca2+) release channels localized predominantly in the endoplasmic reticulum (ER) membrane of all cell types (1). Inositol 1,4,5-triphosphate (IP3) generated by phospholipase C upon G protein- or tyrosine kinase-coupled receptor activation binds to IP3Rs and opens the channel, leading to transfer of Ca2+ from the ER lumen to the cytoplasm (1). Ca2+ released by IP3Rs act as universal messengers required to regulate diverse physiological processes including fertilization, muscle contraction, apoptosis, secretion, and synaptic plasticity (2, 3). Deregulation of IP3Rs results in abnormal Ca2+ signaling, leading to a broad spectrum of pathologies including cancer, neurodegenerative, autoimmune, and metabolic diseases (4).

In mammals, there are three different subtypes (1–3) of IP3Rs, which share 60–70% sequence identity, can form homo- or heterotetramers, exhibit different spatial expression profiles, and are involved in diverse signaling pathways. The type 3 receptors (IP3R-3s) are predominantly expressed in rapidly proliferating cells and are involved in taste perception and hair growth (5–7). Additionally, deregulation of IP3R-3 is implicated in diseases with deficiencies in cell fate decisions such as cancer and degenerative diseases (8–10). For example, the expression of the IP3R-3 is up-regulated in several cancer types including glioblastoma, breast, gastric, and colorectal cancer (11–14). Furthermore, many tumor suppressors and oncoproteins such as protein kinase B, protein phosphatase 2A, promyelocytic leukemia protein, phosphatase and tensin homolog (PTEN), and BRCA1-associated protein 1 tightly regulate the stability and activity of IP3R-3s (10, 15–17). Inhibiting IP3R-3 degradation in PTEN-regulated cancers was shown to be a valid therapeutic strategy (15). Although IP3R-3s are responsible for regulating distinct biological processes compared with types 1 and 2, it is unclear whether there is any mechanistic difference in their operation.

IP3Rs function as signaling hubs where signals from different pathways and metabolic states are integrated to allosterically modulate IP3R gating. Receptor activity is tightly controlled by many factors including second messengers (e.g. IP3, Ca2+), other small molecules (e.g. ATP), modulatory proteins, and posttranslational modifications such as phosphorylation and

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2 This work was supported by the Start-up fund from Vanderbilt University and Vanderbilt Diabetes and Research Training Center Grant DK020593 (to E.K.), National Institutes of Health Grant R01HD061543 (to T.N.) and the Vanderbilt University, and by Molecular Biophysics Training Program Grant T32 GM008320 (to Walter Chazin) (to C. M. A. and E. A. L.). The authors declare that they have no conflicts of interest with the contents of this article. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.
3 The atomic coordinates and structure factors (code 6UQK) have been deposited in the Protein Data Bank (http://wwpdb.org/).
4 The cryo-EM maps reported in this paper have been submitted to the EM Density Maps database under accession numbers EMD-20849 and EMD-20850.
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ubiquitination. Despite recent advances in the structural studies of IP₃Rs, molecular understanding of receptor gating and regulation remains largely unknown.

Structural investigation of IP₃Rs was pioneered by using the IP₃R-1 obtained from native tissues, due to its abundant expression in the cerebellum and well-established purification strategy (18–26). Recently, cryo-EM structures of recombinant IP₃R-3 have been reported and the global architectures of both IP₃R-1 and IP₃R-3 share common features (27). Due to the large size of the IP₃Rs, different structures contain nonoverlapping information resulting, primarily, from variations of local resolution within the 3D map. In fact, the atomic view of the complex is still incomplete. Published structures in the unliganded (apo) and liganded states provide a basis to develop mechanistic hypotheses on the channel gating. However, the current conformational ensemble falls short in revealing the full gating cycle, modulation, as well as mechanism of inhibition by known chemical reagents.

Here we present a cryo-EM structure of the human IP₃R-3 (hIP₃R-3) in a ligand-free conformation revealing a loop extending from the regulatory ARM2 domain that occupies the IP₃-binding site and thus may function as a regulator of IP₃ binding. In addition, our structure identified previously unresolved local structures of the complex, and the location of lipid-binding sites in the transmembrane domain. Collectively, our structural characterization of the hIP₃R-3 provides novel insight into the mechanistic function of IP₃Rs.

**Results**

**Structure of hIP₃R-3**

We expressed recombinant hIP₃R-3 using the Sf9 insect cell/baculovirus expression system and purified detergent-solubilized protein in the absence of any known ligands (Fig. S1). After initial analysis of the protein sample using negative-stain EM, we solved its structure using cryo-EM to an overall resolution of 3.8 Å (Fig. 1 and Figs. S2–S4). We used high resolution crystal structures for the ligand-binding core (PDB IDs 3JRR and 3UJ4) to interpret the map and modeled the rest of the structure manually (28, 29). The overall structure of the hIP₃R-3 expressed in Sf9 cells is consistent with the structures of hIP₃R-3 expressed in HEK GnTI(−) cells, and very similar to the structure of rat IP₃R-1 purified from native tissues (18, 26, 27).
Subunits that form the tetrameric ion channel can be divided into 3 regions: the large, N-terminal cytoplasmic domain (CD), the channel-forming transmembrane domain (TMD), and the C-terminal cytoplasmic domain (CTD) (Fig. 1C). The CD of each subunit resembles a tripod with a hinge-like central linker domain (CLD) (residues 790–1100 and 1587–1697) connected to 3 Armadillo solenoid domains (ARM1–3) (Fig. 1C). The CLD is located at the outer perimeter of the tetrameric receptor. The N-terminal domain (ARM1) extends toward the central 4-fold symmetry axis and connects to 2 contiguous β-trefoil domains (β-TF1 and β-TF2), forming the ligand-binding domain (LBD). The JD is formed by assembly of two fragments separated by symmetry expansion around the C4 symmetry axis that of the neighboring subunit forming a ring around the 4-fold symmetry axis. The second ARM domain (ARM2) bulges from the CLD and is oriented parallel to the membrane surface. It interacts with ARM1 of the neighboring subunit and forms the outer periphery of the receptor together with the CLD. The third ARM domain (ARM3) connects the cytoplasmic domains to a juxtamembrane domain (JD) positioned at the cytoplasmic face of the TMD. The JD is formed by assembly of two fragments separated by symmetry expansion around the C4 symmetry axis. A U-motif composed of a β-hairpin and a helix-turn-helix motif located at the C-terminal end of the ARM3 domain encapsulates a latch-like domain extending from the C-terminal end of the TMD. The JD is further stabilized by a H2C2 zinc finger domain formed by the residues Cys-2538, Cys-2541, His-2558, His-2563, and a zinc ion (Fig. 1C).

**IP$_3$-binding site is occupied by a loop extending from ARM2**

We observed substantive density at the IP$_3$-binding site, despite our initial intention to obtain the structure in a ligand-free conformation (Fig. 1, A–D). The density for the residues forming the binding pocket was well-resolved indicating that the additional density is from a potential ligand that occupies the IP$_3$-binding site; hereafter we will refer to this density as “ligand-like” density (Fig. 1D). To improve the quality of the density at the IP$_3$-binding site, we first treated each individual subunit as a single particle and artificially expanded the dataset by symmetry expansion around the C4 symmetry axis that increased the number of particles 4-fold from 82,511 to 330,044 (Fig. S3) (30). Then, we performed partial signal subtraction from the experimental images to reduce the signal to a region that would only cover the first ~1,800 residues (IP$_3$R-3 NTD) including the LBD as well as ARM1, ARM2, CLD, and part of ARM3 domains of a single protomer (Fig. S3) (30). The subtracted particles were then classified into six 3D classes using a mask that covers the IP$_3$R-3 NTD without performing angular or translational alignment (Fig. S3). Although all six maps from this classification scheme contained the ligand-like density at the IP$_3$-binding site, two of the classes revealed continuous density connecting the “ligand-like” to the first and second helices of ARM2 ($\alpha_{\text{ARM2–1}}$ and $\alpha_{\text{ARM2–2}}$) (Fig. 2A and Fig. S5, A and B). We observed similar results from the 3D classifications performed using different strategies as discussed under “Experimental procedures.” Further investigation of the surrounding area in the model led us to surmise this extended, connecting ligand-like density could, in fact, be an unmodeled loop of the ARM2 domain. In our model, based on the 3D reconstruction of the intact receptor, residues connecting $\alpha_{\text{ARM2–1}}$ to $\alpha_{\text{ARM2–2}}$ are not modeled due to lack of interpretable density potentially resulting from the intrinsic flexibility of this loop (Fig. 2, B and C). This loop was also left unmodeled, presumably due to flexibility, from previously published IP$_3$R-3 structures (27). Within this region there are two patches enriched in acidic residues (Fig. 2E). Therefore, it is plausible that this loop forms a self-binding peptide (SBP) extending toward the IP$_3$-binding site and bringing one or both of these patches in close proximity to basic residues in the pocket of the IP$_3$-binding site (Fig. 2B–D). SBPs are peptide segments that specifically recognize and interact with their cognate targets, while being incorporated to the target in the primary sequence via a flexible polypeptide linker (31).

Although the presence of a density representing a loop extending from the ARM2 domain is unambiguous, modeling of specific amino acids forming the loop was not possible due to weak features observed resulting from the flexibility of the loop and ARM2. Each 3D class had a different ARM2 arrangement relative to the rest of the CD through a rigid body rotation on a pivotal point where ARM2 is connected to the CLD (Fig. S5C). In addition, density at the IP$_3$-binding site is not uniform among different 3D classes implying a dynamic interaction between the SBP and the IP$_3$-binding site (Fig. S5, A and B).

**IP$_3$R-3 SBP competes against IP$_3$ binding**

We reasoned the SBP loop would compete against IP$_3$ binding if it is interacting with the IP$_3$-binding site. To test this hypothesis, we took two approaches. In the first approach, we expressed and purified the hIP$_3$R LBD (residues 4–602) and a larger N-terminal domain (NTD; residues 4–1799), containing ARM2, as GFP fusion proteins and performed microscale thermophoresis (MST) experiments to measure the binding affinity of IP$_3$ (Fig. 3, A and B). IP$_3$ affinity for the NTD was over 7-fold lower than for the LBD alone ($K_d = 1.31 \pm 0.46$ and $0.18 \pm 0.028 \mu M$, respectively) (Fig. 3B). Deletion of the putative SBP (residues 1133–1155) from the NTD construct increased the affinity for IP$_3$ ($K_d = 0.25 \pm 0.09 \mu M$), not significantly different from that of the LBD alone (Fig. 3B). Furthermore, mutagenesis of the four glutamate residues at both acidic patches to alanine (E1136A, E1137A, E1153A, and E1154A) in the putative SBP of the full-length NTD protein caused a similar increase in the IP$_3$ affinity ($K_d = 0.44 \pm 0.20 \mu M$). Taken together, these observations support the hypothesis that residues 1133–1155 of hIP$_3$R form a SBP that competes for binding of the major agonist, soluble IP$_3$.

In the second approach to test this hypothesis, we prepared a construct where the SBP was fused via a flexible linker to the C-terminal end of the LBD so it would be in close spatial proximity to the IP$_3$-binding site (Fig. 3A). We hypothesized this would behave like a gain-of-function construct, where the LBD-SBP fusion would have lower affinity for IP$_3$, similar to the NTD construct used above. We performed ITC experiments to test if the presence of the SBP affects the protein’s affinity for IP$_3$. Similar to the MST experiments, the LBD’s affinity for IP$_3$ (Fig. 3A) decreased nearly 3-fold in the presence of the SBP ($K_d = 0.19 \pm 0.02$ to $0.52 \pm 0.02 \mu M$) (Fig. 3C). This effect was again abolished when the acidic residues in the SBP were mutated to alanine.

Cryo-EM structure of type 3 IP$_3$ receptor

J. Biol. Chem. (2020) 295(6) 1743–1753

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nines (Fig. 3, A–C). These results further support the hypotheses that the SBP competes against IP₃ binding and that the acidic residues are important for this effect, as suggested by the models of our IP₃R-3 structure.

**IP₃R-3 SBP binding**

When compared with the apo-LBD (PDB ID 6DQJ) (27), with no visible density at the IP₃-binding site, the LBD of the SBP-bound hIP₃R-3 adopts a very similar overall conformation with a few local differences at the loops forming the IP₃-binding site at the β₁-H9252-β₂-TF2 domain (Fig. 4A). The most apparent difference involves the loop formed by Leu-269 and Arg-270, which is positioned closer to the SBP density, and the side chain of Leu-269, which points in the opposite direction (Fig. 4A). This slight movement does not seem to affect the overall arrangement of the IP₃R-3 CD, as the whole CD can be superimposed with an r.m.s.d value of 1.1 Å over 1,728 aligned residues. However, there is a subtle but noticeable counter-clockwise rotation (~3°) of the entire tetrameric CD relative to the TMD in the SBP-bound hIP₃R-3 compared with the apo-hIP₃R-3, when the TMDs of both structures are aligned (Fig. 4C).

Previous structures of hIP₃R-3 in complex with IP₃ revealed two different LBD conformations; apo-like class 1 and class 2 with substantial conformational changes (27). The LBD in SBP-bound hIP₃R-3 along with a docking pose of IP₃, placed based on the alignment of the LBDs of IP₃- and SBP-bound IP₃R-3s. D. electrostatic surface representation of the IP₃-binding site with a modeled loop in red. E. sequence alignment of hIP₃R subtypes around the sequence covering α₁₁₂₁ and α₁₁₂₂. Cylinders represent α-helices. Dashed lines indicate the disordered region in the reconstruction of entire receptor with C₄ symmetry. Acidic residues within the loop are colored red.

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Figure 2. IP₃-binding site is occupied by a loop extending from ARM2. A, density maps of hIP₃R-3 NTD class 5 (left) and class 3 (right) after focused 3D classification. Arrows point to the density extending from ARM2 domain. B, ribbon representation of hIP₃R-3 NTD along with transparent surface rendering of the density map of hIP₃R-3 NTD- class 5. Dashed lines represent the putative path for the Cα atoms of the residues forming the loop between α₁₁₂₁ and α₁₁₂₂. C, close up view of the IP₃-binding site of the SBP-bound hIP₃R-3 along with a docking pose of IP₃, placed based on the alignment of the LBDs of IP₃- and SBP-bound hIP₃R-3s. D, electrostatic surface representation of the IP₃-binding site with a modeled loop in red. E. sequence alignment of hIP₃R subtypes around the sequence covering α₁₁₂₁ and α₁₁₂₂. Cylinders represent α-helices. Dashed lines indicate the disordered region in the reconstruction of entire receptor with C₄ symmetry. Acidic residues within the loop are colored red.
to overlap of residues in the ARM1 domain of the class 2 structure with our SBP density. Therefore, it is not possible for the LBD to adopt a similar conformation when the SBP occupies the IP3-binding site due to steric hindrance, suggesting that the SBP would act as an antagonist.

Transmembrane domain

The TMD structure is consistent with previous IP3R structures and has the overall architecture of voltage-gated ion channels with a central pore domain surrounded by voltage sensor-like domains at the periphery (Figs. 1C and 5). Unlike voltage-gated ion channels, we observe two additional helices (S1' and S1") per subunit penetrating through the membrane from the luminal side, similar to those observed in the previous cryo-EM studies of hIP3R-3s as well as rabbit type-1 ryanodine receptors (RyR-1s) (27, 32). Thus, these auxiliary TM helices seem to be a common feature of intracellular calcium release channels. Primary sequences of these two helices are the most diverse region within the TMD among 3 subtypes of IP3Rs and could potentially be involved in subtype-specific regulation and/or localization of the IP3Rs.

Through the ion permeation path of the channel, from the cytoplasmic side, there is an upper vestibule, the narrowest constriction of the channel, and a lower vestibule followed by
an architecture similar to the selectivity filter seen in potassium channels (Fig. S7). In agreement with a closed channel conformation in the resting state, the shortest pore diameter along the channel was 1.1 Å where residues Phe-2513 and Ile-2522 are located (53, 54; Fig. S7). At the lower vestibule side of this constriction, there is a π-helix (residues 2501–2509) located at the middle of the S6 helix (Fig. S7). As suggested previously, transition from π- to α-helix within this region is potentially coupled to gating similar to TRPV6 channels where channel opening is accompanied by a local α- to π-helical transition in S6 (27, 33).

We observed two strong, nonprotein densities per subunit at the TMD. The true identity of the molecules occupying these positions cannot be determined with certainty from the current data, but they potentially derive from either nonannular lipid molecules co-purified with the receptor or well-ordered detergent molecules (Fig. 5). In either case, these sites are likely to be occupied by lipids in biological membranes, as they are embedded in the TMD core and form extensive interface with the protein residues. The density located in the cytoplasmic leaflet of the bilayer is located at the cavity formed by the S3, S4, and S4–5 helices and is in the vicinity of residues Tyr-2322, Ile-2349, Tyr-2350, and Phe-2356 (Fig. 5, A and B). The tip of the density facing the cytoplasmic side extends toward Glu-2353, suggesting that the lipid molecule contains a positively charged head group that can form a salt bridge with the side chain of Glu-2353. The second lipid density is located at the interface of three subunits in the luminal leaflet of the bilayer and could be critical for proper assembly (Fig. 5, C and D). The binding site is formed by S1 and S1’ of one subunit together with the P helix and S6 helix of two neighboring subunits (Fig. 5D).

C-terminal cytoplasmic domain

Unlike RyRs, the C-terminal ends of IP₃Rs extend through the central 4-fold axis and form a left-handed helical bundle at the core of the receptor (18). In our structure, density for the CTD is less resolved compared with the rest of the receptor, but sufficient to model a polyaniline peptide that forms a left-handed coiled-coil motif (Fig. 6, A and B). Connection of the coiled-coiled motif to the JD is only visible at very low threshold levels, and was not built into the model. The C-terminal side of the helices forming the coiled-coiled domain extend toward the β-TF ring and interact with the β-TF2 domain of the neighboring subunit (Fig. 6). The interaction is mediated through a hydrophobic patch formed by Val-287, Val-288, Leu-303, Ile-363, Leu-366, and Leu-392 on the surface of the β-TF2 domain and potentially involves residues Leu-2660, Gly-2661, Phe-2662, Val-2663, Asp-2664, and Val-2665 at the C-terminal end of the receptor (Fig. 6, C and D).

Discussion

The identified direct interaction between the SBP and the ligand-binding site may have a critical physiological role in IP₃₃-3 activity and regulation. By occupying the IP₃ binding site, the SBP reduces the sensitivity of the IP₃₃-3 to its physiological agonist IP₃. Similar mechanisms of receptor regulation were observed for other protein families as well. For example, fibroblast growth factor receptor autoinhibition is mediated by electrostatic interaction of a subregion rich in acidic residues, known as an acid box, with the heparin-binding site of the same
subunit, reducing receptor affinity for heparin and fibroblast growth factor (34). Of note, heparin is an antagonist of IP₃R Rs and interacts with the IP₃-binding site.

Among the IP₃R subtypes, IP₃R-3 has the lowest affinity to IP₃. The subtype differences in IP₃ affinity are mainly attributed to sequence variations at the loop sequence among members of the IP₃R family. However, the current data indicate that the SBP also has a role in modulating binding affinity because of sequence variability within the SBP among the IP₃R subtypes, especially in the number and positioning of the acidic residues (Fig. 2E). In addition, we speculate that the SBP is a plausible target for many proteins that are known to modulate the sensitivity to IP₃. For example, phosphorylation of the Ser/Thr residues within the SBP would increase the net negative charge on the SBP and potentially lead to tighter binding to the IP₃-binding site reducing the receptor sensitivity to IP₃. Moreover, any protein that interacts with the SBP may restrict its interaction with the IP₃-binding site, sensitizing the IP₃R to IP₃.

In addition to a potential physiological role in regulation of IP₃R activity, identification of the SBP as a competitive inhibitor of IP₃ binding opens potential avenues in development of pharmacological agents targeting these important families of proteins. Further research to identify the structural determinants of the SBP interaction with the IP₃-binding site will pave the road for development of novel inhibitors of IP₃ Rs.

One puzzling question is why the additional density from the SBP at the IP₃-binding site was not observed in a previously reported ligand-free hIP₃R-3 structure. One plausible explanation is that the larger number of particles used in our study (82,511 compared with 26,325) provided additional information that permitted the resolution of the SBP. In addition, this could arise from technical differences, such as the protein expression system (mammalian versus insect cells), purification methods, and/or sample preparation for cryo-EM analysis. Furthermore, cryo-EM studies of large macromolecular complexes provide opportunities to identify novel features through different image-processing strategies even with samples prepared in similar conditions. IP₃R Rs are regulated by numerous factors, some of which are not well-understood at the moment, and exhibit multiple conformational rearrangements. Thus, it is likely that further structural studies of IP₃R Rs will continue to uncover additional features providing further functional insights.

In conclusion, the data presented here reveal a previously unanticipated regulatory mechanism of IP₃R where a loop distantly located from the LBD in the primary sequence occupies the IP₃-binding site and competitively inhibits IP₃ binding. Regulation by the SBP is likely to confer subtype-specific biological function to IP₃-mediated calcium signaling due to divergence in the loop sequence among members of the IP₃R family. Our structural data will facilitate design of modifications on the SBP of the intact receptors to functionally test their effect in channel activity to determine the molecular mechanism of the SBP regulation and its physiological role.

Experimental procedures

Expression and purification of hIP₃R-3

The gene encoding hIP₃R-3 (accession number BC172406) was purchased from Dharmaco (36), subcloned (residues 4–2671) with C-terminal OneStrep tag into pFL vector, and incorporated into baculovirus using the Multibac expression system (37). Sf9 cells (4 × 10⁶ cells/ml) infected with the bacu-
loivirus were harvested by centrifugation (4,000 × g) 48 h after infection. Cells resuspended in a lysis buffer of 200 mM NaCl, 40 mM Tris-HCl, pH 8.0, 2 mM EDTA, pH 8.0, 10 mM β-mercaptoethanol (β-ME), 1 mM phenylmethylsulfonyl fluoride were lysed using Avastin Emulsiflex-C3. The cell lysate was centrifuged at 6,000 × g for 20 min and the membrane was pelleted by centrifugation at 40,000 rpm (Ti45 rotor) for 1 h. Membrane pellets were resuspended and homogenized in ice-cold resuspension buffer (200 mM NaCl, 40 mM Tris-HCl, pH 8.0, 2 mM EDTA, pH 8.0, 10 mM β-ME), and solubilized using 0.5% lauryl maltose neopentyl glycol (LMNG) and 0.1% glyo-diosgenin (GDN) at a membrane concentration of 100 mg/ml. After 4 h of stirring, the insoluble material was separated by centrifugation at 40,000 rpm (Ti45 rotor) for 1 h and the supernatant was passed through Strep-Xt-Superflow resin (IBA Biotagnology). The resin was washed with the wash buffer composed of 200 mM NaCl, 20 mM Tris-HCl, pH 8.0, 1 mM EDTA, 10 mM β-ME, 0.005% LMN, 0.005% GDN, and the protein was eluted using the wash buffer supplemented with 100 mM d-biotin, pH 8.2. Protein was further purified by size exclusion chromatography using Superose 6 (10/300 GL, GE Healthcare) equilibrated with 200 mM NaCl, 20 mM Tris-HCl, pH 8.0, 1 mM EDTA, pH 8.0, 2 mM TCEP, 0.005% LMN, and 0.005% GDN. Fractions corresponding to hIP3R-3 were concentrated to 2.3 mg/ml, centrifuged at 70,000 rpm using a S110-AT rotor (Thermo Scientific) for 10 min, and used immediately for cryo-EM imaging. After centrifugation, concentration of the protein decreased to 1.3 mg/ml.

**Negative stain data collection and analysis for hIP3R-3**

Four hundred mesh copper grids were coated with carbon. 4 μl of 0.05 mg/ml of hIP3R-3 was applied to each glow discharged grid (2 min on Quorum K1000X) and allowed to absorb for 30 s. The grid was blotted on filter paper, washed twice in MilliQ water, and negatively stained with 0.75% (w/v) uranyl acetate (Gatan). Data were collected at a nominal magnification of ×31,000 at a defocus range of −1.4 to −3.5 μm under low dose conditions. Specimens were exposed for 10 s at −11 e−/pix/s over 50 frames resulting in a total dose of ~70 e−/Å² using SerialEM automated data collection (38). The pixel size of the image was 1.247 Å.

**Cryo-EM image processing for hIP3R-3**

Motion correction and CTF parameter determination was done using the on-the-fly processing software Focus (40). All images were motion corrected from frames 0–44 using motioncor2 (41) with a dose weighting parameter of 1.4 e−/Å²/frame. CTF parameters for each image was calculated using Gctf (42) and used to remove micrographs with low maximum resolution or high astigmatism. Motion-corrected images were then imported into RELION-2 (39). RELION-3 was used as the new version became available (43). Autopicking was done using representative class averages obtained from 757 manual picked particles as templates. Particles were extracted at a box size of 350 × 350 pixels and binned to 64 × 64 pixels. 2D class averages were determined using 25 iterations of classification. Particles that generated class averages showing well-defined domain structure were re-extracted at 340 × 340 pixels without binning and were subject to 2D classification. Particles that generated 2D class averages showing clear secondary structure subparticle features were subject to 3D classification. The EM density map of the IP3R-1 (EMD-6369) was scaled and clipped, using e2proc3d.py (EMAN) (44), to match our pixel and box size, filtered to 60 Å, and used as an initial model for 3D classification into 6 classes with no symmetry imposed. Two classes had impaired density for one of the subunits and excluded from further analysis. Refinement and reconstruction of the particles belonging to the other four classes were performed using

**Table 1**

| Data collection and processing | Microscope | FEI Polara TF30 |
|------------------------------|-----------|----------------|
| Detector                     | Gatan K2 summit |
| Nominal magnification        | ×31,000   |
| Voltage (kV)                 | 300       |
| Electron exposure (e−/Å²)    | 70        |
| Defocus range (μm)           | −1.4 to −3.5 |
| Pixel size (Å)               | 1.247     |
| Symmetry imposed             | C4        |
| Number of micrographs        | 3,899     |
| Initial particle images      | 110,510   |
| Final particle images        | 82,511    |
| Map resolution (Å)/FSC threshold | 3.8/0.143 |

| Refinement                  | Resolution (Å) | 3.8 |
|----------------------------|----------------|-----|
|                             | B-factor used for map sharpening (Å²) | −90 |
| Model composition           | Non-hydrogen atoms | 51,564 |
|                             | Protein residues | 8,216 |
|                             | Zinc | 4 |
|                             | Mean B factors (Å²) | 155.0 |
|                             | R.m.s. deviations | 115.7 |
|                             | Bond lengths (Å) | 0.002 |
|                             | Bond angles (°) | 0.532 |
| Validation                  | Molprobity score | 0.94 |
|                             | Clashscore | 0.26 |
|                             | Poor rotamers (%) | 0 |
|                             | Ramachandran plot | 95.13 |
|                             | Favor (β) (%) | 4.87 |
|                             | Allowed (β) (%) | 0.0 |
|                             | Disallowed (β) (%) | 0.0 |

**Cryo-EM sample preparation and data collection for hIP3R-3**

2.0 μl of 1.3 mg/ml of hIP3R-3 was applied to a 200-mesh C-flat holey carbon 2/1 grid (Protochips) that was glow discharged for 2 min at 25 mA. The grid was blotted for 3 s at force 1 before being plunged into liquid ethane using an FEI MarkIV Vitrobot at 8 °C and 100% humidity. Micrographs were collected using an FEI Polara F30 microscope operated at 300 keV in counting mode on a K2 Summit direct electron detector (Gatan). Data were collected at a nominal magnification of ×31,000 at a defocus range of −1.4 to −3.5 μm under low dose
cisTEM by imposing C4 symmetry (45) (Table 1). The resulting map was sharpened by applying a B-factor of $-90 \text{ Å}^2$ using Rosenthal and Henderson’s method (46). The final average resolution at the “gold standard” 0.143 cutoff was 3.8 Å. Half-maps were generated using the 3D-generated module in cisTEM. Local resolution was also calculated in ResMap (47) (Fig. S4). Chimera (48), COOT (49) and The PyMOL Molecular Graphics System (Version 2.0, Schrödinger, LLC) were used for visualization and figure preparation.

**Symmetry expansion, partial signal subtraction, and focused 3D classification**

To resolve the unaccounted density better, we first treated each subunit as a single particle and expanded the dataset by using “relion_particle_symmetry_expand” command based on the C4 symmetry and the refined orientation parameters calculated during 3D refinement using RELION-3 for the particles. This process increased the number of particles to 330,044. We created a mask around the hIP₃R-3 tetramer using MaskCreate in RELION-3 and removed the part that corresponds to the IP₃R-3 NTD of one of the subunits using the “volume erase” function of Chimera. The new mask was then used to subtract the signal from the expanded particles using RELION-3 resulting in 330,044 particle images containing a signal for only the IP₃R-3 NTD of one of the subunits. These particles were then subjected to 3D classification using a mask covering the IP₃R-3 NTD, and orientation parameters for the particles from symmetry expansion step. At this stage, we tried several different classification strategies: 3D classification into 4, 6, or 8 classes, 3D classification using a mask excluding the ARM2, and re-classification of the 3D classes into subclasses. The results mostly agreed with each other, and we used the maps from the classification into 6 classes for further analysis (Fig. S5A). Refinement of the particles belonging to Class 5 was performed using cisTEM. The final average resolution at the gold standard 0.143 cutoff was 4.5 Å.

**Model building**

High resolution crystal structures of the β-TF1 domain of mouse IP₃R-3 (PDB ID 3JRR) (28) and β-TF2 and part of the ARM1 domain of the rat IP₃R-1 (PDB ID 3UJ4) (29) were docked into the cryo-EM map followed by rigid-body fitting of the individual β-TF1, β-TF2, and ARM1 domains into the cryo-EM map using COOT (49). The resulting model was manually modified to have the correct hIP₃R-3 residue assignment and fit to the cryo-EM map. The rest of the model was built manually using COOT and refined against the cryo-EM map using Phenix real space refinement (50). Residues without clear density for their side chains were built without their side chains (i.e. as alanines), while maintaining their correct labeling for the amino acid type.

**Expression and purification of soluble hIP₃R-3 constructs**

The gene encoding the hIP₃R-3 LBD (Met-4 to Asn-602) was subcloned into pAceBac1 vector with a N-terminal OneStrep tag followed by a tobacco etch virus protease cleavage site. This construct was modified to prepare the hIP₃R-3 LBD + SBP fusion construct that encodes residues 4 to 603 followed by the Ala-Gly-Pro-Gly-Gly linker and residues 1128 to 1168. For MST experiments, the gene encoding the hIP₃R-3 LBD (residues 4 to 602) and the hIP₃R-3 NTD (residues 4–1799) were subcloned into pAceBac1 vector with an N-terminal OneStrep tag followed by the gene encoding eGFP and tobacco etch virus protease cleavage site. The truncation construct hIP₃R-3 NTD-ΔSBP was prepared by removing residues between Gly-1132 and Gly-1156 with Asn-Gln-Ala. All constructs were incorporated into baculovirus using the Multibac expression system (37). The constructs were expressed using the SF9/Baculovirus system (DH10multibac). Sf9 cells (~4.0 x 10⁶ cells/ml) were harvested by centrifugation (1952 x g, 20 min) 48 h post-infection. The cell pellet was resuspended in lysis buffer composed of 200 mM NaCl, 20 mM Tris-HCl, pH 8.0, 10% glycerol (v/v), 10 mM β-ΜΕ, and 1 mM phenylmethylsulfonyl fluoride. Cells were lysed using Avestin EmulsiFlex-C3 system (greater than 10,000 p.s.I) and centrifuged at 40,000 rpm (Ti45 rotor) for 45 min. Supernatant was recovered and incubated with Strep-XT-Superflow resin (IBA Biotagnology) for 2 h at 4 °C. The resin was then washed with the wash buffer (200 mM NaCl, 20 mM Tris-HCl, pH 8.0, 10% glycerol, and 10 mM β-ΜΕ) and the protein was eluted with the elution buffer (200 mM NaCl, 50 mM Tris, pH 8.2, 100 mM d-biotin, and 10 mM β-ΜΕ). The proteins used for ITC experiments were further purified by SEC using Superdex 200 column (GE Healthcare) equilibrated with the SEC buffer composed of 200 mM NaCl, 20 mM Tris-HCl, pH 8.0, 10% glycerol (v/v), and 0.5 mM TCEP. SEC for the proteins used in MST experiments were performed using a buffer composed of 200 mM NaCl, 20 mM Tris-HCl, pH 8.0, and 0.5 mM TCEP.

**Isothermal titration calorimetry (ITC)**

All proteins used in ITC experiments were dialyzed against the same buffer solution composed of 200 mM NaCl, 20 mM Tris-HCl, pH 8.0, 10% glycerol (v/v), and 0.5 mM TCEP to avoid the possible changes in the salt concentration and pH. IP₃ solution was prepared by dissolving the powder in the dialysis solution. ITC experiments were conducted on an Auto ITC 200 instrument at 20 °C by successive injections of 3 μL of 0.8 mM IP₃ to 0.08 mM of the protein solutions at 200-s intervals following an initial injection of 0.2 μL of 0.8 mM IP₃. Data analysis was performed using the software Origin, version 7.0, with the MicroCal ITC analysis module.

**Microscale thermophoresis (MST)**

MST experiments were conducted on a Monolith NT.115 series (Nanotemper Technologies). Proteins were diluted to 200 nM in 1× MST buffer (50 mM Tris-HCl, 150 mM NaCl, 10 mM MgCl₂, 0.05% Tween 20). IP₃ stock solution (2 mM in water) was diluted to 15 μM using 1× MST buffer. A 2-fold serial dilution of IP₃ in 18 NT.115 standard capillaries was prepared, with 15 μM IP₃ as the highest concentration and 100 nM protein per capillary. Excitation power was set to 20% and MST laser power was set to medium. Data analysis was performed using the software MO analysis (Nanotemper Technologies).
Cryo-EM maps and atomic coordinates have been deposited in the EMDB and PDB under the accession codes EMD-20849 (tetramer with C4 symmetry, PDB ID 6UQK) and EMD-20850 (IP3R-3 NTD, focused refinement with no imposed symmetry). All other source data are available from the corresponding authors upon request.

Acknowledgments—EM data were collected by C. M. A. using FEI Polara and FEI TF20 at the Center for Structural Biology’s Molecular Cryo-EM facility at Vanderbilt University. We thank Drs. Scott Coller and Elad Blainshtein for their support at the facility. We thank Dr. Tim Grant for suggestions for data processing. We thank Drs. James Crowe and Lauren P. Jackson for kindly sharing the ITC instrument and Drs. Roger J. Colbran and Hassane Mchaourab for critically reviewing the manuscript. This work was supported in part using the CPU and GPU resources of the Advanced Computing Center for Research and Education (ACCRE) at Vanderbilt University. We used the DORS storage system supported by National Institutes of Health Grant S10RR031654 (to Jarrod Smith). We acknowledge the use of SBGrid supported software (51).

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