Molecular Basis of the Isoform-specific Ligand-binding Affinity of Inositol 1,4,5-Trisphosphate Receptors

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Three isoforms of the inositol 1,4,5-trisphosphate (IP$_3$) receptor (IP$_3$R), IP$_3$R1, IP$_3$R2, and IP$_3$R3, have different IP$_3$-binding affinities and cooperativities. Here we report that the amino-terminal 604 residues of three mouse IP$_3$R types exhibited $K_d$ values of 49.5 ± 10.5, 14.0 ± 3.5, and 163.0 ± 44.4 nM, which are close to the intrinsic IP$_3$-binding affinity previously estimated from the analysis of full-length IP$_3$Rs. In contrast, residues 224–604 of IP$_3$R1 and IP$_3$R2 and residues 225–604 of IP$_3$R3, which contain the IP$_3$-binding core domain but not the suppressor domain, displayed an almost identical IP$_3$-binding affinity with a $K_d$ value of ~2 nM. Addition of 100-fold excess of the suppressor domain did not alter the IP$_3$-binding affinity of the IP$_3$-binding core domain. Artificial chimeric proteins in which the suppressor domain was fused to the IP$_3$-binding core domain from different isoforms exhibited IP$_3$-binding affinity significantly different from those of the proteins composed of the native combination of the suppressor domain and the IP$_3$-binding core domain. Systematic mutagenesis analyses showed that amino acid residues critical for type-3 receptor-specific IP$_3$-binding affinity are involved in Glu-39, Ala-41, Asp-46, Met-127, Ala-154, Thr-155, Leu-162, Trp-168, Asn-173, Asn-176, and Val-179. These results indicate that the IP$_3$-binding affinity of IP$_3$Rs is specifically tuned through the intramolecular attenuation of IP$_3$-binding affinity of the IP$_3$-binding core domain by the amino-terminal suppressor domain. Moreover, the functional diversity in ligand sensitivity among IP$_3$R isoforms originates from at least the structural difference identified on the suppressor domain.

The inositol 1,4,5-trisphosphate (IP$_3$)$_3$ receptors (IP$_3$Rs) function as IP$_3$-gated Ca$^{2+}$ release channels located on intracellular Ca$^{2+}$ stores, such as the endoplasmic reticulum (1). Mammalian IP$_3$R family consists of three isoforms (IP$_3$R1, IP$_3$R2, and IP$_3$R3), and they form homotetrameric or heterotetrameric channels (2). There is evidence of a functional difference among the three isoforms of IP$_3$R in terms of their IP$_3$ sensitivity (3–5) and cooperativity with respect to IP$_3$ binding (5). The intrinsic association constants of mouse IP$_3$R1, IP$_3$R2, and IP$_3$R3 expressed in Sf9 cells are estimated to be 3.5 × 10$^7$, 1.7 × 10$^8$, and 3.4 × 10$^8$ M$^{-1}$, respectively (5). IP$_3$R2 exhibits both negative and positive cooperativity, whereas IP$_3$R3 exhibits negative IP$_3$ binding cooperativity (5). This diversity of responsiveness to IP$_3$ observed among the three IP$_3$R isoforms may contribute to the generation of the different degree of IP$_3$ sensitivity of the Ca$^{2+}$ store. The molecular basis of the isoform-specific IP$_3$-binding affinity, however, is not well understood.

The IP$_3$-binding domain of IP$_3$R1 is composed of two functional domains, the amino-terminal suppressor domain and the carboxyl-terminal IP$_3$-binding core domain (6). The IP$_3$-binding core domain is the minimum region required for specific IP$_3$ binding and is mapped within residues 226–578 of mouse IP$_3$R1, a polypeptide of 2749 residues (6). The amino-terminal 225 amino acid residues of IP$_3$R1 function as the suppressor for IP$_3$ binding, and deletion of these residues results in significant enhancement of IP$_3$ binding (6). The atomic resolution structures of both the suppressor domain (7) and the IP$_3$-binding core domain (8) of mouse IP$_3$R1 were solved by x-ray crystallography to 1.8- and 2.2-Å resolution, respectively. The IP$_3$-binding core domain comprises two asymmetric domains, the $\beta$-domain and $\alpha$-domain. A highly positive-charged pocket is created at the interface of these two domains to which an IP$_3$ molecule binds. The 11 amino acid residues in the IP$_3$-binding core domain of IP$_3$R1 are responsible for the coordination of IP$_3$ (8), and all of them except Gly-268 are conserved in other isoforms. The suppressor domain contains a $\beta$-trefoil fold and a helix-turn-helix structure inserted between two $\beta$-strands of the $\beta$-trefoil fold (7). The conserved 7 amino acid residues, which are clustered on one side of the suppressor domain, were found to be critical for the suppression of IP$_3$ binding (7). These structural and functional analyses of the IP$_3$-binding domain have been carried out mainly using the type-1 isoform, and the suppression ability of the amino-terminal regions of IP$_3$R2 and IP$_3$R3 has not been well characterized.
In the present study, we analyzed the IP₃-binding affinity of the IP₃-binding domain of three isoforms and found that the intramolecular suppression of IP₃-binding specifically tunes the IP₃-binding affinity of IP₃R isoforms. Systematic analyses of chimeric mutants and site-directed mutants created based on the three-dimensional structures of the suppressor domain provide us with a novel insight into the structural basis of the isoform-specific IP₃-binding affinity of the three IP₃R types.

EXPERIMENTAL PROCEDURES

Gene Construction—Plasmids carrying the amino-terminal 604 amino acid residues of mouse IP₃R1, IP₃R2, and IP₃R3 (T604m₁, T604m₂, and T604m₃, respectively) were constructed as follows. The 1.8-kbp fragments of the three IP₃R genes were amplified with the PCR method from pBact-STneoB-C1, pBluescriptII-C2, and pBact-STneoB-C3 (5), with three pairs of primers, P1/P4, P2/P5, and P3/P4 (supplemental Table S1), respectively. The fragments were digested with NdeI and EcoRI and were subcloned into the modified pRSET-A (Invitrogen), in which the hexa-histidine (His₆) tag coding region was removed, to generate pRSET-T604m₁, pRSET-T604m₂, and pRSET-T604m₃, respectively. To express amino acid residues 224–604 of IP₃R2 and 225–604 of IP₃R3 (supplemental Fig. S1), plasmids were constructed as follows. The 1.1-kbp fragments were amplified from pBluescriptII-C2 and pBact-STneoB-C3 with pairs of primers, P6/P5, and P7/P4 (supplemental Table S1), respectively. The fragments were digested with NdeI and EcoRI and were subcloned into the His₆ tag-removed pRSET-A to generate pRSET-(224–604)m₂ and pRSET-(225–604)m₃, respectively. For the expression of residues 224–604 of mouse IP₃R1, pET-D-(1–223)/T604 (9) was used. For the expression of the His₆-tagged suppressor domain (residues 2–223) of mouse IP₃R1 (supplemental Fig. S1), pET23a-T7-IP₃Rsup-6His (7) was used. Plasmids carrying chimeric proteins, in which the suppressor domains were fused to the IP₃-binding core domain from different isoforms (supplemental Fig. S1), were constructed using the technique of splicing by overlap extension with PCR (10) and primers, P1, P3–5, P8, and P10–18 (supplemental Table S1). The combination of templates and primers used is summarized in supplemental Table S2. Mixtures of two DNA fragments separately produced in the first PCRs were used as templates for the second PCR (supplemental Table S2). The products of the second PCR were digested with NdeI and EcoRI and were subcloned into the His₆ tag-removed pRSET-A to generate pRSET-(1–223)m₂-(224–604)m₁, pRSET-(1–224)m₃-(224–604)m₁, pRSET-(1–223)m₁-(224–604)m₂, pRSET-(1–224)m₃-(224–604)m₂, pRSET-(1–223)m₁-(225–604)m₃, and pRSET-(1–223)m₂-(225–604)m₃, respectively.

Site-directed mutagenesis within the suppressor domain of T604m₃ or (1–223)m₁-(225–604)m₃ (supplemental Fig. S2) to engineer systematic chimeric proteins as summarized in supplemental Fig. S2 was performed with a Quick Change site-directed mutagenesis kit (Stratagene) and primers containing the appropriate substitution (M1–M13, supplemental Table S3). Multiple mutants were generated by sequential mutagenesis. Only sense primers used for the site-directed mutagenesis are shown in supplemental Table S3. Substitution of amino acid residues 61–122 of T604m₃ with amino acid residues 62–121 of T604m₁ was carried out using the technique of splicing by overlap extension with PCR (supplemental Table S2) and primers P3, P19, P20, and P21 (supplemental Table S1). The product of the second PCR was digested with NdeI and EcoRI and was subcloned into the His₆ tag-removed pRSET-A to generate pRSET-(1–223)m₁-(224–604)m₃, pRSET-(1–223)m₂-(224–604)m₃, and pRSET-(1–223)m₃-(224–604)m₃, respectively.

FIGURE 1. Comparison of IP₃-binding affinity of the amino-terminal 604 residues among the three IP₃R isoforms. A, SDS-PAGE analysis of T604s. Purified proteins (1 μg each) of T604m₁ (lane 1), T604m₂ (lane 2), and T604m₃ (lane 3) were separated on 7.5% SDS-PAGE followed by Coomassie Brilliant Blue R-250 staining. Arrowheads indicate T604 proteins. Molecular size markers are shown on the left (×10³). B, equilibrium IP₃-binding activity of the IP₃-binding domains of the three IP₃R isoforms. Values are averages of 3 (T604m₁, circle), 3 (T604m₂, triangle), and 9 (T604m₃, square) measurements. Error bars correspond to the standard deviation. C, comparison between the apparent dissociation constants of T604 and the intrinsic dissociation constants estimated from the measurements using full-length IP₃Rs expressed in Sf9 cells (5). Circle, IP₃R1; triangle, IP₃R2; and square, IP₃R3. Error bars correspond to the standard deviation.
ucts and site-directed mutants used in this study were confirmed by DNA sequencing with a 3130 Genetic analyzer (Applied Biosystems).

**Expression and Purification of Recombinant Proteins**—Recombinant proteins were expressed in *Escherichia coli* BL21-codonplus (Stratagene) as described previously (9). Protein purification was performed with a HiTrap heparin HP column (GE Healthcare) according to the method described previously (5). The His6-tagged suppressor domain of mouse IP3R1 (amino acid residues 2–223) was purified with a ProBond resin (Invitrogen) as described previously (5). Protein concentrations were determined with a protein assay kit (Bio-Rad) and bovine serum albumin as a standard.

**IP3 Binding Assay**—An equilibrium IP3 binding analysis of purified soluble proteins was performed as described previously (9), except for the reaction condition with a cytosol-like medium (110 mM KCl, 10 mM NaCl, 5 mM KH2PO4, and 50 mM Heps-KOH, pH 7.4, at 4 °C). Purified protein (0.02–0.8 μg) was incubated with 0.14–8.68 nM [3H]IP3 (New England Nuclear/DuPont) and various concentrations of unlabeled IP3 (Dojindo) in a binding buffer (cytosol-like medium containing 1 mM dithiothreitol and 0.5 mM EGTA). To avoid tracer depletion, the amount of the protein and the concentration of [3H]IP3 were adjusted for each experiment. Nonspecific binding was measured in the binding buffer without adding the protein. Nonlinear regression of IP3 binding data with the Hill-Langmuir equation,

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F = \frac{[IP3]}{K_d + [IP3]} 
\]  
(Eq. 1)

where F is the fraction of the recombinant protein that binds IP3, [IP3] is the concentration of IP3, and Kd is the apparent dissociation constant, was performed with Igor Pro (version 4.04, Wavematrics) software.

**Modeling of the Suppressor Domain in IP3R3**—The structure of the IP3R1 suppressor domain (residues 7–223) (7) was used for homology modeling of the suppressor domain structure of IP3R3, residues 6–224. The sequence alignment between IP3R1 and IP3R3 was generated with ClustalW (11) and used for modeling with Modeller (12). Because the loop linking the two helices in the Arm sub-domain of IP3R1 (residues 76–81) was not defined in the original structure, this portion of the suppressor domain was also modeled in the type-3 isoform. Thirty models were calculated and superimposed with Molmol (13) (root mean square deviation = 1.2 Å). Only the mean model is shown.

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**FIGURE 2.** Comparison of IP3-binding affinity of the IP3-binding core domain among the three IP3R isoforms. A, SDS-PAGE analysis. Purified proteins (0.75 μg) of (224–604)m1 (lane 1), (224–604)m2 (lane 2), and (225–604)m3 (lane 3) were separated on 10% SDS-PAGE followed by Coomassie Brilliant Blue R-250 staining. Arrowheads indicate the proteins containing IP3-binding core domains. Molecular size markers are shown on the left (×103). B–D, the relationship between normalized IP3 binding and IP3 concentrations applied. Filled circles: (224–604)m1 (B), (224–604)m2 (C), and (225–604)m3 (D). IP3-binding activity of T604s shown in Fig. 1B is plotted as open circles (B, T604m1; C, T604m2; and D, T604m3). E, comparison of the apparent dissociation constants of (224–604)m1, (224–604)m2, and (225–604)m3. Averages of three independent measurements are shown. Error bars correspond to the standard deviation. Statistical analysis was performed using one-way analysis of variance followed by a post-hoc comparison using Dunn’s multiple comparison procedure. N.S., not significant.
RESULTS

Characterization of IP3 Binding to the Amino-terminal 604 Residues of Three IP3R Isoforms—Nontagged amino-terminal 604 amino acid residues of the three isoforms of mouse IP3Rs, T604m1, T604m2, and T604m3 (supplemental Fig. S1), were expressed in E. coli and were purified on a HiTrap heparin HP column. Recombinant T604 proteins showed an apparent molecular mass of 65 kDa (Fig. 1A). We measured the IP3-binding activity of these purified proteins using 3H-labeled IP3. Fig. 1B shows the relationship between the normalized amount of IP3 bound to the purified proteins and the IP3 concentration applied. Because the data of the three isoforms were well fitted with the Hill-Langmuir equation (see “Experimental Procedures”) and each protein possesses a single IP3-binding site, IP3 binding to the purified amino-terminal 604 amino acid residues seemed to occur independently. The apparent dissociation constants of T604m1, T604m2, and T604m3 were estimated to be 49.5 ± 10.5 nM (n = 3), 14.0 ± 3.5 nM (n = 3), and 163.0 ± 44.4 nM (n = 9) (mean ± S.D.), respectively. These values are well consistent with the intrinsic dissociation constants estimated from the analyses of homotetrameric IP3R channels expressed in Sf9 cells under the same experimental condition (28.6 nM for IP3R1, 5.9 nM for IP3R2, and 294.0 nM for IP3R3) (5) (Fig. 1C). These results suggest that the type-specific IP3-binding affinity originates from the amino-terminal 604 amino acid residues of each IP3R type.

Comparison of the IP3-binding Affinity of the IP3-binding Domain of Three Isoforms without the Suppressor Domain—The IP3-binding domain of IP3R1 is functionally divided into two parts: the amino-terminal suppressor domain and the carboxyl-terminal IP3-binding core domain (6, 14). The IP3-binding core domain has been defined as a minimum essential region for specific IP3 binding, which resides within amino acid residues 226–578 of IP3R1 (6). Because the amino acid residues 224–604 of mouse IP3R1, designated as (224–604)m1 (supplemental Fig. S1B), are expressed well in E. coli (9), we used it for the measurement of the IP3-binding affinity of the IP3-binding domain without the suppressor domain. Supplemental Fig. S1A shows portions of the amino acid sequence alignments among the three mouse IP3R isoforms. To compare the unsuppressed IP3-binding affinity among the three isoforms, we purified the bacterially expressed (224–604)m1, residues 224–604 of mouse IP3R2 ((224–604)m2), and the residues 225–604 of mouse IP3R3 ((225–604)m3) (Fig. 2A). We found that all of the IP3 binding data of these proteins were well fitted with the Hill-Langmuir equation (Equation 1) (Fig. 2, B–D) with statistically indistinguishable apparent dissociation constants, 1.78 ± 0.63 nM (n = 3) for (224–604)m1, 1.51 ± 0.01 nM (n = 3) for (224–604)m2, and 2.04 ± 0.65 nM (n = 3) for (225–604)m3 (mean ± S.D.) (Fig. 2E). These results indicate that the presence of the amino-terminal 223, 223, and 224 amino acid residues of mouse IP3R1, IP3R2, and IP3R3 results in the suppression of IP3 binding by 27.8-fold (Fig. 2B), 9.3-fold (Fig. 2C), and 81.1-fold (Fig. 2D), respectively. Moreover, the isoform-specific IP3-binding affinity of the
native IP₃Rs reflects the different degree of the suppression of IP₃ binding by the suppressor domain of each isoform.

**Mechanism of IP₃ Binding Suppression**—To assess the mechanism for the suppression, we investigated the effect of the addition of the bacterially expressed His₉-tagged amino acid residues 2–223 of mouse IP₃R1 (H(2–223)m₁) (supplemental Fig. S1) on the IP₃-binding activity of (224–604)m₁. As shown in supplemental Fig. S3 the addition of 100-fold excess (molar ratio) purified H(2–223)m₁ did not significantly alter the IP₃-binding activity of (224–604)m₁. These results indicate that the suppression of IP₃ binding requires a covalent bond between the suppressor domain and the IP₃-binding core domain.

**Functional Difference among the Suppressor Domains of the Three IP₃Rs Isoforms**—We created six chimeric proteins composed of a suppressor domain fused with an IP₃-binding core domain from different isoforms (supplemental Fig. S1B) to analyze the mechanism underlying the generation of isoform-specific IP₃-binding affinity. All of the equilibrium IP₃-binding data obtained for these chimeric proteins showed good fits with the Hill-Langmuir equation (Equation 1, data not shown) with apparent dissociation constants of 44.2 ± 13.4 nM (n = 3) for (1–223)m₂-(224–604)m₁, 73.0 ± 17.3 nM (n = 3) for (1–224)m₃-(224–604)m₁, 15.8 ± 3.6 nM (n = 3) for (1–223)m₁-(224–604)m₂, 40.7 ± 2.6 nM (n = 3) for (1–224)m₃-(224–604)m₂, 65.9 ± 7.4 nM (n = 9) for (1–223)m₁-(225–604)m₃, and 53.7 ± 5.2 nM (n = 3) for (1–223)m₂-(225–604)m₃ (mean ± S.D.). The results of the statistical analyses of these data are shown in Fig. 3. All of the chimeric proteins showed >10-fold lower IP₃-binding affinity (Fig. 3, A–C) compared with the proteins that do not possess the suppressor domain (Kₐ ≈ 2 nM) (Fig. 2). The artificial chimeric proteins, however, revealed apparent dissociation constants that significantly differed from those of the proteins composed of the native combination of the suppressor domain and the IP₃-binding core domain (Fig. 3, A–C). This suggests that the suppressor domain alone is not a prime determinant of the affinity for IP₃. When we compare the same data in respect of the IP₃-binding core domain (Fig. 3, D–F), the different character of the IP₃-binding core domain of the three isoforms became obvious. The IP₃-binding core domain of the type-1 isoform exhibited an almost identical IP₃-binding affinity despite the type of the suppressor domain connected (Fig. 3D). The type-2 IP₃-binding core domain showed indistinguishable affinity for IP₃ when it was fused with the type-1 and type-2 suppressor domains, but the chimeric protein with the type-3 suppressor domain showed an affinity ~3-fold lower compared with the native combination of the type-2 isoform (Fig. 3E). The IP₃-binding core domain of the type-3 isoform strictly required the type-3 suppressor domain to generate its native IP₃-binding affinity (Fig. 3F). These results indicate that: 1) the isoform-specific IP₃-binding affinity is predominantly determined by the IP₃-binding core domain rather than the suppressor domain; 2) the suppressor domains of the type-1 isoform and type-2 isoform are mutually interchangeable for both the IP₃-binding core domains of IP₃R1 and IP₃R2 to generate their native IP₃-binding affinity; and 3) the type-3-specific site(s) in the suppressor domain may be critical for the proper suppression of the IP₃-binding core domain of IP₃R3.

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**Critical Loop Regions in the Suppressor Domain for the Suppression of IP₃R3**—To elucidate the structural basis for the suppression of the type-3 isoform, we created a series of mutated proteins based on the amino acid sequence difference between IP₃R1 and IP₃R3 and on the three-dimensional structure of the suppressor domain of mouse IP₃R1. Twelve β-strands and eleven loop regions are shown in red and blue, respectively. Boundaries of the β-strands and loop regions are determined as described previously (7). The equilibrium IP₃-binding activity of (b1,b4,b8,b9,b12)m₃/T604m₃ (n = 3, closed circle), T604m₃ (open diamond), (1–223)m₁-(225–604)m₃ (open circle), and (225–604)m₃ (open triangle) is shown. Error bars correspond to the standard deviation.

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**FIGURE 4. IP₃-binding activity of a mutant protein in which all of type-3-specific amino acid residues within the β-strands of the suppressor domain were replaced with those appearing in IP₃R1.** A, three-dimensional structure of the suppressor domain of mouse IP₃R1. Twelve β-strands and eleven loop regions are shown in red and blue, respectively. Boundaries of the β-strands and loop regions are determined as described previously (7). B, equilibrium IP₃-binding activity of (b1,b4,b8,b9,b12)m₃/T604m₃ (n = 3, closed circle), T604m₃ (open diamond), (1–223)m₁-(225–604)m₃ (open circle), and (225–604)m₃ (open triangle) is shown. Error bars correspond to the standard deviation.
Identification of the loop regions critical for the suppression of IP₃ binding to the type-3 IP₃-binding core domain. A, apparent dissociation constants of (1–223)m₁-(225–604)m₃ (lane 1), T604m₃ (lane 2), L₁m₁/T604m₃ (lane 3), L₃m₁/T604m₃ (lane 4), L₄m₁/T604m₃ (lane 5), L₅m₁/T604m₃ (lane 6), L₇m₁/T604m₃ (lane 7), L₈m₁/T604m₃ (lane 8), and L₁₀m₁/T604m₃ (lane 9). B, apparent dissociation constants of (1–223)m₁-(225–604)m₃ (lane 1), T604m₃ (lane 2), and (L₅,L₇)m₁/T604m₃ (lane 3). C, apparent dissociation constants of (1–223)m₁-(225–604)m₃ (lane 1), (L₁,L₅,L₇)m₁/T604m₃ (lane 2), (L₃,L₅,L₇,L₈)m₁/T604m₃ (lane 3), (L₄,L₅,L₇,L₈)m₁/T604m₃ (lane 4), (L₅,L₇,L₈)m₁/T604m₃ (lane 5), and (L₅,L₇,L₁₀)m₁/T604m₃ (lane 6). D, apparent dissociation constants of T604m₃ (lane 1), (L₅,L₇,L₈,m₁-(1–223)m₁-(225–604)m₃ (lane 2), and (L₅,L₇,L₈,L₁₀,m₁-(1–223)m₁-(225–604)m₃ (lane 3). The number of measurements is shown in parentheses. *, p < 0.05; **, p < 0.01; N.S., not significant (Student’s t test). The mutants used in this study are summarized in supplemental Fig. S2.

To identify critical sites within the suppressor domain for the suppression of IP₃ binding to the type-3 IP₃-binding core domain, we created seven mutant proteins in which the amino acid sequences of individual type-3-specific loop regions, L₁, L₃, L₄, L₅, L₇, L₈, and L₁₀, were substituted with residues appearing in IP₃R₁ (supplemental Fig. S2). We found that both L₅m₁/T604m₃ (97.4 ± 26.2 nM, n = 7) and L₇m₁/T604m₃ (105.0 ± 7.8 nM, n = 6) exhibited an apparent dissociation constant, which was significantly different from that of T604m₃ (Fig. 5A). These results showed that both L₅ and L₇ are required for the suppression of IP₃ binding to the IP₃-binding core domain of IP₃R₃. We then constructed a mutant protein in which type-3-specific amino acid residues within both L₅ and L₇ in T604m₃ were simultaneously changed to the residues that appear in IP₃R₁ (supplemental Fig. S2). This mutant protein, (L₅,L₇)m₁/T604m₃, exhibited an apparent dissociation constant (80.6 ± 9.9 nM, n = 6) that was lower than those of L₅m₁/T604m₃ and L₇m₁/T604m₃ (Fig. 5B), but the effect of the simultaneous replacement was not statistically significant when compared with the effects of the single loop replacements. The apparent dissociation constant of (L₅,L₇)m₁/T604m₃ is significantly different from that of (1–223)m₁-(225–604)m₃ (Fig. 5B).

We then created five mutant proteins, (L₁,L₅,L₇)m₁/T604m₃, (L₃,L₅,L₇,L₈)m₁/T604m₃, (L₄,L₅,L₇,L₈)m₁/T604m₃, and (L₅,L₇,L₁₀)m₁/T604m₃, in which the type-3-specific amino acid residues in three loop regions, including L₅ and L₇, were substituted with residues appearing in IP₃R₁ (supplemental Fig. S2). Among these mutants, (L₃,L₅,L₇)m₁/T604m₃ and (L₅,L₇,L₈)m₁/T604m₃ exhibited an apparent dissociation constant (69.2 ± 10.6 nM (n = 5) and 77.4 ± 12.7 nM (n = 5), respectively) that was not different significantly from that of (1–223)m₁-(225–604)m₃ (p = 0.525 and 0.066, respectively, Fig. 5C). To confirm these results, we created mutant proteins in which all of the type-1-specific amino acid residues within L₅, L₇, and L₈ in (1–223)m₁-(225–604)m₃, or within L₅, L₇, and L₈ in (1–223)m₁-(225–604)m₃ were substituted with residues appearing in IP₃R₃ (supplemental Fig. S2). The mutants, (L₃,L₅,L₇,m₁-(1–223)m₁-(225–604)m₃ and (L₅,L₇,L₈,m₁-(1–223)m₁-(225–604)m₃, exhibited an apparent dissociation constant of 149.0 ± 5.8 nM (n = 6) and 158.0 ± 11.5 nM (n = 6), respectively, both of which are indistinguishable from that of T604m₃ (Fig. 5D). We therefore concluded that L₅ and L₇ plus either L₃ or L₈ in the suppressor domain are essential and sufficient for the generation of type-3-specific IP₃-binding affinity of the IP₃₁-binding core domain of IP₃R₃.

**Type-3-specific Amino Acid Residues Critical for the Suppression of IP₃ Binding to the Type-3 IP₃-binding Core Domain**—Fig. 6 shows amino acid sequence alignments of L₃, L₅, L₇, and L₈ of the suppressor domain of the three IP₃R types. The amino acid residues specific for IP₃R₃, but not for IP₃R₁, are Glu-39, Ala-41, and Asp-46 in L₃, Met-127 in L₅, Ala-154, Thr-155, and Leu-162 in L₇, and Trp-168, Asn-173, Asn-176, and Val-179 in L₈ (Fig. 6). All of the amino acid residues except Leu-162 are located at the surface of the suppressor domain of IP₃R₃ (Fig. 7).
Isoform-specific IP$_3$-binding Affinity of IP$_3$Rs

**DISCUSSION**

The IP$_3$ sensitivity of the intracellular Ca$^{2+}$ stores seems to be heterogeneous within the cytosol, and spatially restricted Ca$^{2+}$ releases, such as Ca$^{2+}$ puffs, have frequently been observed in various cell types (16, 17). Multiple intracellular Ca$^{2+}$ stores with variable IP$_3$ sensitivities in single cells may contribute to the generation of the complex spatiotemporal patterns of intracellular Ca$^{2+}$ dynamics (18–21). We have previously reported that three isoforms of the IP$_3$R have different IP$_3$-binding affinities and cooperativities (5). A non-cooperative IP$_3$ binding model did not fit the equilibrium IP$_3$ binding data obtained from tetrameric IP$_3$R2 or IP$_3$R3 complexes expressed in Sf9 cells (5), indicating that the IP$_3$-binding affinity of each subunit within single tetrameric complexes depends on the number of occupied subunits within a homotetrameric complex composed of IP$_3$R2 or IP$_3$R3. The intrinsic IP$_3$-binding affinities of IP$_3$R1 and IP$_3$R2, but the suppressor domain from IP$_3$R3 (residues 1–225) can physically interact with the IP$_3$-binding core domain of IP$_3$R1 (residues 226–604), the interdomain interaction may cause changes in the IP$_3$-binding affinity of the IP$_3$-binding core domain. The amino-terminal 604 residues of IP$_3$R types bind IP$_3$ in a non-cooperative manner (Fig. 1B), indicating that the suppression of IP$_3$ binding occurs independently in each molecule. Because the addition of a 100-fold excess of the suppressor domain did not significantly alter the IP$_3$-binding affinity of the IP$_3$-binding core domain (supplemental Fig. S3), the suppressor domain is not effective for separating molecules. We therefore suspect that the suppression of IP$_3$ binding is an intramolecular event that requires a covalent bond between the suppressor domain and the IP$_3$-binding core domain. The present results suggest that 1) the isoform-specific IP$_3$-binding affinity originates from the intramolecular suppression of IP$_3$ binding, but not from the difference in the intrinsic IP$_3$-binding affinity of the IP$_3$-binding core domain, and 2) the nature of the interaction between the suppressor domain and the IP$_3$-binding core domain is different among the three isoforms, thereby resulting in their different IP$_3$-binding affinities. In other words, the IP$_3$-binding affinity of IP$_3$Rs is generated by the precisely controlled attenuation of IP$_3$-binding affinity caused by the intramolecular interaction between the suppressor domain and the IP$_3$-binding core domain, and the IP$_3$-binding affinity is a tunable parameter rather than a stable constant.

**Molecular Basis of the Type-specific Suppression of IP$_3$ Binding**—To test domain compatibility among the three IP$_3$R types, we have engineered chimeric proteins in which the amino-terminal suppressor domain was fused to the IP$_3$-binding core domain from different isoforms and found that the suppressor domain is not a prime determinant of the IP$_3$-binding affinity (Fig. 3, A–C). The IP$_3$-binding core domain of IP$_3$R1 showed almost the same apparent dissociation constants regardless of the type of the suppressor domain connected (Fig. 3D). The IP$_3$-binding core domain of IP$_3$R2 did not discriminate the suppressor domain from IP$_3$R1 and IP$_3$R2, but the suppressor domain from IP$_3$R3 failed to produce the native IP$_3$-binding affinity of IP$_3$R2 (Fig. 3E). The IP$_3$-binding core domain of IP$_3$R3 strictly required the suppressor domain from the same isoform to produce its native IP$_3$-binding affinity (Fig. 3F). These results indicate that 1) conserved amino acid residues in the suppressor domain of IP$_3$R1 and IP$_3$R2 are involved in the interaction with the type-1 and type-2 IP$_3$-binding core do-

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**FIGURE 6. Amino acid sequence alignments of the third, fifth, seventh, and eighth loop regions of the suppressor domain.** Amino acid residues identified to be critical for the suppression of IP$_3$ binding to type-3-IP$_3$-binding core domain are shown in red. Type-2-unique residues are shown in blue.
mains, and 2) type-3-specific amino acid residues are strictly required for the proper interaction with the type-3 IP$_3$-binding core domain. Type-3-specific residues may also interfere in the proper interaction with the type-2 IP$_3$-binding core domain.

Identification of Amino Acid Residues Critical for the Type-3-specific IP$_3$-binding Affinity—Systematic mutagenesis analyses (Figs. 4 and 5) showed that at least L5 and L7 in the suppressor domain are essential for the generation of the type-3-specific IP$_3$-binding affinity. Interestingly, (L3,L5,L7)$_{m1}$/T604$_{m3}$ and (L5,L7,L8)$_{m1}$/T604$_{m3}$ exhibited an apparent dissociation constant indistinguishable from that of (1–223)$_{m1}$(225–604)$_{m3}$ (Fig. 5C) and (L3,L5,L7)$_{m3}$(1–223)$_{m1}$(225–604)$_{m3}$ and (L5,L7,L8)$_{m3}$(1–223)$_{m1}$(225–604)$_{m3}$ exhibited an apparent dissociation constant indistinguishable from that of T604$_{m3}$ (Fig. 5D). These results suggest that, in addition to L5 and L7, either L3 or L8 is required for the generation of the native IP$_3$-binding affinity of IP$_3$R3. This interpretation seems to be supported by the results shown in Fig. 5A. The replacement of either L5 or L7 from T604$_{m3}$ resulted in a significant enhancement of the IP$_3$-binding affinity, whereas, single replacement of either L3 or L8 did not result in a significant alteration of the IP$_3$-binding affinity (Fig. 5A). The position of L5 and L7 are relatively close on the suppressor domain and the three type-3-specific amino acid residues, Met-127, Ala-154, and Thr-155, within these regions are nearby the previously identified conserved residues essential for the suppression of IP$_3$ binding to IP$_3$R1 (Fig. 7). L3 is located on the same side of L5 and L7 on the suppressor domain, whereas L8 is placed on the opposite side (Fig. 7). These results predict a dynamic nature of the intramolecular interaction for the suppression of IP$_3$ binding. More specifically, there are at least two different conformational states of the complex comprising the suppressor domain and IP$_3$-binding core domain with both states contributing toward the suppression of IP$_3$ binding. One possible interpretation is that one of the complexes employs an interface formed by L3, L5, and L7 on the suppressor domain, whereas the other uses an interface formed by L5, L7, and L8. The amino acid sequences of these critical loops might also indirectly affect the structure of the interface.

**FIGURE 7.** Surface and ribbon diagram representations of critical sites for the IP$_3$ binding suppression. Surface (A and C) and ribbon diagram (B and D) representations of the suppressor domain of mouse IP$_3$R1 and surface (E and G) and ribbon diagram (F and H) representations of the suppressor domain of mouse IP$_3$R3 are shown. The amino acid residues critical for the type-specific suppression are depicted in red. The conserved amino acid residues essential for the IP$_3$ binding suppression (7) are labeled in pink. Amino acid residues in blue are involved in the determination of the IP$_3$-binding affinity of IP$_3$R1 (7). C, view in A rotated by 180°. D, view in B rotated by 180°. G, view in E rotated by 180°. H, view in F rotated by 180°.

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between the suppressor domain and the IP$_3$-binding domain. According to the prediction, the essential amino acid residues for the generation of the type-3 receptor-specific IP$_3$-binding affinity are Glu-39, Ala-41, Asp-46, Met-127, Ala-154, Thr-155, Leu-162, Trp-168, Asn-173, Asn-176, and Val-179 (Figs. 6 and 7). Szlufcik et al. showed that the deletion of amino acid residues 76–86 of IP$_3$R1 or the replacement of these residues by Ala resulted in an increase in IP$_3$ binding and the sensitivity of IP$_3$-induced Ca$^{2+}$ release (22). In our experiments, the replacement of L4, which includes residues 76–86 of IP$_3$R1 or residues 75–86 of IP$_3$R3, did not induce a significant change in the apparent dissociation constant of T604$_{m3}$ (Fig. 5A). Because Szlufcik et al. (22) measured the amount of [H]$IP_3$ bound to crude membrane fractions prepared from cell lines with different expression levels of recombinant receptors, it is not clear whether the mutations on residues 76–86 significantly affected the IP$_3$-binding affinity of the actual receptor proteins. Because our results reported here are mainly based on an analysis of the IP$_3$-binding domain fragments expressed in bacteria, the critical sites for determining isoform-specific IP$_3$-binding affinity in full-length receptor proteins should be confirmed. Identification of the interface between the suppressor domain and the IP$_3$-binding core domain by x-ray or NMR analyses should help our better understanding of the nature of the molecular interaction between these two domains.

A Possible Mechanism for the Suppression of IP$_3$ Binding—Addition of 100-fold excess of the type-1 suppressor domain did not result in a significant reduction of the IP$_3$-binding affinity of the type-1 IP$_3$-binding core domain (supplemental Fig. S3). This result indicates that the suppression of IP$_3$ binding is not a simple competitive inhibition of IP$_3$ binding to the IP$_3$-binding core domain by the suppressor domain. Recently, we have measured the reaction kinetics of the fluorescence resonance energy transfer of the IP$_3$ sensor protein upon the IP$_3$ binding (24). The IP$_3$ sensor protein, designated IRIS, is composed of the type-1 IP$_3$-binding core domain fused to two fluorescent proteins, enhanced cyan fluorescent protein and Venus. Analysis of the reaction kinetics of IRIS shows that there are at least two conformational states of the IP$_3$-binding core domain in the absence of IP$_3$ and that IP$_3$ binding fixes the conformation of the IP$_3$-binding core domain to one state (24). This reaction model is consistent with the result of the NMR studies, which suggests a dynamic equilibrium exists between two or more conformations in the apo state of the IP$_3$-binding core domain (25). According to this model, not only the rate constants for ligand-association and dissociation but also the rate constants for conformational changes of the receptor protein determine the equilibrium dissociation constant between the receptor and ligand. Evaluation of the effect of the suppressor domain on the rate of ligand binding and the rate of conformational changes of the IP$_3$-binding domain should help us to understand the mechanism of the IP$_3$ binding suppression.

Functional Significance of the Intramolecular Turning of IP$_3$-binding Affinity—What is the functional significance of the intramolecular attenuation of the intrinsic IP$_3$-binding affinity of IP$_3$Rs except for the generation of the isoform-specific IP$_3$-binding affinity? We have previously shown that the IP$_3$ binding to the tetrameric complex of IP$_3$R2 or IP$_3$R3 is not a random process, and the occupation of the IP$_3$-binding site changes the IP$_3$-binding affinity of vacant sites on the neighboring subunits (5). During the sequential binding of four IP$_3$ molecules to single tetrameric complexes of IP$_3$R2 and IP$_3$R3, the dissociation constants of vacant sites are estimated to be changed to 5.8 × 10$^{-9}$ M (0 IP$_3$ molecule/tetramer), 3.7 × 10$^{-8}$ M (1 IP$_3$ molecule/tetramer), 1.3 × 10$^{-6}$ M (2 IP$_3$ molecules/tetramer), and 3.4 × 10$^{-7}$ M (3 IP$_3$ molecules/tetramer) for IP$_3$R2 and to 2.9 × 10$^{-7}$ M (0 IP$_3$ molecule/tetramer), 7.0 × 10$^{-7}$ M (1 IP$_3$ molecule/tetramer), 8.2 × 10$^{-7}$ M (2 IP$_3$ molecules/tetramer), and 2.8 × 10$^{-6}$ M (3 IP$_3$ molecules/tetramer) for IP$_3$R3, respectively (5). These flexible changes of IP$_3$-binding affinity may be generated from the molecular interaction between occupied subunits and vacant subunits within a single tetrameric channel complex through the suppressor domain of vacant subunits. We have also pointed out that the suppressor domain is a focal point for the interaction with various modulatory proteins, including calmodulin, CaBP1, and RACK1 (7). These proteins may have some potential for the modulation of the IP$_3$-binding affinity of IP$_3$Rs by changing the degree of the suppression of IP$_3$ binding. Our recent analysis of cytosolic IP$_3$ dynamics in single living cells suggest that IP$_3$ sensitivity of the intracellular Ca$^{2+}$ stores continuously change during Ca$^{2+}$ oscillations (24). The unique ligand binding machinery, composed of the suppressor domain and the IP$_3$-binding core domain of IP$_3$Rs, may account for the generation of the dynamic change of the sensitivity of the receptor for IP$_3$ in living cells.

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