Inositol 1,4,5-trisphosphate receptor L3-2 loop 부분과 chromogranin B의 잘 보존된 N말단 부분 사이의 상호작용에 참여하는 residue들의 확인

Identification of residues participating in the interaction between an inositol 1,4,5-trisphosphate receptor L3-2 loop region and a conserved N-terminal of chromogranin B
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Identification of residues participating in the interaction between an inositol 1,4,5-trisphosphate receptor L3-2 loop region and a conserved N-terminal of chromogranin B

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Abstract

Inositol 1,4,5-trisphosphate receptor (IP$_3$R) is an intracellular calcium channel that mediates calcium release from endoplasmic reticulum (ER). IP$_3$R is involved in many biological processes and diseases. It is regulated by Ca$^{2+}$ and IP$_3$, and modulated by many additional molecules, such as calmodulin, chromogranin, IRBIT, Homer and ERp44. IP$_3$R is composed of heterotetramer or homertetramers and the pore of channel is formed by the transmembrane helices and loops, located in region between the 5th and the 6th transmembrane helices. The bindings between the L3–2 and the chromogranin A and B, well-known calcium storage proteins, were reported previously. Whereas much information is known about the cytoplasmic regulation of IP$_3$R, very little is known about its regulation of the luminal side regulator. Here, through the NMR titration experiments, we confirmed the interaction between the recombinant IP$_3$R L3–2 and the conserved N terminal region of the chromogranin B. The conserved region in the N-terminal region of chromogranin B has been shown to competitively inhibit calcium signaling by IP$_3$R. Understanding the detailed regulatory mechanisms of these regulators requires the identification of the residues directly involved in the binding. NMR resonance assignments analysis showed that the 14th–20th residues of L3–2 loop is a specific interaction site with the chromogranin B peptide, with a dissociation constant (Kd) of 300µM. However, the interaction between an L3–2 loop of IP$_3$R and chromogranin A peptide could not to be observed. The results also suggest that a mechanism of IP$_3$R regulation by chromogranin B is induction of the conformation exchange of the L3–2 loop region. These findings should help further studies on regulation of IP$_3$R by other luminal regulators.

Keywords: IP3R, Chromogranin, NMR, Interaction
Korean Abstract

Inositol 1,4,5-trisphosphate receptor (IP₃R)은 endoplasmic reticulum(ER)로부터 calcium 유리를 매개하는 intracellular calcium channel 이다. IP₃R은 많은 생리학적 과정과 질병에 연관되어 있다. IP₃R은 Ca²⁺과 IP₃에 의해 조절되며, calmodulin이나 chromogranin, IRBIT, Homer, 그리고 ERp44와 같은 많은 추가적인 분자들에 의해 조절된다. IP₃R은 homertetramer 또는 heterotetramer을 형성하며, channel의 pore는 transmembrane helices와 loop에 의해 형성되고 5번째 transmembrane helices와 6번째 transmembrane helices 사이에 위치한다. IP₃R의 L3-2 loop과 calcium storage protein인 chromogranin A와 B 사이의 binding은 이전에 보고되었다. 그러나 IP₃R의 cytoplasmic regulation에 대해서는 많이 알려져 있는 반면, chromogranin과 같은 calcium storage protein에 의한 IP₃R의 intraluminal regulation에 대해서는 잘 알려져 있지 않다. 우리는 NMR titration 실험을 통해 IP₃R L3-2와 chromogranin B의 잘 보존되어있는 N-terminal region 사이의 상호작용을 알아보았다. chromogranin B의 N-terminal 부위의 잘 보존되어 있는 peptide 부분은 IP₃R에 의한 calcium signaling을 경쟁적으로 억제한다고 알려져 있었다. 이러한 regulator의 자세한 조절 메카니즘을 이해하기 위해서는 binding에 직접적으로 연관된 residue의 확인이 필요하다. 우리는 NMR resonance assignments 분석을 통하여 L3-2 loop의 14번째에서 20번째 residue가 chromogranin B peptide와 300µM의 Kd를 가지고 특이적으로 결합할 수 있다는 것을 보였다. 하지만 IP₃R과 chromogranin A peptide와의 상호작용은 관찰되지 않았다. 이러한 결과는 chromogranin B에 의한 IP₃R 조절 메카니즘이 L3-2 region의 conformation 변화를 통해 이루어진다는 것을 보여준다. 우리의 이러한 발견은 다른 luminal regulator에 의한 IP₃R의 regulation 연구에 더 많은 도움을 줄 것이다.
1. Introduction

Calcium, well-known as a second messenger, is linked to numerous physiological processes of cells, including muscle contraction, secretion, membrane excitability, gene expression, cell division and apoptosis. The calcium storage organelle in most cells is endoplasmic reticulum (ER). The ER contains a free Ca\(^{2+}\) concentration of about 100~700µM. In contrast, the concentration of Ca\(^{2+}\) in the cytoplasm of inactive cells is about 100nM [1].

The intracellular calcium concentration is regulated by a Ca\(^{2+}\)-ATPases pump and Inositol 1,4,5-trisphosphate receptor (IP\(_3\)R), located in an ER membrane and/or plasma membrane. The Ca\(^{2+}\)-ATPases accumulate Ca\(^{2+}\) in the ER lumen to high levels [1,2]. Whereas, IP\(_3\)R releases Ca\(^{2+}\) from ER lumen by a regulators, such as Inositol 1,4,5-trisphosphate(IP\(_3\)) and Ca\(^{2+}\).

Thus, IP\(_3\)R plays important roles in maintaining cellular calcium homeostasis [3,4].

The IP\(_3\) that acts as a major regulator of IP\(_3\)R is made by phospholipase C (PLC)–β and PLC–γ. The PLC hydrolyzes phosphatidylinositol 4,5-bisphosphate(PIP\(_2\)). As a result, IP\(_3\) and diacylglycerol are generated [5]. IP\(_3\)R is a large protein comprised of about 2700 amino acid residues. It has a very long N-terminal cytoplasmic region composed of about 2100 amino acids, including a suppressor domain, an IP\(_3\) binding core and modulatory domain, a membrane channel domain with 6 transmembrane helices and loops, followed by the C-terminal region in the cytoplasmic compartment.

The N-terminal region of IP\(_3\)R is involved in ligand–binding and channel regulation, whereas the C-terminal region is involved in the oligomerization of the receptor (Fig. 1). Recently, it was reported that C-terminals of IP\(_3\)R were required for channel activity [6]. There are three IP\(_3\)R subtypes, IP\(_3\)R1, IP\(_3\)R2, and IP\(_3\)R3.
Figure 1. Schematic representation of the type 1 IP$_3$R. N-terminal region of IP$_3$R including suppressor domain (a.a 1-226), IP$_3$ binding core (a.a 226-578), modulatory domain (a.a 578-2250) is shown as dotted line. The channel domain is shown as solid line, and e-terminal region is shown as paired line. The binding sites of each domain of IP$_3$R are shown as square box.
Functional Ca\(^{2+}\) channels are formed from homo- or heterotetramer arrangements of these IP\(_3\)Rs [7,8].

The pore of the channel is formed by the transmembrane helices and loops, particularly by the region between the 5th and the 6th transmembrane helices. The region between the 5th and the 6th is subdivided into L3-1 and L3-2 loops. The two loops are exposed to ER lumen. The amino acid sequences of the two helices are very conserved in a closely-related calcium channel, ryanodine receptor (RyR), but the loop region (L3-1, L3-2) between them is missing in RyR [9]. Therefore, it seems that the loop region is involved in IP3R-specific regulation by being the primary target of the luminal regulators. As shown in figure 1, it is known that the N-terminal region of IP3R can bind to regulatory molecules, such as calmodulin, IRBIT, IP\(_3\), Homer, RACK1, and Ca\(^{2+}\) [10]. There has been much research done on these regulators. For example, the regulation of IP3R by calcium is very complex. At low concentrations, such as a hundred nanomolar range, calcium activates the channel, but at higher concentration, such as a millimolar range, it inhibits the channel [11,12]. Thus, IP3R channel activity is generally a bell-shaped function (biphasic regulation) of cytosolic Ca\(^{2+}\) concentration [13-15]. However, research done on the regulation of IP3R by an intraluminal regulator has seldom been reported, except for chromogranins which are comparatively well-known as positive regulators.

Chromogranins are major Ca\(^{2+}\) storage proteins in secretory granules. Also, chromogranins have been found in the lumen of the ER of numerous cell types. Chromogranins A and B are composed of about 400 amino acid residues and 600 amino acid residues, respectively. There are conserved regions in the N-terminal side and C-terminal side of chromogranin A and B [16]. The conserved C-terminal region of both chromogranins is involved in the oligomerization. Therefore, these molecules can form homotetramers and heterotetramers.
In addition, conserved N-terminal regions of chromogranins A and B are involved in the interaction with the L3-2 loop region of IP$_3$Rs. Therefore, chromogranins and IP$_3$Rs would interact with the homo- and/or heteroteramer of both molecules [18-20]. These interactions between homo- and/or heteroterameric IP$_3$R and homo- and/or heteroterameric chromogranin could explain the varying degrees of released Ca$^{2+}$ by equivalent amounts of IP$_3$ [17]. The chromogranins A and B exist in about a 2mM range in secretory granules and they have a low affinity and a high capacity for Ca$^{2+}$ [16]. The low affinity of chromogranins seems to respond to the Ca$^{2+}$ concentration change on the lumen. The regulation of IP$_3$R by chromogranins has been characterized in pancreatic cells, chromaffin cells, and neuroendocrine cells [19,21]. The granin-familly proteins, such as secretogranin and chromogranin are processed by endoprotidases to form peptides displaying a variety of biological activities [21, 23].

The binding of IP$_3$Rs with chromogranins (CgAB) enhances Ca$^{2+}$ release. In addition, it is known that the conserved N-terminal region of chromogranin B can disrupt the interaction between full length CgAB and the IP3R, as well as intracellular Ca$^{2+}$ signaling, by the competition with the full-length chromogranins [24]. Recently, there were reports about a new intraluminal inhibitor called the ERp44. The ERp44 binds to the L3-1 of IP$_3$R and regulates negative regulation of IP$_3$R, dependent on the luminal environment, such as redox states [25]. Although there was much research done for IP$_3$R/chromogranin binding, the detailed binding site of IP$_3$R-L3-2 to chromogranin remains unknown.

In this study, to further understand the detailed regulatory mechanisms between the IP$_3$R-L3-2 and the conserved N-terminal region of the chromogranins, we investigated the direct binding residues of IP$_3$R-L3-2 involved in the binding with the chromogranin.
A titration experiment, based on NMR, showed a specific binding with an L3–2 region of IP₃R and a conserved N-terminal region of chromogranin B, but not a conserved N-terminal of chromogranin A. The L3–2 residue of IP₃R participating in binding to a conserved N-terminal of chromogranin B were identified by triple-resonance experiments and resonance assignments, yielding the first detailed insight into the interaction between the IP₃R and its regulators. The analysis of the binding data suggested an interaction mechanism involving a conformational exchange of the IP₃R intraluminal loop region.
2. Materials and methods

2-1. Materials

The reagents and enzymes used in this study were purchased from Takara Korea (Seoul, Korea), Becton Dickinson Korea (Seoul, Korea), AMERICAN BIOANALYTICAL (Natic, MA, USA), Sigma Korea (Soul, Korea) and Cambridge Isotope Laboratories, Inc (Andover, MA, USA). The Bacto(TM) Tryptone and Bacto(TM) Yeast Extract, etc., the component of LB media, was purchased from Becton Dickinson Korea Company (Seoul, Korea). The Ammonium chloride (15N) and D-glucose (U-13C6) required for protein labeling was purchased from Cambridge Isotope Laboratories, Inc (Andover, MA, USA). The other reagents, including Sodium chloride (NaCl), 2-(n-morpholino) ethanesulfonic acid (MES), Acrylamide:Bis (37.5:1) 40% solution, TEMED, a 20% sodium dodecyl sulfate (SDS) solution, ammonium persulfate (APS), Isopropyl-β-D-Thiogalactopyranoside (IPTG), an ethidium bromide (EtBr) solution, Coomassie brilliant blue R-250, Kanamycin sulfate and Agarose GPG/LE, etc. were purchased from the AMERICAN BIOANALYTICAL (Natic, MA, USA) company. The restriction enzyme and ligation kit (DNA Ligation Kit Ver.2.1) for cloning were manufactured by Takara Korea Company (Seoul, Korea). The primer needed for PCR was obtained from Bioneer (Daejun, Korea). Ni-NTA agarose and the PCR purification kit (QIAquick® PCR purification Kit) were manufactured by QIAGEN (Valencia, CA, USA). The GB1 fusion vector was obtained from Dr. Gerhard Wagner at Harvard Medical School (Boston, MA, USA). The Escherichia coli host Rosetta (DE3) used to clone gene expression was acquired from Novagen (Madison, WI, USA).
2-2. Cloning of type 1 IP₃R L3-2 gene

The gene of the IP₃R L3-2 (amino acid 2510~2529) region was amplified from a bovine type1 IP₃R gene using PCR. The IP₃R L3-2 gene was pre-denatured for 5 minutes at 95°C, and then denatured for 1 minute at 95°C. It was annealed for 1 minute at 55°C and extended for 1 minute at 72°C. This process was repeated 33 times. Finally, it was extended for 10 minutes at 72°C. In this process, the sense primer has a GACAAGGGATCC ATGTGCTCAGAAGCCATCCAAAG sequence, and the antisense primer has a CATAGATCAGCTGAGCAGCAAAGAGG sequence.

The amplified PCR product was purified and digested with BamHI and XhoI, followed by a double digestion. The double digestion product ligated into the GB1 fusion vector (GB1–6X His). This combination of the restriction sites enables one to take advantage of the 6X–His tag, present in the original pET30a vector. The ligation product (GB1–L3-2–6X His) was transformed into competent Rosetta (DE3) E. coli. For transformation, the competent Rosetta (DE3) E. coli and ligated DNA were mixed at a ratio of 10 to 1 (40ul : 4ul) ratio, respectively. The mixed solution was put on ice for 5 minute, and then it was subjected to a heat shock for 30 seconds at 42°C and put on ice for 2 minutes. The mixture was mixed with 100ul LB media and allowed an outgrowth for 1 hour at 37°C. The cell was selected after incubation on the LB plate with 50µg/ml kanamycin at 37°C. For final confirmation, plasmids from the selected colonies were sequenced (Macrogen, Seoul, Korea).

2-3. Expression test of GB1-IP₃R-L3-2

To confirm whether there was a selected colony expression of GB1-IP₃R-L3-2, we did an expression test. The colony was inoculated to 300ul LB media with 50µg/ml of kanamycin. The colony was grown at 37°C until their OD₆₀₀ reached 0.4 and were expressed for 3 hours by the addition
of IPTG at a final concentration of 1mM. The cell was centrifuged at 16000xg for 5 minutes. The pellet was mixed with 75µl of distilled water (DW). Suspended cells were mixed with a 6X SDS-sample buffer 15µl, and then heated for 5 minutes at 100°C. The extent of expression of protein was evaluated by 18% SDS-PAGE electrophoresis.

2-4. Solubility test of GB1–IP₃R–L3–2

The cells confirmed in the expression test were tested for solubility. The recombinant cells were inoculated to 5ml LB media with 50µg/ml of kanamycin. The colony was grown at 37°C until their OD₆₀₀ reached 0.4 and were expressed for ~5 hours by the addition of IPTG at a final concentration of 1mM. The cells were centrifuged at 2800xg for 10 minutes. The pellet was mixed with a 600µl His-lysis buffer (50mM NaH₂PO₄, 300mM NaCl, 10mM imidazole, pH8.0). The suspended cells were lysed by sonication, for 10 seconds at 4°C. The lysed cell was centrifuged at 16000xg to obtain a soluble fraction. A insoluble fraction was obtained by suspension of the pellet with 600µl His-lysis buffer. Both the soluble fraction and the insoluble fraction were mixed with a 6X SDS-sample buffer (150µl) and then heated for 5 minutes at 100°C. The solubility extent of the sample was confirmed by 18% SDS-PAGE electrophoresis.

2-5. Expression and Isotope labeling of GB1–IP₃R–L3–2

The GB1–IP₃R–L3–2 cells were grown in LB or M9 media with 50µg/ml kanamycin. M9 media was used for isotope labeling. M9 media was composed of 0.1mM CaCl₂, 5.6mM glucose, 1mM kanamycin, 17.2mM K₂HPO₄, 1mM MgSO₄, 8.6mM NaCl, 56.7mM NaH₂PO₄, 18.7mM NH₄Cl, and 0.001% Thiamine. The ¹⁵N–labeled ammonium chloride and ¹³C–labeled glucose were used as sources to label nitrogen and/or carbon in amino acids. The GB1–IP₃R–L3–2 cells were grown at 37°C until their OD₆₀₀ reached 0.4, and to increase the
solubility level, the temperature was lowered to 20°C. After that, to initiate the expression, we added IPTG at a final concentration of 1 mM. When the OD<sub>600</sub> reached 1.4, the GB1-IP<sub>3</sub>R-L3-2 cells were harvested by centrifugation at 4427xg for 10 minutes. The pellet was frozen at −70°C.

2-6. Purification of GB1-IP<sub>3</sub>R-L3-2

The frozen pellet was thawed at 4°C and mixed with 20 ml of a His-lysis buffer (50 mM NaH<sub>2</sub>PO<sub>4</sub>, 300 mM NaCl, 10 mM imidazole, pH 8.0). Suspended cells were lysed by sonication three times for 3 minutes at 4°C. After sonication, the soluble fraction was obtained by centrifugation at 17000xg for 10 minutes at 4°C. The supernatant containing GB1-IP<sub>3</sub>R-L3-2 was incubated with Ni-NTA agarose resin for 10 minutes at 4°C. The resin holding GB1-IP<sub>3</sub>R-L3-2 was washed with 200 ml of a His-wash buffer (50 mM NaH<sub>2</sub>PO<sub>4</sub>, 300 mM NaCl, 20 mM imidazole, pH 8.0). Following the wash, the GB1-IP<sub>3</sub>R-L3-2 from the resin was eluted with 40 ml of a His-elution buffer (50 mM NaH<sub>2</sub>PO<sub>4</sub>, 300 mM NaCl, 250 mM imidazole, pH 8.0). The purity of the sample was confirmed by an 18% SDS-PAGE electrophoresis. The affinity-purified GB1-IP<sub>3</sub>R-L3-2 by Ni-NTA agarose was concentrated using an Amicon Ultra centrifugal filter with a 5000 MW cut-off (Amicon, Billeica, MA, USA) at 2800xg. The concentrated GB1-IP<sub>3</sub>R-L3-2 sample was buffer exchanged with 20 mM Tris-HCl, pH 7.5 buffer using a PD-10 desalting column (GE Healthcare, UK), and then the sample was purified by anion exchange chromatography with a Mono Q 5/50 GL column. The anion exchange chromatography was performed using a buffer of 20 mM Tris-HCl, pH 7.5 (A buffer) and 20 mM Tris-HCl, 1 M NaCl, pH 7.5 (B buffer). The sample was injected into the column pre-equilibrated with the A buffer. The sample was eluted with a gradient (0 to 25%) using the B buffer at 1 ml/min for 40 minutes. The peak of samples was monitored with absorbance at 280 nm and the fraction of the peak was analyzed by an 18% SDS-PAGE electrophoresis.
The fractions containing the GB1–IP3R L3–2 were pooled and concentrated by centrifugation at 2800xg using an Amicon Ultra centrifugal filter with a 5,000 MW cut-off (Amicon, Billeica, MA, USA).

2–7. Mass spectrometry
The mass sample of GB1–IP3R-L3–2 was dialyzed with DW to remove salt. Mass spectrometry was taken with the Voyager-DE-STR in linear mode (Applied Biosystems, Foster City, CA).

2–8. Chromogranin A, B peptide synthesis
The N-terminal peptide of chromogranins A and B, with the sequence of IVEVISDTLSKPSMPVSKE and IIEVLSNALLKSSAPPITPE, respectively, was synthesized using standard Fmoc chemistry at Peptron (Daejun, Korea). The tryptophan (W) was added to the N-terminal of each peptide, and because tryptophan has absorbance at 280nm, the quantity of the peptide can be measured with UV spectroscopy.

2–9. Purification of synthetic peptide
The synthetic peptide was purified by HPLC with a C18 reverse-phase column (Grace Vydac, Atlanta). The buffers used were A buffer: DW and B buffer: 80% acetonitrile (ACN). The peptide sample was injected into the column pre-equilibrated with the A and B buffers, and then was eluted for 50 minutes with gradients from 20% to 40% ACN at 1ml/min. The peak of the peptides was monitored with an absorbance at 280nm. The fraction of the peak was concentrated by a speed vac concentrator (Savant, Minnesota).
2–10. NMR spectroscopy

The $^{13}$C-labeled and/or $^{15}$N-labeled GB1–IP$_3$R–L3–2 sample was buffer exchanged with 50mM MES, pH 6.0 buffer using a dialysis tubing (Pierce, Rockford, IL, USA) and the purified peptide sample was solubilized in the same buffer. The concentration of the buffer exchanged GB1–IP$_3$R–L3–2 sample in the NMR spectroscopy was used ~200µM. The NMR sample was made by adding 5% D$_2$O for volume. For binding titration experiments, chromogranin peptides were added to the GB1–IP$_3$R–L3–2 sample at the specified molar ratio. First, the spectra were taken when only the GB1–IP$_3$R–L3–2 existed without the chromogranin peptide. Thereafter, the GB1–IP$_3$R–L3–2 spectra with the chromogranin peptide were taken by titration of the chromogranin peptide. The GB1–IP$_3$R–L3–2 spectrum was compared with spectra having the chromogranin peptide by overlapping.

The spectra were taken using a Varian 400 MHz machine equipped with a triple-resonance Z-gradient probe (collaborative instruments center of Inha University). For resonance assignments, HNCA, CB, and CBCA (CO)NH spectra were taken using a Bruker Avance 800 MHz machine equipped with a triple-resonance triple-axis gradient probe (Korea Basic Science Institute). All of the NMR data were processed with nmrPipe and analyzed with nmrVier software.
3. Results

3-1. Optimization of IP₃R L3-2 expression using GB1 vector

For NMR studies using proteins, how do we increase solubility and stability of the target proteins? These are important points. For these purposes, fusion tags, such as GST and MBP, have been used extensively to enhance the expression of soluble recombinant proteins. However, these tags were known to be large, which hinders direct NMR studies of the fusion proteins. In contrast the tag made by the protein in the GB1 domain was known to be especially suited for NMR studies of protein domains with molecular weight below 30KDa.

Figure 2. Schematic diagram of GB1 Vector. The GB1 pant is indicated in the lower diagram.
The protein of the GB1 domain consists of 56 amino acids. It is a highly stable and soluble molecule [26]. Therefore, we used the GB1 vectors to enhance solubility of IP₃R-L3-2.

The pET30-GB1 Fusion vector was constructed by cloning GB1 with a C-terminal His-tag into the pET30a (+) vector between NdeI and EcoRI sites. The IP₃R L3-2 target protein was inserted between BamHI and XhoI sites of GB1 vector (Fig. 2).

3-2. Expression test of GB1-IP₃R-L3-2

The expression level of the inserted IP₃R-L3-2 in the GB1 vector was measured by 1mM of IPTG induction at 37°C. Figure 3 shows the result of the fusion protein expressed in the Rosetta (DE3) E. coli.

![Figure 3. SDS-PAGE analysis of expressed GB1-IP₃R-L3-2 fusion protein in the Rosetta(DE3) E. coli. The black bars on the right are shown as molecular mass marker. The GB1–IP₃R–L3–2 band is indicated by an arrow.](image)
The target protein was expressed in the presence of 1mM IPTG, but is not in the absence of 1mM IPTG (Fig. 3). The molecular weight of the expressed target protein in the presence of 1mM IPTG was shown on the figure and matched well with the molecular weight determined by protein sequence analysis (Fig. 3 lane2).


The confirmed cells were investigated for the amount of soluble protein. The GB1–IP₃R–L3–2 fusion protein with 1mM of IPTG was expressed for about 5 hours at 37°C and the pellet and supernatant were analyzed on the 18% SDS-PAGE gel (Fig. 4).

Figure. 4 Solubility analysis of GB1–IP₃R–L3–2 fusion protein on the SDS–PAGE gel. GB1–IP₃R–L3–2 fusion protein was expressed by 1mM IPTG for 5hr at 37°C. The black bars on the right show molecular mass marker. The GB1–IP₃R–L3–2 band is indicated by an arrow.
As the soluble GB1–IP$_3$R–L3–2 fusion protein and insoluble GB1–IP$_3$R–L3–2 fusion protein existed at about 50:50 ratio, we lowered the expression temperature from 37°C to 20°C to further increase the amounts of soluble GB1–IP$_3$R–L3–2 fusion protein. There are reports that soluble fusion protein levels can be increased with IPTG amounts and different temperatures. Low temperatures (20°C) and lower IPTG (0.5mM) amounts increased the soluble fusion protein levels [27,28].


The GB1–IP$_3$R–L3–2 protein has a 6X–His tag. Therefore, GB1–IP$_3$R–L3–2 was purified by Ni–NTA agarose. The purified fusion protein from the affinity column has not only GB1–IP$_3$R–L3–2 but also has impurities. (Fig. 5A). Thus, we tried anion exchange chromatography to remove the impurities (~26KD). The peaks were separated by an increase of B buffer (20mM of Tris–HCl, 1mM of NaCl pH 7.5) concentration. The peak corresponding to GB1–IP$_3$R–L3–2 eluted at about 12 minutes on the Mono–Q chromatogram (Fig. 5B). Although the peak of GB1–IP$_3$R–L3–2 has an asymmetric shape, it showed very good separation. The peak of GB1–IP$_3$R–L3–2 was confirmed by 18% SDS–PAGE gel (Fig. 5C). The purified GB1–IP$_3$R–L3–2 fusion protein by Mono–Q much purer when compared with the GB1–IP$_3$R–L3–2 fusion protein from the affinity column, without any impurities (about 26KD).
Figure 5. Purification of GB1–IP₃R–L3–2. SDS–PAGE analysis of purified GB1–IP₃R–L3–2(A) by Ni–NTA affinity region and (C) by Mono–Q. The black bars on the left and right indicate molecular mass marker. The GB1–IP₃R–L3–2 band is indicated by an arrow. (B) Mono–Q anion exchange chromatogram. Dot line shown gradient of B buffer by time.
3–5. Mass spectrometry

To confirm the integrity of the purified fusion peptide by Mono-Q, we examined the molecular weight by MALDI-TOF mass spectrometry. The mass spectrum by MALDI-TOF spectrometry showed the molecular ion peak at 9749(M+H^+). The obtained 9749 value by mass was in good agreement with the calculated value of 9750 (Fig. 6). Also, the mass spectrum showed a 4875 peak corresponding to one half of the peak of 9749. The 4875 peak indicates a doubly charged ion peak of the GB1–IP₃R–L3–2 fusion protein.

![Figure 6. Mass spectrum of GB1–IP₃R–L3–2 by MALDI-TOF. The peak of 9,749 shows monomeric ion peak of GB1–IP₃R–L3–2. The peak of indicates doubly charged ion peak of GB1–IP₃R–L3–2.](image-url)
3–6. Purification of chromogranin A, B peptide by HPLC

The conserved N-terminal region of chromogranin A and B were synthesized. The synthesized peptides sometimes contain impurities. Therefore, we purified synthesized peptides using C18 reverse-phase chromatography. As shown in Figure 4, synthesized peptides of chromogranin A and B were separated from impurities by increasing the B buffer. The purified chromogranin A and B peptides were concentrated using a speed vac concentrator.

3–7. NMR spectroscopy

For the NMR titration experiment, GB1-IP\textsubscript{3}R-L3-2 was labeled with \textsuperscript{15}N, and then obtained by using a previously established preparation method. Several groups reported that fusion tags, such as GB1, can be used as reporters for the nativeness of the target proteins \cite{29,30}. Also, chemical shifts in the assignment of GB1 were reported \cite{31}. The NMR can be applied to structure, dynamics, protein folding, and ligand binding studies. Specifically, the protein folding and ligand binding can be easily confirmed by peak analysis of the spectrum. The folded protein shows a large dispersion on the spectrum. Whereas, unfolded protein shows a small dispersion on the spectrum. Also, when ligand is added to some proteins, the ligand binding can cause peak shifts or disappearance. Since the folding of fusion peptides by the GB1 tag can be easily confirmed by NMR, we recorded the HSQC spectrum of the GB1-IP\textsubscript{3}R-L3-2 (Fig. 8). Each peak of the spectrum indicates a single amino acid. Thus, the peaks of the spectrum shows both the GB1 amino acids and IP\textsubscript{3}R-L3-2 region amino acids. As shown in Figure 8, the native GB1 spectrum compared with the GB1 fused IP\textsubscript{3}R-L3-2 spectrum showed a clear distinction between the GB1 and L3-2 peaks.
Figure 7. Purification of chromogranin A and B synthetic peptides by HPLC. (A) Chromatogram of conserved N-terminal region of chromogranin A. (B) Chromatogram of conserved N-terminal region of chromogranin B. The arrow indicate peak of chromogranin A and B.
Figure 8. HSQC spectrum of GB1–IP$_3$R–L3–2 fusion protein. The GB1 peaks are shown in black blank circle shapes and IP$_3$R–L3–2 peak are shown in black filled circle shapes. The IP$_3$R–L3–2 includes 6X His Tag.
In addition, the good match of the GB1 peak in the two spectra showed that the GB1 region in the GB1–IP₃R–L3–2 fusion protein is folded natively and independently, showing that it does not interfere with the IP₃R–L3–2 region. The nativeness of GB1 indicated that the IP₃R–L3–2 region is also in the native environment. Nevertheless, the IP₃R–L3–2 region peak showed a very narrow dispersion, indicating the unfolded state compared with GB1. These results were consistent with the idea that the luminal loop region has an unfolded form. To test the nativeness of the IP₃R–L3–2 and confirm the previously reported interaction between the IP₃R–L3–2 and the chromogranin A, B peptide, we carried out NMR titration experiments.

When we added the chromogranin A peptide to GB1–IP₃R–L3–2, we could not observe any changes of GB1–IP₃R–L3–2 peaks. As shown in Figure 9, the peaks of GB1–IP₃R–L3–2 did not change at all when the chromogranin A peptide is added. These results indicates that chromogranin A peptide does not bind to the IP₃R–L3–2 loop. However, with the addition of the chromogranin B peptide, the GB1–IP₃R–L3–2 decreased the intensities of several IP₃R–L3–2 peaks, compared to those in GB1–IP₃R–L3–2 alone (Fig. 10). The result confirms the nativeness and the binding activity of the recombinant IP₃R–L3–2 for the chromogranin B peptide. To obtain deeper insights into the binding phenomena, we added chromogranin A and B peptides to the IP₃R–L3–2 at different ratios. We did not observe peak intensity changes in the IP₃R–L3–2 by chromogranin A peptide. However, we observed peak intensities changing in the IP₃R–L3–2 by chromogranin B peptide.
Figure 9. HSQC binding experiments between the GB1-IP$_3$R-L3–2 and the chromogranin A peptide. The spectrum with GB1-IP$_3$R-L3–2 only is shown in filled green circle shapes and that with added chromogranin A peptide is shown in red blank dotted circle shapes.
Figure 10. HSQC binding experiments between the GB1-IP$_3$R-L3-2 and the chromogranin B peptide. The spectrum with GB1-IP$_3$R-L3-2 only is shown in filled green circle shapes and that with added chromogranin B peptide is shown in red blank dotted circle shapes. The peaks corresponding to the IP$_3$R-L3-2 region are marked with asterisks.
Figure 11 shows that the intensities of the peaks of IP$_3$R-L3-2 mentioned in the Figure 10 decrease by an increased amount of the chromogranin B peptide. In contrast, the intensity decreases of the GB1 peaks and/or other peaks of the IP$_3$R-L3-2 were not observed (Fig. 11B). These findings indicate that the binding between the IP$_3$R-L3-2 and the chromogranin B peptide is specific for a part of the IP$_3$R-L3-2 region of the fusion peptide. The concentration-dependent decrease of the IP$_3$R-L3-2 peak intensities suggest that binding involves an intermediate time-scale exchange. Also, the decrease tendency of intensity among binding residues were fairly similar (Fig. 11A), which enabled us to estimate the overall Kd value by the law of mass action. The extracted value was 300µM. The Kd value agreed well with the previously reported value (480µM) [20].

Figure 11. Titration of the GB1-IP$_3$R-L3-2 with the chromogranin B peptide. (A) Peak intensities of the IP$_3$R-L3-2 binding residues. (B) Peaks intensities of the IP$_3$R-L3-2 non-binding residues and the GB1 residues.
Moreover, the relative signal intensities, back-calculated from the obtained Kd value, matched very well with the observed signal decay of the binding residue (a dotted thick line in Fig. 11A).

3-8. NMR spectral assignment

To identify the peaks of the GB1-IP-R-L3-2 that participated in binding, the GB1-IP-R-L3-2 was labeled with $^{15}\text{N}$ and $^{13}\text{C}$. Triple-resonance spectra were obtained using the HNCACB and CBCA (CO) NH spectra, we were able to assign all of the backbone amide resonance, except E10 (Table 1). The HNCACB strip plot shows the inter-residue connectivities (Fig. 12).

Figure 12. Strip plot of the HNCACB spectrum of IP-R-L3-2 part. Each strip represents that part of the $^{15}\text{N}$ plane corresponding to the indicated residues. Interresidue connectivities are indicated as dotted lines.
<table>
<thead>
<tr>
<th>Residue</th>
<th>HN</th>
<th>N</th>
<th>CA</th>
<th>CB</th>
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<td>122.605</td>
<td>51.558</td>
<td>38.414</td>
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<tr>
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<td>119.701</td>
<td>60.321</td>
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<tr>
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<td>52.654</td>
<td>39.509</td>
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<tr>
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<td>122.225</td>
<td>53.201</td>
<td>28.008</td>
</tr>
<tr>
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<td>124.746</td>
<td>51.558</td>
<td>29.651</td>
</tr>
<tr>
<td>6 Pro</td>
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<td>N/A</td>
<td>60.565</td>
<td>29.508</td>
</tr>
<tr>
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<td>116.811</td>
<td>55.392</td>
<td>61.417</td>
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<tr>
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</tr>
<tr>
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<tr>
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<td>N/D</td>
<td>N/D</td>
<td>N/D</td>
</tr>
<tr>
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<td>51.558</td>
<td>38.414</td>
</tr>
</tbody>
</table>

HN shifts were obtained from HSQC spectrum and the $^{15}$N, $^{13}$C chemical shifts were obtained from the HNCACB spectrum. N/D stands for non-detectable residue and N/A for non-existent proline amides.
The E10 resonance does not seem to show a peak on the HSQC spectrum, which is sometimes the case with a residue prior to a proline, due to the possible conformational heterogeneity. The analysis results indicated that the binding residue of the IP₃R-L3-2 for the chromogranin B peptide corresponded to residues 14–20. Therefore, we concluded that the IP₃R interacts with the chromogranin B peptide by 7 residues (14th–20th) of L3–2 loop. The sequential residues assignment also confirmed the identities of the peaks that we initially assigned to the L3–2 by simple comparison of the spectra of the GB1–IP₃R–L3–2 fusion protein with those of the GB1 alone.
4. Discussion

For protein studies, target protein production often confronts problems associated with expression, solubility level, and stability. In actuality, our initial production of the IP₃R with a 6X–His tag did not result in any expression, possibly due to the easy degradation of small peptides. Therefore, to enhance solubility and stability of target proteins, we selected the GB1 domain. Commonly, the tags, such as GST, MBP and Trx, are used to increase solubility and stability of proteins. The MBP and Trx tags are well-known to be the most effective solubilizing agent for large and small proteins, respectively, because they are very stable and can be expressed at high levels in E. coli [22]. But the MBP tag is large. Also, Trx tag can directly affect the target proteins. The GST, a known dimer in most physiological conditions, can induce non-native dimerization of the target proteins. Therefore, these tags suitable for NMR studies. However, the GB1 tag is very well-known as an independently folding unit and has favorable solubility-enhancing properties [26].

There have been reports that full length IP₃ receptors interact with chromogranin B in the native cellular environment [32]. Also, the L3–2 region of GST–fused IP₃ receptors was reported to bind to the chromogranin B peptide [20]. In the present study, we used the GB1–fused L3–2 and identified the binding residue of L3–2. In addition, the GB1 moiety is in the N-terminal part of our construct and the binding occurs in the C-terminal part of the L3–2. Therefore, we believe the presence of GB1 does not affect the binding much between the L3–2 and the chromogranin B peptide. Here, we have shown that the GB1 indeed folded independently of our target peptide, IP₃R–L3–2, and also does not interfere with IP₃R–L3–2. GB1 serves as an internal control for monitoring possible non–specific binding in NMR titration studies.
We showed that the GB1 residues were not affected by the chromogranin B peptide (Fig. 11B). Therefore, as previously reported [26], the fusion system by the GB1 tag should be a generally applicable technique in structural studies of poorly behaving and/or easily degraded peptides.

Figure 13. Schematic diagram to IP3R L3-2 loop. This figure magnifies pore region between TM5 and TM6. The L3-2 loop used in this study is shown as the amino acid codes. The square box indicates binding molecules to L3-2 loop.
According to previous studies, the chromogranin A peptide has a pH-dependent binding with L3–2 at pH 5.5, but not at pH 7.5, whereas chromogranin B peptide interacts at both pH’s [20,33]. We carried out binding experiments between the L3–2 loop and chromogranin A, B peptides under the same conditions (20mM MES pH 6.5 buffer). When chromgranin B peptide was added to the GB1–IP₃R–L3–2, we observed a change of the L3–2 peak on the GB1–IP₃R–L3–2 spectrum. These results indicate that chromogranin B peptide interacts with the L3–2 loop. However, when the chromgranin A peptide was added to the GB1–IP₃R–L3–2, we could not observe any changes on the spectrum. Therefore, our results were compatible with previously reported results, although the binding experiment was not carried out in the pH condition mentioned above. Both results indicate that the binding of the chromogranin A peptide to the L3–2 loop required a narrow range of around pH 5.5, and that the chromogranin B peptide can bind to the L3–2 loop over a wider range of pH.

The IP₃R has been shown to be present in secretory granules, endoplasmic reticulum and nucleus [34, 35]. The pH of secretory granule and ER (nucleus) is around pH 5.5 and around pH 7.4, respectively. Thus, our results suggest that both the chromogranin A and B peptide interact with the L3–2 loop in secretory granules to regulate the IP₃R, because both peptides interact with the L3–2 loop at around pH 5.5, and that the chromogranin B peptide specifically interacts with the 14th–20th residue of the L3–2 loop in ER and nucleus (Fig. 13).

It can be easily shown that, in diffusion-controlled molecular association, Kd of hundreds of micromolar range induces a fast exchange phenomenon of the NMR chemical shift time scale. For our L3–2 and chromgranin B peptide case, the 300µM of Kd would yield a k_{off} of 30000s⁻¹ and an average complex lifetime of 33us, which are within the fast exchange regime in the diffusion limit (k_{on}=10⁸ M⁻¹ s⁻¹) [36]. However, this fast exchange is not compatible with the observed peak intensity decrease, as the fast exchange
should cause a peak shift rather than disappearance. These analyses indicate that the IP$_3$R L3-2 and the chromogranin peptide binding cannot be explained by the simple diffusion-controlled association that assumes single-step rigid-body interaction [36]. Therefore, it might well require an additional step for finite-time conformation exchange. The suggestion of the conformational exchange is consistent with the previous thermodynamic analyses that the IP$_3$R and chromogranin binding is driven mainly by entropic changes and that the binding occurs even when the enthalpy change is positive [20]. By considering the extra time required for the conformational changes in the association process, we can readily explain the peak intensity decrease in the observed hundred- micromolar range binding. The ligand-concentration-dependent decrease of the peak intensities indicates that there is an intermediate time scale exchange, with the exchange rate being about the same as the chemical shift differences. With our experiments at 400MHz proton frequency, the exchange rate should be about several hundred times per second. Therefore, together with the experimentally measured Kd (300uM), the analysis suggests that the conformational change takes some time, resulting in the one hundred fold decreased association rate constant compared with that in the diffusion limit situation. The pore region of IP$_3$R is located in between the 5th and the 6th transmembrane helices. The L3-2 loop region is an essential part of pore form. As yet, not much is known about the luminal regulators of IP$_3$R. Therefore, the L3-2 loop may be a prime target for unidentified luminal regulators. As confirmed in this study using NMR, the L3-2 region is natively unfolded and only one third of the loop is involved in interaction with the chromogranin B peptide. Therefore, the rest of the part of the L3-2 loop is available for the binding of other intraluminal regulators. Also, the natively unfolded proteins can bind to many different molecules in the same part [37]. Our NMR–resonance assignments and peptide preparation approaches should contribute to elucidation of the complex luminal regulation of the IP$_3$R.
References


