Structural Studies of Inositol 1,4,5-Trisphosphate Receptor COUPLING LIGAND BINDING TO CHANNEL GATING*§

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The three isoforms of the inositol 1,4,5-trisphosphate receptor (IP₃R) exhibit distinct IP₃ sensitivities and cooperativities in calcium (Ca²⁺) channel function. The determinants underlying this isoform-specific channel gating mechanism have been localized to the N-terminal suppressor region of IP₃R. We determined the 1.9 Å crystal structure of the suppressor domain from type 3 IP₃R (IP₃RSUP, amino acids 1–224) and revealed structural features contributing to isoform-specific functionality of IP₃R by comparing it with our previously determined structure of the type 1 suppressor domain (IP₃RSUP). The molecular surface known to associate with the ligand binding domain (amino acids 224–604) showed marked differences between IP₃RSUP and IP₃RSUP. Our NMR and biochemical studies showed that three spatially clustered residues (Glu-20, Tyr-167, and Ser-217 in IP₃RSUP) interact directly with their respective C-terminal fragments. Together with the accompanying paper (Yamazaki, H., Chan, J., Ikura, M., Michikawa, T., and Mikoshiba, K. (2010) J. Biol. Chem. 285, 36081–36091), we demonstrate that the single aromatic residue in this region (Tyr-167 in IP₃R and Trp-168 in IP₃R) plays a critical role in the coupling between ligand binding and channel gating.

The inositol 1,4,5-trisphosphate (IP₃) receptor (IP₃Rs) are tetrameric intracellular Ca²⁺ release channels on the endoplasmic membrane that are activated by the ligand IP₃. In mammals, the IP₃R family consists of three genes, which encode for isoforms type 1 (IP₃R1), type 2 (IP₃R2), and type 3 (IP₃R3) (1). The full-length isoforms are 60–70% identical in primary sequence, with the N-terminal ligand-binding and C-terminal channel domains sharing the highest similarity (2). IP₃Rs are ubiquitously expressed, and most cells express more than one IP₃ isoform (3–5).

IP₃R1 is widely expressed in the central nervous system, in particular the cerebellar Purkinje cells (6). The importance of IP₃R1 in embryonic development is demonstrated by the fact that the majority of IP₃R1 knock-out mice die in utero (7). IP₃R1-null animals that survive until birth have 40% brain sizes and 50% body mass compared with wild type littermates. In contrast, neither IP₃R2 nor IP₃R3 knock-out mice display any abnormal phenotype (8). However, double IP₃R2⁻/⁻·IP₃R3⁻/⁻ knock-out animals show defects in exocrine functions. For example, saliva production is greatly reduced in double knock-out mice as a consequence of defective IP₃-mediated Ca²⁺ release (8).

Although functional differences among IP₃ isoforms in vivo remain unclear, in vitro studies of IP₃R have revealed that one of the most significant differences between the isoforms is in their IP₃ binding affinities. IP₃R2 exhibits the highest IP₃ binding affinity, followed by IP₃R1 and then IP₃R3 (Kₐ = 5.9, 28.6, and 294 nM, respectively) (9, 10). Using chimeric proteins, we have shown that different IP₃ binding affinities of IP₃Rs isoforms are not solely dependent on the ligand binding core (IP₃RCORE; amino acids 224–578) but also involve the suppressor domain (IP₃RSSUP; amino acids 1–223) (9). The molecular mechanism underlying these isoform-specific affinities is not well understood, but recent studies suggest that distinct IP₃ sensitivities of the three isoforms are a product of the suppressor and ligand binding domain interaction (9).

Earlier biochemical studies (11) show that there is a direct coupling between the N and C termini in IP₃R1, believed to occur in an intermolecular manner (i.e. aa 1–340 of the N-terminal fragment of one subunit interact with sites located between aa 2418 and 2749 of the C-terminal fragment of an adjacent subunit). Introduction of mutations within a cytoplasmic loop that connects transmembrane helices 4 and 5 in the C-terminal fragment (aa 2418–2437; M4–M5 linker) prevents the co-immunoprecipitation of N and C termini, suggesting that this region is involved in the N-terminal-to-C-terminal coupling ligand binding and channel gating.
intersubunit interaction (N-C interaction). The M4-M5 linker has been implicated in IP₃R gating function because mutations to this region inhibit channel activity (12). Moreover, we have previously demonstrated that deletion of the suppressor domain abolishes channel activity while simultaneously increasing IP₃ binding affinity (13), thus suggesting the suppressor domain is essential to IP₃R gating function. Despite this evidence of the functional significance of the suppressor domain in the channel regulation, little is known about the structural determinants responsible for the N-C interaction of IP₃R.

In our accompanying paper (31), we report the identification of critical amino acid residues in the suppressor domain for IP₃R channel gating function. Specifically, a single mutation of Tyr-167 to alanine in IP₃R1 or the corresponding mutation in IP₃R3(W168A) was sufficient to abolish channel activity, suggesting that this residue is crucial to the gating mechanism of IP₃R. In this paper, we report structural evidence for the significance of this aromatic residue in the physical contact between the N-terminal suppressor domain and the C-terminal channel domain. We determined the high resolution crystal structure of the suppressor domain from type 3 IP₃R and characterized the interaction between the suppressor domain and synthetic peptides of the M4-M5 linker by NMR and biochemical approaches. Our structural data provide further evidence for the involvement of not only the aromatic residue (i.e. Trp-168 in IP₃R3) but also some neighboring residues within the suppressor domain in this functionally essential interaction. This region responsible for the interaction with the C terminus of the receptor is adjacent to but distinct from the sites of IP₃ binding modulation and IP₃ binding core interaction.

**EXPERIMENTAL PROCEDURES**

**Expression and Protein Purification—**Mouse IP₃R3SUP (aa 1–224) was expressed as an N-terminal tagged GST fusion protein in BL21-CodonPlus(DE3)-RIL Escherichia coli strain (Stratagene). Freshly transformed cells were first grown in 4 liters of LB medium at 37 °C until A₆₀₀ reached ~0.6. Cells were then harvested, washed by resuspending in M9 medium, harvested again, and then transferred into 1 liter of minimal M9 medium supplemented with 1 g/liter [¹⁵N]NH₄Cl and/or 2 g/liter [¹³C₆]glucose (Cambridge Isotope Laboratories). Additionally, for triple resonance experiments, the protein was deuterated by growing cells in 99% [²H]D₂O-based M9 medium. Cellally, for triple resonance experiments, the protein was deuterated by growing cells in 99% [²H]D₂O-based M9 medium. Cell lysates were prepared with APBS (17).

**Data Processing**—Crystals of IP₃R3SUP, which was concentrated to 10 mg/ml, were grown by hanging drop vapor diffusion at 22 °C by combining 2 μl of protein with an equal volume of reservoir solution (100 mM HEPES, pH 7.6, 150 mM NaCl, 14% PEG 4000, 2 mM TCEP, 1% dioxane, 50 mM EDTA). Crystal clusters appeared within 2 weeks, and following 2–3 rounds of microseeding, single plate-like crystals with dimensions 0.2 × 0.1 × 0.02 mm were obtained. They were flash-cooled in Paratone-N. Two rounds of annealing improved diffraction, and native data were collected at 100 K on a 19-BM beam line at the Advanced Photon Source Synchrotron facility (Argonne, IL). Data processing was carried out with HKL2000 (14). Crystals belonged to the space group C2 with cell dimensions a = 79.4 Å, b = 59.9 Å, c = 111.4 Å, β = 97.88° with two molecules in the asymmetric unit. Phase information was obtained by molecular replacement using the mouse IP₃R1SUP crystal structure (Protein Data Bank code 1XZZ) as the search model. Refinement was carried out in CNS (15), and manual model building was performed with Coot (16). Electrostatic potentials were calculated using APBS (17).

**Nuclear Magnetic Resonance—**NMR spectra were recorded at 25 °C using a 600-MHz Varian INOVA spectrometer or an 800-MHz Bruker spectrometer. ¹H-¹⁵N heteronuclear single quantum coherence (HSQC) spectra were measured on a uniformly ¹⁵N-labeled sample. HNCA CB (18) and CBCA(CO)NH experiments (20, 21) were performed with Coot (16). Electrostatic potentials were calculated using APBS (17).

**NMR Peptide Titration Studies—**Titration experiments of unlabeled peptide into a sample containing uniformly ¹⁵N-labeled protein were performed by acquisition of ¹H-¹³C HMQC spectra at various peptide/protein ratios. Initially, a ¹H-¹⁵N HMQC spectrum of protein at ~0.16 mM concentration was recorded in the absence of peptide. The 350-μl sample was then removed from the NMR tube and mixed with 14 μl of 2 mM peptide solution to achieve a peptide/protein molar ratio of 0.5:1. The mixed sample was returned to the NMR tube, and a new ¹H-¹³C HMQC spectrum was recorded. More peptide solution was incrementally added to acquire ¹H-¹³C HMQC spectra.
Crystal Structure of IP₃R3 Suppressor Domain

at peptide/protein molar ratios of 1:1, 2:1, and 3:1. The reported results are for the spectra measured in the absence of peptide compared with the presence of a 3-fold molar excess of peptide. Data were analyzed by first normalizing raw peak volumes with the peak volume of a reference peak (peak unaffected by peptide addition) in the same spectrum. The peak volume values were then compared between the no-peptide and 3-fold molar excess peptide-added conditions.

**GST Pull-down Assays**—GST-T3S₄S₅ and GST-T1S₄S₅ (pGEX-4T vectors (GE Healthcare)) were expressed as described above. Suppressor domain proteins were subcloned into pET28-a expression vectors (Novagen) and expressed as N-terminal His fusion proteins in BL21-CodonPlus(DE3)-RIL E. coli strain (Stratagene) in 1 liter of LB medium grown at 15 °C overnight after induction with 0.5 mM IPTG (final concentration). Harvested cells were lysed in buffer (20% (v/v) glycerol, 20 mM Tris-HCl, pH 7.1, 500 mM NaCl, 20 mM imidazole, 0.2% Nonidet P-40, 10 μg/ml DNase I, EDTA-free protease inhibitor mixture (Roche Applied Science), 0.5 mM TCEP), and the clarified supernatant was subjected to affinity chromatography by passing it through a pre-equilibrated nickel-NTA resin (Qiagen). After washing with 3 column volumes of wash buffer (10% (v/v) glycerol, 20 mM Tris-HCl, pH 7.1, 300 mM NaCl, 20 mM imidazole, 0.5 mM TCEP), the His fusion proteins were eluted in buffer containing 3% (v/v) glycerol, 20 mM Tris-HCl, pH 7.1, 300 mM NaCl, 300 mM imidazole, 0.5 mM TCEP and dialyzed against the incubation buffer, which is identical in composition to the NMR titration buffer (3% (v/v) glycerol, 20 mM Tris-HCl, pH 7.1, 300 mM NaCl, 2 mM TCEP). Prior to incubation with suppressor domain proteins, the GST columns with immobilized GST-T3S₄S₅ or GST-T1S₄S₅ were pre-equilibrated with incubation buffer. The bed volume of the GST column was 250 μl. 500 μg of purified IP₃R₁SUP or IP₃R₃SUP were incubated with 200 μg of GST fusion proteins in the column for 2 h at 4 °C. Following five 1-ml washes with high salt buffer (3% (v/v) glycerol, 15 mM Tris-HCl, pH 7.1, 500 mM NaCl, 2 mM TCEP), proteins were eluted using 250 μl of elution buffer (50 mM Tris-HCl, pH 7.1, 300 mM NaCl, 10 mM DTT, and 10 mM reduced glutathione). The 30 μl of elution samples were separated on a 15% SDS-polyacrylamide gel and then analyzed by Western blotting using a 1:1000 dilution of a primary penta-His anti-peptide antibody (Qiagen). Bands were quantitated by a densitometer, and controls were normalized to 1.

**RESULTS**

Crystal Structure of the Type 3 IP₃R Suppressor Domain—The three-dimensional structure of IP₃R₃SUP consists of a hammer-like overall shape with a globular "head" domain comprising a β-trefoil fold and a helix-turn-helix motif, which makes up the “arm” domain (Fig. 1 and Table 1). The overall structure of IP₃R₃SUP superimposes very well with that of IP₃R₁SUP reported previously (root mean square deviation ~1.3 Å) (Fig. 1B) (25). The 12-β-strand β-trefoil fold of IP₃R₃SUP is characterized by three trefoils, which together form a barrel and cap structure. Each trefoil is made up of two two-stranded hairpins, with one hairpin contributing to the barrel structure and the other forming the cap structure. Poorly defined electron density prevented us from modeling one stretch of the protein that is a part of the arm domain (Gln-76 to Glu-82).

**Structural Comparisons with Type 1 IP₃R Suppressor Domain and Implications for Isoform-specific IP₃ Binding Affinity**—To understand the molecular basis of isoform-specific IP₃ binding affinity, we performed a careful inspection of the structures of IP₃R₃SUP and IP₃R₁SUP. Comparison of IP₃R₃SUP and IP₃R₁SUP...
Crystal Structure of IP3R3 Suppressor Domain

FIGURE 2. Sequence alignment of members of the IP3R family and electrostatic surface potential diagrams of crystal structures of type 1 and 3 IP3R suppressor domain. A, sequence alignment with conserved residues highlighted in green (accession numbers: mIP3R3, NP_542120.2; hIP3R3, NP_002215.2; mIP3R1, NP_034715.3; mIP3R2, NP_064307.2; dIP3R, NP_730942.1). Secondary structural elements of mIP3R3SUP identified from the crystal structure are displayed: β-strands as arrows and α-helices as rectangles (a dotted line represents the unresolved portion). Structural elements from the head subdomain are colored blue, and those from the arm subdomain are colored purple. Asterisks denote residues involved in M4-M5 linker interaction. Surface electrostatics of IP3R3SUP (B and D) and of IP3R1SUP (C and E) are shown. The acidic patch of IP3R3SUP and the corresponding area on IP3R1SUP are highlighted (yellow dotted oval). Views in D and E are 180° rotated along the y axis with respect to those in B and C, respectively.

In Vitro Interaction between the Suppressor Domain and the M4-M5 Linker—Previous reports have shown a direct interaction between the N-terminal 42-kDa and C-terminal 94-kDa fragments of IP3R (11). More recently, a 20-αmino acid stretch between transmembrane helices 4 and 5, the M4-M5 linker, has been implicated in this N-C termini interaction (12). Notably, mutations to the M4-M5 linker that perturb its interaction with the N-terminal fragment result in a non-functional channel (12). Based on these pioneering studies, we sought specific amino acids within the suppressor domain, which can interact with the M4-M5 linker. In our accompanying study (31), we observed that mutation to a single residue (Tyr-167 or Trp-168 in type 1 or type 3 IP3R, respectively) was sufficient to abolish channel activity.
To examine whether the suppressor domain could interact with the M4-M5 linker in vitro, we performed GST pull-down assays using immobilized GST-M4-M5 linker and N-terminal His-tagged type 1 or type 3 IP3RSUP wild type proteins as well as their respective mutant proteins, Y167A or W168A. The results show that the M4-M5 linker interacts with wild type IP3R1SUP or IP3R3SUP in vitro but not to the GST control (Fig. 3). The Y167A or the W168A mutant proteins, on the other hand, displayed considerably weaker binding under the same conditions (Fig. 3, lane 4). These data confirm that Tyr-167 from type 1 or Trp-168 from type 3 are critical to the N-C interaction and that mutation of this single residue is sufficient to disrupt this interaction.

Mapping the Residues within the Suppressor Domain Responsible for Interaction with the M4-M5 Linker—We performed NMR titrations using chemically synthesized peptides corresponding to the M4-M5 linker into 15N-labeled IP3R3SUP and IP3R1SUP proteins and monitored changes to their respective 1H-15N HSQC spectra. We recorded the 1H-15N HSQC spectra of both IP3R1SUP (aa 1–223) and IP3R3SUP (aa 1–224). The 1H-15N correlation peaks observed for IP3R1SUP were considerably broader in comparison with IP3R3SUP, which behaved well during long three-dimensional triple resonance experiments; consequently, we were able to sequentially assign ~70% of the backbone of IP3R3SUP using these data (supplemental Fig. 4).

Due to the low solubility of the chemically synthesized M4-M5 linker peptide (aa 2418–2437 in IP3R1; aa 2346–2365 in IP3R3) in aqueous solution, we utilized the half-peptides, which displayed better solubility. To identify residues involved in the N-C interaction of IP3R, we titrated either the N-terminal half (T1S4S5N, aa 2418–2427 for IP3R1; T3S4S5N, aa 2346–2355 for IP3R3) or C-terminal half (T1S4S5C, aa 2428–2437 for IP3R1; T3S4S5C, aa 2356–2365 for IP3R3) of the M4-M5 linker to IP3R1SUP or IP3R3SUP proteins (Fig. 4 and supplemental Fig. 4). We recorded 1H-15N HSQC spectra of IP3R1SUP or IP3R3SUP with successive additions of each peptide at peptide/protein ratios of 0.5:1, 1:1, 2:1, and 3:1. For IP3R3SUP, at low peptide/protein molar ratios (i.e. less than 1:1), no significant change was observed. However, at molar ratios of 1:1 or above, peak broadening was notable for a number of specific residues (data not shown). By comparing peak volumes before and after a 3-fold molar excess of peptide was added, we noticed that at least three peaks (Glu-19, Trp-168, and Ser-218) showed a reduction of greater than 20% in the presence of either
T3S4S5N or T3S4S5C peptide. Specifically, the resonance peak corresponding to Trp-168 was broadened in the presence of either T3S4S5N or T3S4S5C peptides, whereas the Trp-19 peak volume was only reduced in the presence of T3S4S5N, and the Ser-218 peak volume was only reduced with the addition of T3S4S5C peptide. These results implicate that both N- and C-terminal halves of the M4-M5 linker are involved in the interaction with the suppressor domain. We generated E19A and S218A mutant proteins (as well as E20A and S217A mutants in type 1 IP3R) and confirmed by GST pull-down assays that their absence disrupted the M4-M5 linker interaction with the suppressor domain (Fig. 3).

**DISCUSSION**

IP₃Rs are a tetrameric receptor with a combined molecular mass of ~1.2 MDa, whose cytoplasmic region harbors various regulatory functions of the Ca²⁺ channel’s gating. Among these regulatory processes, such as those involving protein phosphorylation, ATP binding, interactions, with cellular proteins, IP₃ binding by the N-terminal region plays a crucial and universal role in the channel gating. This ligand-dependent activation is conserved among the three isoforms, but the ligand sensitivity and the channel response are different from one isoform to another. In parallel to these channel properties, these isoforms are expressed to varying degrees in different mammalian cells. IP₃R1 is the major isoform expressed in the nervous system, whereas IP₃R2 and IP₃R3 are predominant in most other tissues. At the amino acid sequence level, the isoforms are modestly conserved, based on *Mus musculus* sequence comparisons: 68% identity between IP₃R1 and IP₃R2, 64% identity between IP₃R1 and IP₃R3, and 61% identity between IP₃R2 and IP₃R3. In an effort to gain insight into the general mechanism of ligand-induced gating of IP₃Rs and the structural differences and similarities between the isoforms, we have investigated the structure of the suppressor domain of type 3 IP₃R and the interaction of this domain with the putative binding site on the channel domain. Our structural studies presented in this paper augment the functional and biochemical data described in the accompanying paper (31), highlighting the significance of the interaction of the suppressor domain with the channel domain in the gating function of both type 1 and type 3 IP₃Rs.

Despite various EM structures of full-length IP₃R, the exact positions of the suppressor domain and ligand binding domain remain ill defined. Hence, the exact orientation and distance between the suppressor domain and the M4-M5 linker are currently unknown. Our NMR titration studies identified three residues (Glu-19, Trp-168, and Ser-218) within IP₃R₃SUP that are either directly in contact with or in close proximity to the interaction interface with the M4-M5 linker domain. In our accompanying paper (31), the corresponding residues (Glu-20, Tyr-167, and Ser-217) in IP₃R₁ have been mutated to alanine. Although single substitution at Glu-20 and Ser-217 did not show significant change in the Ca²⁺ release activity of the channel, the E20A/Y167A/S217A triple substitution exhibited a significant change in the gating function. Moreover, this mutant displayed significantly lower sensitivity against trypsin compared with...
that of Y167A. Using our Ca\(^{2+}\) flux assays (31), we have found that Lys-168 and Leu-169 in IP\(_{3}\)R1 were also functionally important. The corresponding residues in IP\(_{3}\)R3 are Lys-169 and Leu-170, respectively. We mapped these conserved residues to our crystal structure of IP\(_{3}\)R3\(^{SUP}\) (Fig. 5) and found that they localize to one face of the head domain, on which Trp-168 is situated in the center of the binding site. Because the corresponding residue in IP\(_{3}\)R1 is Tyr-167, we have denoted this region the Tyr/Trp contact region (Figs. 1 and 5). This M4-M5 linker binding site is distinct from the C1 region of the structure (25), which has been shown to interact with the ligand binding core domain (26). The distinction is clearly shown in the structure of IP\(_{3}\)R3\(^{SUP}\), where the Tyr/Trp contact region is located at an opposite face of the C1 region (Fig. 1, B and C). This observation is consistent with the interaction of the suppressor domain with the M4-M5 linker does not mutually exclude its interaction with the core domain suggests that the two events potentially act together in a cooperative manner. Further studies are required to deduce the precise binding mechanism of the M4-M5 linker to the suppressor domain and its relationship with IP\(_{3}\) binding. Because the structures of IP\(_{3}\)R1\(^{SUP}\) and IP\(_{3}\)R3\(^{SUP}\) are very similar, it is not surprising that the corresponding residues in the type 1 suppressor domain (Glu-20, Tyr-167, and Ser-217) are localized to the same face of the structure. Moreover, the surface electrostatic potentials of the suppressor domain (Fig. 5) and amino acid sequence of the M4-M5 linker (Fig. 4) are very similar between type 1 and type 3 IP\(_{3}\)R, suggesting that the interaction with the M4-M5 linker is conserved between the two isoforms.

Specific mutations to either the M4-M5 linker or to the IP\(_{3}\)R suppressor domain result in a non-functional channel (12, 31). Interestingly, we note that the IP\(_{3}\)R gating-deficient mutation (Y167A in the type 1 isoform, W168A in the type 3 isoform) coincides with a large basic region in the N-terminal domain of type 1 ryanodine receptor (RyR1) (27) to which a “hot spot” of mutations resulting in malignant hyperthermia and/or central core disease are localized. These disease-associated mutations in RyR1 and the IP\(_{3}\)R gating-deficient mutations are found in the loop segment between \(\beta 8\) and \(\beta 9\) (loop 8) (supplemental Fig. 6). In the case of RyR, it has been suggested that the TM6-TM7 loop (analogous to the M4-M5 linker in IP\(_{3}\)R) interacts with a region within its central domain (28). The present experimental data from both functional and structural studies strongly argue that the suppressor domain provides a key interaction for the N-C coupling required for the ligand-operated Ca\(^{2+}\) channel gating function of IP\(_{3}\)Rs. The presence of other interaction points (possibly including the 160 most C-terminal residues of IP\(_{3}\)R) between the M4-M5 linker and the cytoplasmic region of IP\(_{3}\)R cannot be excluded.

The present study and the accompanying paper (31) have provided compelling evidence for a critical role of the IP\(_{3}\)R suppressor domain in the channel gating function as well as the IP\(_{3}\) sensitivity. More specifically, the Tyr/Trp contact residue (Tyr-167 in IP\(_{3}\)R1 and Trp-168 in IP\(_{3}\)R3) is essential in the communication between the N-terminal ligand-binding region and the C-terminal channel domain. The crystal structure of the type 3 suppressor domain revealed that this region is structurally conserved, and our NMR data suggested that these aromatic residues in type 1 and 3, together with Glu-20 and Ser-217 in type 1 and Glu-19 and Ser-218 in type 3, are involved in the interaction with the M4-M5 linker within the transmembrane domain in each isoform. Although this interaction is essential to the channel gating, we believe that the Tyr/Trp contact region is not the sole contributor to the gating mechanism. Instead, we propose that there are multiple “contact sites” between the transmembrane channel region and the rest of the molecule, which includes the central modulatory domain and the C-terminal “gatekeeper” domain. Together with the interaction involving the Tyr/Trp contact region within the N-terminal suppressor domain, other interactions involving a larger portion of the receptor must participate in this protein-protein interaction network, which positively and negatively regulate the activity of the Ca\(^{2+}\) channel in response to various external stimuli, including the natural agonists IP\(_{3}\) and Ca\(^{2+}\), ATP (29), and phosphorylation events (reviewed in Ref. 30). Further studies are needed to obtain a full picture of the protein-protein interaction network within the functional tetrmeric receptor.

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