The Pro\(^{335}\) → Leu Polymorphism of Type 3 Inositol 1,4,5-Trisphosphate Receptor Found in Mouse Inbred Lines Results in Functional Change*

Received for publication, February 16, 2005, and in revised form, May 4, 2005 Published, JBC Papers in Press, May 11, 2005, DOI 10.1074/jbc.M501777200

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Inositol 1,4,5-trisphosphate receptor (IP\(_R\)) is an intracellular Ca\(^{2+}\) channel involved in various cellular signaling. Type 3 IP\(_R\) (IP\(_R3\)) retains ligand-gated Ca\(^{2+}\) channel properties differing from other subtypes in terms of IP\(_3\)-binding affinity and regulation of its channel activity by effector molecules. In this study, we found the natural Pro\(^{335}\) → Leu polymorphism of mouse IP\(_R3\) between BALB/c and C57BL/6J. We investigated the functional differences between Pro\(^{335}\)IP\(_R3\) and Leu\(^{335}\)IP\(_R3\) purified receptors reconstituted into proteoliposomes as well as with soluble ligand binding domains. Pro\(^{335}\)IP\(_R3\) exhibited significantly higher IP\(_3\)-binding affinity and IP\(_3\)-induced Ca\(^{2+}\) release than those of Leu\(^{335}\)IP\(_R3\) in both forms of the receptor. Moreover, the polymorphic change caused differences in the effect of external Ca\(^{2+}\) on IP\(_3\)-induced Ca\(^{2+}\) release. The Pro\(^{335}\) → Leu substitution alters the conformation of soluble ligand binding domain as revealed by intrinsic fluorescence and circular dichroism spectra with or without Ca\(^{2+}\). The results indicate that the polymorphism of IP\(_R3\) causes changes in receptor function, presumably affecting intracellular Ca\(^{2+}\) signaling.

* This work was supported in part by the Glycomics Program and Grant BK21 (to C. P.) from the Ministry of Education, Korea. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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The abbreviations used are: IP\(_3\), inositol 1,4,5-trisphosphate; IP\(_R\), inositol 1,4,5-trisphosphate receptor; IP\(_R1\), type 1 IP\(_R\); IP\(_R3\), type 3 IP\(_R\); ET, reverse transcription; PMSF, phenylmethylsulfonyl fluoride; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid; GST, glutathione S-transferase; LBD, ligand-binding domain; BC, BALB/c; B6, C57BL/6J; ICR, IP\(_3\)-induced Ca\(^{2+}\) release.
Materials and Methods

Mice—Inbred strains of mice, BC and B6, were purchased from Daehan Biolink Co. Ltd. (Korea). All mice were raised in housing with five per cage in a specific pathogen-free and temperature-controlled facility at 22 °C under a 12-h light-dark cycle with light on at 07:00. Humidity was maintained at 55% with food and water freely available. Male mice at the age of 5–10 weeks were used for this study. All experimental procedures with animals followed the National Institutes of Health Guideline for the Care and Use of Laboratory Animals.

Full-length Nucleotide Sequences of Mouse IP3R3—The full-length sequences of mouse IP3R3 cDNA were obtained from plasmid, G431004L16 (RIKEN). Based on the nucleotide sequences obtained, we designed specific primers for mouse IP3R3. The entire coding sequences of IP3R3 were amplified between BC and B6 mouse by RT-PCR. All total RNA was extracted mouse brain followed by reverse transcription. After PCR amplification of an ~2–3 kb DNA fragment with specific primers, the DNA fragments were sequenced on an ABI 3700 sequencer (PerkinElmer Life Sciences).

RT-PCR was conducted as described previously with minor modifications (21). After 2 μg of total RNA were purified from whole brains and intestine, first strand cDNAs were synthesized by using the total RNA as template. PCR amplification was performed with the first cDNAs as templates. PCR conditions were as follows: 1 cycle at 94 °C for 3 min, followed by 27 cycles at 94 °C for 30 s, 55 °C for 1 min (for subtypes of I/III), 52 °C for 1 min (for subtypes of I/III), or 72 °C for 1 min. The oligonucleotides used for PCR were as follows: for type I/III, 5'-GT(G/C)ACCAGGGACATGG-3' and 5'-GCCATATGAATGAAATGTCCAGC-3'; for type II/III, 5'-CCGAGTCAAGAACAAGACGTTGAC-3' and 5'-CCAGGTCAAGAACAGACGTTGAC-3'. The PCR products were electrophoresed on a 1.5% agarose gel and then transferred to nylon membranes. Blots were hybridized with 32P-labeled type-specific oligonucleotide probes (type I, 5'-AGGGAAGAAGAATCTGTAT-3'; type II, 5'-GAAATGTCCAGC and the reverse primer, 5'-GCTCGAGAAGCTTCCGGTTGTTGT by using plasmid pGEX-LBD). The bands were visualized under a Bioimager (Fujifilm).

Construction of pGEX-LBD and pET-LBDHis Vector—The cDNA encoding the N-terminal 605 amino acids of the mouse Pr335IP3R3 was amplified by PCR with the forward primer ATCCCGGGAATGATGAAATGTCCAGC and the reverse primer GCCATATGAATGAAATGTCCAGC, followed by subcloning into the pGEM-T Easy vector. For the normalization of the IP3R signals, glyceraldehyde-3-phosphate dehydrogenase was amplified with specific primers (forward primer, 5'-ACCACTGCTCATGCGACAC-3' and reverse primer, 5'-TCCACCCCTTTGGTTGTA-3'). Then glyceraldehyde-3-phosphate dehydrogenase signals detected by Southern blot analysis were used as standard. The radioactivity of the membrane was analyzed using a PhosphorImager (Fujifilm).

Purification of Recombinant GST-LBD and LBD-His Protein—The full-length mouse IP3R cDNA were synthesized by using the total RNA as template, and subcloned into the pGEM-T Easy vector. The resulting proteoliposomes were pelleted by centrifugation at 100,000 x g for 1 h and then centrifuged at 32,000 x g for 1 h at 4 °C. The supernatant obtained was mixed with an equal volume of buffer C (20 mM Tris-HCl, pH 7.5, 0.1 mM NaCl, 1 mM β-mercaptoethanol, 10 μM leupeptin, and 10 μM pepstatin) and were broken in a blender, followed by homogenization in a glass-Teflon homogenizer. The homogenates were then centrifuged at 100,000 x g for 1 h to precipitate the membrane pellet. The pellet was resuspended in buffer B (50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1 mM β-mercaptoethanol, 0.1 mM PMSF, 10 μM leupeptin, and 10 μM pepstatin) containing 1% (w/v) Triton X-100 to give the membrane protein concentration of ~2 mg/ml. The membrane solution was stirred for 1 h and then centrifuged at 32,000 x g for 1 h at 4 °C. The supernatant obtained was mixed with an equal volume of buffer C (20 mM Tris-HCl, pH 7.5, 0.1 mM NaCl, 1 mM β-mercaptoethanol, 10 μM leupeptin, and 10 μM pepstatin) and was applied to a type 3 IP3R antibody-coupled immunoaffinity column equilibrated with buffer C as described above. The protein-loaded column was washed with 20 bed volumes of buffer C to remove unbound proteins, and the IP3R was eluted by 10 ml of elution buffer D (0.1 M glycine, pH 2.8, 0.2% (w/v) Triton X-100, 0.5 mM NaCl, 1 mM β-mercaptoethanol, 0.1 mM PMSF, 10 μM leupeptin, and 10 μM pepstatin). The eluate was immediately neutralized by adding 1× Tris-HCl buffer containing 1 M Tris, 0.5 M NaCl, 1% (w/v) Triton X-100, 0.2% (w/v) Triton X-100, 0.5 mM NaCl, and 1 mM β-mercaptoethanol), and then applied to a benzamidine-Sepharose column equilibrated with buffer E to remove any residual proteases from the IP3R sample. The IP3R-containing fraction was collected and stored at −70 °C until use.

Reconstitution of IP3R into Proteoliposomes—Phospholipids were prepared from Avanti Polar Lipids. Cholesterol solutions of lipids were stored in sealed ampules under argon gas at −20 °C. Phosphatidylinoline (from bovine brain), phosphatidylethanolamine (from bovine brain), and phosphatidylserine (from bovine brain) dissolved in chloroform were mixed to give a molar ratio of 50, 30, and 20%, respectively. The final lipid concentration was 5 mg in a total volume of 1 ml. The chloroform was evaporated under a stream of argon. The resulting chloroform was removed by speed vacuuming. The dry lipids were hydrated in buffer F (20 mM HEPES, pH 7.5, 100 mM NaCl, 1 mM CaCl2, 1% CHAPS) containing about 5 μg/ml IP3R. The mixtures were dialyzed for 72 h against an excess volume of buffer G (buffer F without CHAPS). The resulting proteoliposomes were pelleted by centrifugation at 100,000 × g for 30 min at 4 °C, washed with buffer H (20 mM HEPES, pH 7.5, 100 mM NaCl), and then dialyzed against buffer E for 24 h at 4 °C. The resulting proteoliposomes were passed through Chelex 100 to remove free Ca2+.

The formation of proteoliposomes was monitored by measurement of light scattering during analysis with a spectrophotometer (excitation and emission wavelength of 450 nm). The average diameter of the liposomes was about 380 when assayed as described previously (22). The amounts of reconstituted IP3R were determined by using NanoOrange® protein quantitation kit (Molecular Probes). After the reconstitution, the ratio of phospholipid concentration (w/v) to that of protein (L/P) was determined as 890 ± 27 for Pr335IP3R3 and 879 ± 22 for Leu335IP3R, in reaction samples, respectively.

IP3-induced Ca2+ Release Measurement Using Indo-1 Fluorescence—Ca2+ efflux from the proteoliposomes was observed by measuring the external calcium efflux of indo-1. Fluorescent measurements were performed at 30 °C by using a Shimadzu RF-5301 PC spectrofluorometer. The fluorescence intensity was measured at the emission wavelength of 393 nm under an excitation wavelength of 355 nm. The fluorescent intensities of 10 μM indo-1 were calibrated to free Ca2+ concentration using the Ca2+ -EGTA buffering system (24). To quantify the amount of released Ca2+ from proteoliposomes by IP3 binding, the fluorescence intensity of indo-1 after addition of IP3 was compared with an Amicon French pressure cell. Cell lysates were applied to a column of glutathione-agarose pre-equilibrated with lysis buffer. The column was washed with lysis buffer and then eluted with elution buffer (50 mM Tris-HCl, pH 8.0, 10 mM reduced glutathione). The expression of pET-LBDHIs was performed as described previously (22). To purify recombinant LBD-His proteins, harvested bacterial pellets were resuspended in the binding buffer (20 mM Tris-HCl, pH 8.0, 500 mM NaCl, 5 mM imidazole, 1 mM PMSF, 5 μg/ml of benzamidine, leupeptin, and pepstatin). The cells were lysed by passing through an Amicon French pressure cell. Cell lysates were applied to a nickel-nitrilotriacetic acid agarose column pre-equilibrated with the binding buffer. The column was washed with binding buffer and washing buffer (20 mM Tris-HCl, pH 8.0, 500 mM NaCl, and 60 mM imidazole). The LBD-His proteins were eluted in the binding buffer containing 250 mM imidazole. The concentrations of recombinant proteins were quantified using bicinchoninic acid according to the manufacturer’s instruction (Pierce).
the fluorescence intensity after addition of Triton X-100 (1% (w/v), final concentration) instead of IP3.

Effect of External Ca2+ on the IP3-mediated Ca2+ Release Measurement from the Proteoliposomes Using 45Ca2+—The proteoliposomes were produced in the presence of 45Ca2+ to include ~20,000 cpm of 45Ca2+ in buffer H (20 mM HEPES, pH 7.5, 100 mM NaCl, 1 mM β-mercaptoethanol) according to the procedure described. To remove residual Ca2+ bound to the vesicle surface, the sample was applied to Sephadex G-25 column equilibrated with buffer H, and the liposomes were pelleted by centrifugation (100,000 × g, 30 min, 4 °C). The pellet was then redissolved and dialyzed against excess volume of buffer H for 12 h at 4 °C. The proteoliposomes were mixed with each indicated concentration of CaCl2 and incubated for 10 min at 30 °C. After further incubation of the sample for 10 min in the presence of 1 μM of IP3, in the reaction mixtures, the sample was diluted with buffer I (buffer H plus 1.5 x KCl). The liposomes were pelleted by centrifugation (100,000 × g, 30 min, 30 °C). The pellet was then dissolved with 1% (w/v) Triton X-100, and the radioactivity of each fraction (pellet and supernatant) was determined by scintillation counting.

Removal of Ca2+ Contamination—Removal of Ca2+ contamination was conducted according to the method described previously (25). Ca2+ contamination during all experiments was checked using the fluorescence of the Ca2+ indicator, indo-1, before measurements.

Preparation of IP3R3 Antibody-coupled Column—Type 3 IP3R3 antibody-coupled column was prepared by coupling 0.6 mg of affinity-purified anti-peptide type 3 IP3R antibody (Sigma) to 1.2 ml of the immobilized protein A resin from the ImmunoPure protein A IgG orientation kit (from Pierce) according to the instructions. The column was stored in 20 mM Tris-HCl, pH 7.5, containing 0.02% sodium azide until use.

(3H)IP3 Binding to Recombinant GST-LBD Fusion Protein or Constituted IP3R3—10 μg/ml GST-LBD fusion protein or 2.8 μg/ml IP3R3 in buffer J (buffer H plus 1 mM EGTA, 1 mM β-mercaptoethanol) was incubated with various concentrations of IP3 containing 1:1000 as much (3H)IP3. Binding experiments were carried out in the absence of free external Ca2+. After 20 min of incubation at 30 °C, a 0.2 volume of each sample was filtered through a spun concentrator with molecular weight cut-off of 30,000. The radioactivity of each filtrate was determined by a liquid scintillation counter and compared with the control values without proteins. The same method was applied to measure Ca2+ binding on the recombinant GST-LBDs.

Effect of External Ca2+ on the IP3 Binding to Recombinant GST-LBD—10 μg/ml IP3-R-GST fusion protein was incubated with various concentrations of Ca2+ in the presence of a fixed concentration of IP3 containing 1:1000 as much (3H)IP3. After 20 min of incubation at 30 °C, a 0.2 volume of each sample was filtered through a spun concentrator, and the radioactivity of filtrate was determined as described.

Fluorescence and CD Spectroscopy—To measure the intrinsic fluorescence of Trp residues in the recombinant LBD-His protein (1 μM), the emission intensity in the range 310–400 nm was recorded under 295 nm of excitation wavelength using a Shimadzu RF-5301 PC spectrofluorometer at 25 °C. CD spectra in the far-UV and near-UV regions were monitored at room temperature with a Jasco J715 spectropolarimeter (Japan Spectroscopic, Tokyo); the optical path lengths were 0.1 and 1.0 cm, respectively. Measurements were conducted in 100 mM sodium phosphate (pH 7.4) containing 2.45 μM of Pro335LBD-His and 2.5 μM of Leu335-LBD-His for the far-UV and 12.17 μM of Pro335LBD-His and 21.43 μM of Leu335-LBD-His for near-UV regions, respectively. The standard conditions were as follows: bandwidth, 1 nm; response, 1–2 s; step resolution, 0.1–0.2 nm. Blanks (buffer) were routinely recorded and subtracted from the original spectra. On average, data from 30 to 40 scans were accumulated.

SDS-PAGE and Immunoblotting—Immunoprecipitated IP3R samples were dissolved with a 1% SDS solution and then analyzed by 7% SDS-PAGE. This was followed by immunoblotting and detection with anti-IP3R antibody.

RESULTS

Polymorphism of IP3R3 Found in BC and B6 Mice—The coding sequences of the IP3R3 of BC and B6 mice were compared with the search for polymorphism that may potentially be associated with functional variation. The open reading frame consists of 8013 bp and 2670 encoded amino acids, showing 95 and 92% identity with rat and human orthologs, respectively. A total of 11 nucleotide polymorphisms were founded in the open reading frame. One of the polymorphisms at the 335th position resulted in a change in amino acid from proline to leucine (Fig. 1A). Sequencing of genomic DNA obtained from the two mouse strains confirmed the polymorphism. The change is located in the N-terminal LBD (amino acids 1–605) with variations among species (Fig. 1B). On the basis of the crystal structure of the IP3R1 LBD core (amino acids 224–604), we were able to position the 335th residue in a loop between the β6 and β7 strands (26). The LBDs seems to have similar structure regardless of the receptor subtypes, because the sequence homologies among them are fairly high, e.g. 68% identity between types 1 and 3 in the LBD core sequence.

In order to compare the relative expressions of three IP3Rs and the ones between two mouse strains, semi-quantitative RT-PCR (21) was conducted with specific primers designed to amplify two isoforms simultaneously. The expression levels of IP3Rs of BC mice were ~30% higher than those of B6 mice in the brain regardless of the receptor type (Fig. 2A). In the intestine, type 1- and type 2-specific bands were not detected in both mice. On the other hand, IP3R3 mRNA was expressed more in the BC intestine than that of B6 by ~60%. A Western blotting analysis after immunoprecipitation using a specific antibody against IP3R3 showed that more IP3R3 proteins are expressed in the BC intestine than that of B6 (Fig. 2B), which is consistent with the results of semi-quantitative RT-PCR. The results confirmed that IP3R3 is the major isofrom expressed in the mouse intestine, with a slight variation in their expressions among inbred mice.

IP3-binding Affinity and IP3-induced Ca2+ Release (IICR) of the Pro335 and Leu335 Forms of IP3R3—in order to test
whether the polymorphism affects the intrinsic function of IP3R3, an IP3-binding assay was performed with purified Pro335IP3R3 and Leu335IP3R3 reconstituted into proteoliposomes. Considering that IP3R3 was highly expressed (10) and the expression of type 1 or 2 IP3R was not detected in intestines by the semi-quantitative RT-PCR (Fig. 2A), the purified IP3R was expected to be mostly type 3. From the results, Pro335IP3R3 showed 2.4-fold higher IP3-binding affinity than that of Leu335IP3R3 (Fig. 3A). In particular, the difference was more significant at values less than 1 μM IP3, which is the range of physiological concentration after stimulation (27, 28). Above 1 μM IP3 concentration, both IP3R3s exhibited similar IP3-binding affinities. The IP3 dissociation constants \( K_d \) for Pro335IP3R3 and for Leu335IP3R3 were estimated to be 301.5 ± 28.9 and 722.4 ± 27.0 nM, respectively. Hill coefficients of both Pro335IP3R3 and Leu335IP3R3 were 1.1 and 2.7, respectively. These values indicate that IP3 binds to each subunit of Pro335IP3R3 independently, whereas IP3 binding to Leu335IP3R3 exhibits a positive cooperativity.

As we observed an alteration in IP3-binding affinity, we further examined whether the polymorphism influences IICR. In a previous study, purified bovine IP3R1 reconstituted into proteoliposomes showed Ca\(^{2+}\) release in dose-dependent manner of IP3 (29). The dose-dependent Ca\(^{2+}\) release of IP3 from reconstituted proteoliposomes in each IP3R was compared. As shown in Fig. 3B, Pro335IP3R3 released a significantly higher amount of Ca\(^{2+}\) than that of Leu335IP3R3 for all IP3 concentrations tested. In particular, the difference was remarkable in the range less than 3 μM IP3. The IP3 concentration for half-maximal Ca\(^{2+}\) release (EC\(_{50}\)) of Pro335IP3R3 was 4.2-fold lower than that of Leu335IP3R3. EC\(_{50}\) of Pro335IP3R3 and Leu335IP3R3 was 248.6 ± 14.3 and 1019.2 ± 365.6 nM, respectively. This indicates that Pro335IP3R3 forms a more sensitive Ca\(^{2+}\) channel than Leu335IP3R3 in response to the same IP3 concentration.

**Ca\(^{2+}\) Effects on IICR of Pro335- and Leu335IP3R3—External Ca\(^{2+}\) is a well known modulator of IP3R3 Ca\(^{2+}\) channel activity (15). Two Ca\(^{2+}\)-binding sites were identified previously in LBD of IP3R1 exposed to cytosol, and the residues were conserved in LBD between IP3R1 and IP3R3 (11). As the polymorphism is located near the putative Ca\(^{2+}\)-binding sites, we investigated the effects of external Ca\(^{2+}\) on IICR of both receptors. As shown in Fig. 4, external Ca\(^{2+}\) differentially modulated the Ca\(^{2+}\) release of each receptor in the presence of 1 μM IP3, showing a maximum difference in IICR, as illustrated in Fig. 3B. In the case of Pro335IP3R3, IICR exhibited a characteristic biphasic pattern with a maximum release of Ca\(^{2+}\) at 1 μM of Ca\(^{2+}\). A further decrease or increase in external Ca\(^{2+}\) concentration reduced the IICR of Pro335IP3R3 from the maximum value. In contrast, IICR of Leu335IP3R3 was monotonically inhibited up to 3 μM of Ca\(^{2+}\). It has been suggested that a biphasic mode of Ca\(^{2+}\) on the channel activity of IP3R is important in generating characteristic Ca\(^{2+}\) signaling within the cells (30, 31). Therefore, the present biphasic regulation by Ca\(^{2+}\) suggests that Pro335IP3R3 shows different Ca\(^{2+}\) signaling from that of Leu335IP3R3 in vivo.

**IP3-binding Affinities and Ca\(^{2+}\) Effects on IP3 Binding of Recombinant Pro335 and Leu335 of IP3R3 LBD—**In order to investigate further the different modes of IP3 bindings between the receptors, we constructed bacterial expression vectors containing 605 amino acid sequences of each LBD fused to GST. In a previous study, LBD (amino acids 1–576) of rat IP3R3 fused to GST was expressed in bacteria and retained proper IP3-binding affinity (10). Fig. 5A shows that IP3-binding affinity on...

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**Fig. 2. Expression of IP3R3 between B6 and BC mice.** A, ratio of semi-quantitative RT-PCR of total RNA extracted from brain and intestine. After first cDNA was synthesized with total RNA extracted from the indicated tissues, PCR was conducted with primer pairs (type III or type II/III) using the cDNA as a template. The subtype-specific signals were detected by Southern blotting with \(^{32}\)P-labeled type-specific probes. RT and w/o RT indicate that RT-PCRs were performed with and without reverse transcriptase, respectively. EcoRI-digested plasmids containing the PCR fragment of each subtype were loaded on the subtypes lanes. B, Western blot analysis of immunoprecipitated samples compared with lanes 1 and 3, respectively. A and B represent typical results of four and three experiments, respectively. Br, brain; Int, intestine; G3PDH, glyceraldehyde-3-phosphate dehydrogenase.
examined the effect of polymorphism on IP$_3$ binding to GST-LBDs by affinity-purified IP$_3$R3 reconstituted into proteoliposomes. After the reconstitution, the amounts of reconstituted IP$_3$R3 and phospholipid were determined to 302 and 202 (µg/ml), respectively, for Pro$_{335}$IP$_3$R$_3$ and to 0.32 and 281 (µg/ml) for Leu$_{335}$IP$_3$R$_3$. For measuring ICR, the amount of IP$_3$R3 was adjusted to be same in both reaction samples. The liposomes were incubated with various concentrations of IP$_3$ containing 1:1000 as much [3H]IP$_3$. The results indicated that IP$_3$ binding to Pro$_{335}$IP$_3$R$_3$GST-LBD was slightly increased at 0.5 µM Ca$^{2+}$ and then decreased up to 4 µM Ca$^{2+}$ (Fig. 5B). In contrast, the binding to Leu$_{335}$GST-LBD was inhibited progressively with increasing Ca$^{2+}$ concentrations. The external Ca$^{2+}$-induced inhibition was 53% for Pro$_{335}$GST-LBD and 64% for Leu$_{335}$GST-LBD at a 4 µM concentration. These results indicate that the single polymorphism causes different Ca$^{2+}$ effects on IP$_3$ binding to the LBD.

Ca$^{2+}$ Binding to the LBDs and Change in Intrinsic Fluorescence of LBDs by Ca$^{2+}$—In an effort to investigate further the differential IP$_3$ binding by external Ca$^{2+}$ to both LBDs, we compared 45Ca$^{2+}$-binding affinity between two LBDs. From the results of the 45Ca$^{2+}$-binding assay, the $K_a$ values of Pro$_{335}$GST-LBD and Leu$_{335}$GST-LBD were found to be nearly identical at 260.4 ± 25.4 and 287.7 ± 51.4 nM, respectively (Fig. 6A). This indicates that the polymorphism does not affect the intrinsic Ca$^{2+}$-binding property of the LBD.

Next, Ca$^{2+}$-induced conformational changes of the two recombinant LBDs were examined using intrinsic emission fluorescence. The LBD of IP$_3$R$_3$ contains 7 tryptophan and 17 tyrosine residues. Pro$_{335}$- and Leu$_{335}$GST-LBD showed maximum emission peaks ($\lambda_{\text{max}}$) at 355 and 356 nm, respectively, under an excitation at 280 nm (result not shown). Fig. 6B shows that the intensities at $\lambda_{\text{max}}$ gradually increased with increasing Ca$^{2+}$ concentration. The fluorescence values of Pro$_{335}$- and Leu$_{335}$GST-LBD were increased by 75 and 35%, respectively, compared with the Ca$^{2+}$-free case. It suggests that both LBDs have different conformations upon Ca$^{2+}$ binding. To obtain the binding affinity for the Ca$^{2+}$-binding property of the LBD.

In the reconstitution study, external Ca$^{2+}$ induced a different ICR between Pro$_{335}$- and Leu$_{335}$IP$_3$R$_3$. Therefore, we examined the effect of polymorphism on IP$_3$ binding to GST-LBDs by external Ca$^{2+}$-fixed at 0.4 µM IP$_3$, which displayed the most significant difference in IP$_3$ binding, as described in Fig. 5A. The results indicated that IP$_3$ binding to Pro$_{335}$IP$_3$R$_3$GST-LBD was slightly increased at 0.5 µM Ca$^{2+}$ and then decreased up to
the two LBDs, circular dichroism analyses were performed in the far- and near-UV regions. To avoid possible effects of GST protein on the conformational change of LBDs, we used a C-terminal His-tagged LBD instead of the GST fusion. The intrinsic fluorescence change of the Pro335- and Leu335LBD-His by Ca$^{2+}$ was consistent with that of GST-LBDs (result not shown).

Any difference in the secondary structure of LBD-His proteins was not detected in the far-UV region without Ca$^{2+}$ (Fig. 7A). The $\alpha$-helical content estimated from mean residue ellipticity ($\theta_{222}$) at 222 nm (32) was 34.4 and 32.8% with Pro335- and Leu335LBD-His, respectively. In addition, the far-UV CD spectra of both proteins were not significantly different in the absence of Ca$^{2+}$. Data are presented as the mean ± S.E. of three independent experiments. • Pro$^{335}$IP$_3$R3; ○, Leu$^{335}$IP$_3$R3.

![FIG. 5. IP$_3$ binding and Ca$^{2+}$ effect on IP$_3$ binding to the recombinant GST-LBDs. A. IP$_3$-binding curves of Pro$^{335}$GST-LBD and Leu$^{335}$GST-LBD under Ca$^{2+}$-free conditions. B. Ca$^{2+}$ effect on IP$_3$ binding to both recombinant proteins in the presence of 0.4 $\mu$M IP$_3$, are plotted as Ca$^{2+}$ concentration versus the amount of bound IP$_3$. The inset of B represents IP$_3$ binding as percentages of that bound in the absence of Ca$^{2+}$. Data are presented as the mean ± S.E. of three independent experiments. • Pro$^{335}$IP$_3$R3; ○, Leu$^{335}$IP$_3$R3.](image)

![FIG. 6. Ca$^{2+}$ binding on the recombinant GST-LBDs and changes of maximum intrinsic fluorescence at various Ca$^{2+}$ concentrations. A, Ca$^{2+}$-binding curves of Pro$^{335}$GST-LBD and Leu$^{335}$GST-LBD. B, changes of intrinsic fluorescence in the peak at various Ca$^{2+}$ concentrations. The results are typical of three experiments. •, Pro$^{335}$GST-LBD; ○, Leu$^{335}$GST-LBD.](image)

DISCUSSION For the Pro$^{335} \rightarrow$ Leu polymorphism found in the mouse IP$_3$R3, we investigated their functional differences with the whole receptors and their LBDs. The polymorphism affects IP$_3$-binding affinity and IICR of the receptor, i.e. Pro$^{335}$IP$_3$R3 exhibited higher IP$_3$-binding affinity and IICR than those of Leu$^{335}$IP$_3$R3. In particular, the difference was noted in the range of submicromolar IP$_3$ concentrations. It has been reported that IP$_3$ concentration reaches ~100–300 nM during stimulation in lymphocytes (27, 28). Therefore, Pro$^{335}$IP$_3$R3 may respond to stronger cellular Ca$^{2+}$ signals than those of Leu$^{335}$IP$_3$R3 upon stimulation. In addition, Pro$^{335}$IP$_3$R3 exerted a distinct biphasic inhibition on IICR by external Ca$^{2+}$, whereas monotonic inhibition was shown in the case of Leu$^{335}$IP$_3$R3. Moreover, the receptors showed differences in Ca$^{2+}$-induced conformational change when assayed with intrinsic fluorescence and CD. The results obtained from near-UV CD spectroscopy indicate that the Pro$^{335}$LBD retains a more ordered tertiary structure than that of Leu$^{335}$LBD upon Ca$^{2+}$ binding, although the two LBD proteins do not show a significant difference in secondary structure as assayed with far-UV CD. Therefore, it seems plausible that the functional differences between the receptors are mainly attributable to their structural differences caused by single amino acid substitution in the LBD region that is exposed to cytosol. Based on these results, we speculate that the Pro$^{335} \rightarrow$ Leu polymorphism of IP$_3$R3 causes a change in receptor functions, with distinct cellular Ca$^{2+}$-signaling properties. However, implica-
tion on their in vivo function may require further experiments. It has been shown that IP₃R reconstituted into proteoliposomes displays similar functional properties with the native receptors including IP₃-binding affinity, Ca²⁺ selectivity, and cytosolic Ca²⁺ dependence of channel activity (33–35). Therefore, our results from the reconstitution study may indicate actual differences between the two receptors. Recently, the same receptor, Leu₃³⁵IP₃R₃, from the B6 mouse was cloned and characterized (36), in which the $K_d$ value of the purified receptor for IP₃ (340 nM) was 2-fold higher than that of the receptor reconstituted into the proteoliposome. In this study, Leu₃³⁵IP₃R₃ reconstituted into the proteoliposome showed positive cooperativity for IP₃ binding. However, Iwai et al. (36) indicated that IP₃ binds to the receptor with negative cooperativity based on the model of four IP₃-binding sites. The reason for this contradiction is not clear, although we cannot rule out the involvement of various accessory factors. Here we conducted an IP₃-binding assay with purified IP₃R₃ reconstituted into proteoliposomes, excluding the possibility of accessory factors. Iwai et al. (36) used membrane fractions obtained from Sf9 cells expressing mouse IP₃R₃, which may contain additional factors influencing receptor activity.

The $K_d$ values of LBDs for ligand binding were reported for the two mutant forms of mouse IP₃R₃ (10, 37). The IP₃-binding affinity of GST-fused LBD of rat IP₃R₃ ($K_d$ = 66.0 ± 13.2 nM) is close to that of Pro₃³⁵LBD obtained here (95.4 ± 2.9 nM). In the case of human IP₃R₃ using the N-terminal 750 residues of LBD, the $K_d$ for IP₃ was 151 nM (37). Differences in the IP₃-binding affinities of IP₃R₃ are in the order of rat LBD > mouse Pro₃³⁵LBD > human LBD > mouse Leu₃³⁵LBD. It is interesting that the 335th residue of rat, BC mouse, human, and B6 mouse are proline, proline, methionine, and leucine, respectively. Although we cannot rule out the possibility that the difference may lie in other variable residues among species or in different experimental conditions, the association of the 335th residues with IP₃-binding affinities together with our results suggest that the residue is one of the major determinants for the ligand-binding affinity among species.

The putative calcium sensor regions of IP₃R isoforms were localized in the regulatory domain (38). According to the results, Ca²⁺ induced a conformational change of recombinant protein made of that region, and calcium sensitivity was exchanged by swapping the sensor regions of two isoforms (38). Also, mutations within the sensor region (D₂¹₀⁰E and E₂¹₀⁰Q) shifted the bell-shaped Ca²⁺ dependence curve into the high Ca²⁺ range, indicating a reduction of Ca²⁺-binding affinities (39). However, in our results, IP₃ binding to LBD was regulated by conformational changes in the presence of free

![Fig. 7. CD spectra of recombinant Pro335LBD-His and Leu335LBD-His. CD spectra of the Pro335LBD-His (solid lines) and Leu335LBD-His (dashed lines) in the far-UV (A) and near-UV regions were measured in the presence of 1 mM EGTA (B) and 1 mM Ca²⁺ (C). Molar ellipticity [θ] is expressed on the basis of their molar concentration.](http://www.jbc.org/content/early/2018/07/24/jbc.M118.003380/F1.large.jpg)
Ca$^{2+}$. In addition, the Pro$^{335}$ → Leu polymorphism changed the Ca$^{2+}$ effect on IICR. Taken together, the results suggest that LBD of IP$_3$R3 plays an important role in Ca$^{2+}$ sensing. The Ca$^{2+}$ sensor region of IP$_3$R3 in the regulatory domain (Met$^{1835}$-$\text{Arg}^{2199}$) is associated with Ca$^{2+}$ with a $K_{Ca}$ of 100 nM (38). Therefore, the LBD of IP$_3$R3 may function as a low affinity Ca$^{2+}$ sensor as opposed to the regulatory domain.

Based on the crystal structure of the LBD core (amino acids 224–436) of IP$_3$R1, the 335th residue is localized in the undefined loop between the –6 and –7 strands (26). Although the loop structure is not yet determined, the polymorphic residue is unlikely to affect directly the IP$_3$-coordinating region and Ca$^{2+}$-binding site because of the distant configuration in the receptor. The crystallographic structure provides only the LBD core without an N-terminal inhibitory domain (residues 1–225). Many studies have suggested that N-terminal inhibitory domain plays an important role in the functional regulation of LBD by a possible domain-domain interaction (26, 40, 41). Therefore, we cannot exclude the possibility that the polymorphic residue (335th) affects the structure and function of LBD in association with the N-terminal inhibitory domain.

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J. Biol. Chem. 2005, 280:26024-26031.
doi: 10.1074/jbc.M501777200 originally published online May 11, 2005

Access the most updated version of this article at doi: 10.1074/jbc.M501777200

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