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ORIGINAL PAPER

Inositol 1, 4, 5-trisphosphate receptor interacts with the SNARE domain of syntaxin 1B

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Abstract Inositol 1, 4, 5-trisphosphate receptors (IP_3Rs) are intracellular ligand-gated Ca^{2+} channels that mediate Ca^{2+} release from the endoplasmic reticulum (ER) into the cytosol and function in diverse cellular processes including fertilization, muscle contraction, apoptosis, secretion, and synaptic plasticity. The Ca^{2+} release activity of IP_3Rs is tightly regulated by many factors including IP_3R-binding proteins. We show that IP_3Rs interact with syntaxin 1 (Syx1), a membrane trafficking protein that regulates various plasma-membrane ion channels including N-, P/Q, and L-type voltage-gated Ca^{2+} channels, voltage-gated potassium channels, and an epithelial sodium channel. We found that a SNARE-domain of Syx1B, one of the two Syx1 isoforms, directly interacted with the type1 IP_3R (IP_3R1) internal coupling domain, a known modulator for channel opening. These results indicate that Syx1B is an IP_3R-interacting protein and that its interaction may play a crucial role in regulating the channel activity of IP_3Rs in Syx1B-expressing cells.

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Introduction

The inositol 1, 4, 5-trisphosphate receptors (IP_3Rs) are intracellular IP_3-gated Ca^{2+} release channels on the endoplasmic reticulum (ER). They play a critical role in the initiation and propagation of the cytosolic free-Ca^{2+} concentration in response to various physiological stimuli [1, 2]. Three distinct genes encoding three subtypes of IP_3Rs (types 1–3) have been identified in mammals [3, 4]. In the central nervous system, IP_3R1 is predominantly expressed in brain tissue and plays a critical role in the regulation of synaptic plasticity [5–7]. IP_3R1-knockout mice display motor control deficits including ataxia and seizure-like behavior [8]. The other two subtypes, types 2 and 3 IP_3Rs (IP_3R2 and IP_3R3), are co-expressed in various tissues. Although IP_3R2 and IP_3R3 double-knockout mice do not exhibit ataxia, they show severe deficits in saliva, gastric juice secretion [9], and nasal mucus [10] secretions, suggesting that IP_3R2 and IP_3R3 are important for exocrine secretion [2].

Mouse IP_3R1 is composed of 2,749 amino acids and consists of three domains (Fig. 1a): an N-terminal IP_3-binding domain, a C-terminal domain, and an internal coupling domain connecting these two domains [11, 12]. The C-terminal domain has subdomains, a six-membrane-spanning channel pore-forming domain and a short cytoplasmic gatekeeper domain. The Ca^{2+} release activity of the IP_3R channel is regulated by intracellular modulators (ATP, calmodulin, and Ca^{2+}), protein kinases, and IP_3R-binding proteins [2, 13], and the tight regulation of IP_3R channel activity by these factors underlies the spatio-temporal

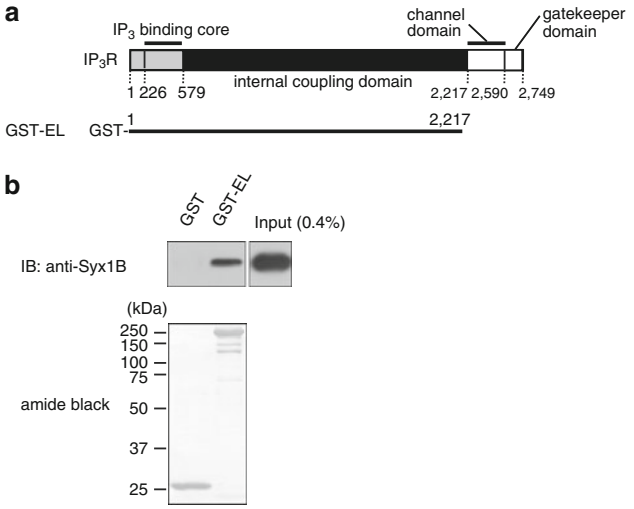

Fig. 1 Binding of Syx1B with IP_3R1N-terminal cytosolic region. **a** A schematic representation of the domain structure of IP_3R1 and its deletion mutant fused to GST (*GST-EL*). N-terminal IP_3-binding domain (amino acids 1–579); the internal coupling domain (amino acids 580–2,217); the C-terminal domain (amino acids 2,218–2,749). The *numbers* refer to amino acids. **b** GST pull-down assays with mouse brain microsomal lysate. The microsomal lysate was incubated with GST or GST-EL, and the eluates from the Glutathione–Sepharose 4B beads were analyzed by immunoblotting with anti-Syx1B. *Upper panel* immunoblot using anti-Syx1B, *lower panel* amido black staining indicating GST and GST-EL proteins

intracellular Ca^{2+} dynamics required for diverse cellular processes [14–20]. Therefore, further identification of IP_3R-binding proteins is crucial for elucidating the molecular mechanism of regulation of IP_3R-mediated Ca^{2+} dynamics and related cellular responses.

Syntaxin 1 (Syx1) is a membrane trafficking protein that regulates secretory vesicle exocytosis [21]. Membrane trafficking is mediated by soluble *N*-ethylmaleimide-sensitive fusion protein (NSF) attachment protein receptors (SNAREs) [22–24]. SNAREs were originally classified into two categories: vesicular SNAREs (v-SNAREs), which are localized in the donor membrane, and target SNAREs (t-SNAREs), which are localized in the acceptor compartment. The pairing of v- and t-SNAREs between two opposing membranes leads to the formation of a parallel alpha-helical bundle composed of four chains, called the SNARE complex, and subsequent membrane fusion [25–28]. Syx1 is the most characterized t-SNARE and forms the SNARE complex with the t-SNARE protein SNAP25 and the v-SNARE protein VAMP2 [27]. In Ca^{2+}-dependent exocytosis, Syx1 interacts with voltage-dependent Ca^{2+} channels (VDCCs), such as the N-, P/Q, and L-type Ca^{2+} channels [29–32], and regulates their channel activity, which is required for rapid and precise Ca^{2+}-dependent vesicle exocytosis [33–35]. Furthermore, recent studies have shown that Syx1 interacts with and regulates other plasma membrane ion channels such as voltage-gated potassium channels (Kv1.1 and Kv2.1) [36–38], an epithelial sodium channel (EnaC) [39–41], and a cyclic AMP-gated chloride channel [cystic fibrosis transmembrane conductance regulator (CFTR)] [42]. Thus, it is generally accepted that Syx1 regulates various plasma-membrane ion channels. However, whether Syx1 regulates the intracellular ion channel IP_3Rs remains to be elucidated. In the present study, to assess a potential Syx1 regulation of the intracellular ion channels, we investigated whether Syx1B binds to IP_3Rs.

Materials and methods

Animals

All animal experiments were performed in accordance with the RIKEN guidelines.

Cell culture

A rat adrenal pheochromocytoma cell line, PC12, was maintained in Dulbecco's modified Eagle's essential medium (DMEM) supplemented with 10% fetal bovine serum, 10% horse serum, 100 U/ml penicillin, and 100 μg/ml streptomycin (Nacalai Tesque, Saitama, Japan). Cells were maintained at 37°C in 5% CO_2.

Antibodies

A polyclonal antibody directed against Syntaxin1B (anti-Syx1B) was generated by immunizing rabbits with a synthetic Syx1B peptide (amino acid residues 171–187) fused with keyhole-limpet hemocyanin (KLH). The amino acid residues 171–187 of Syx1B were selected as the antigen. Other antibodies used in this study were goat anti-glutathione S-transferase (GST) antibody (GE Healthcare, Tokyo, Japan), rabbit anti-MBP antibody (New England Biolabs, Ipswich, MA, USA), mouse anti-IP_3R1 antibody KM1112, mouse anti-IP_3R2 antibody KM1083, and mouse anti-IP_3R3 antibody KM1082 [43].

Plasmid construction

To generate the Baculovirus transfer vectors encoding GST-fused IP_3R1 fragments, the GST-tag was cloned into the *Hin*dIII site of the pFastBac1 vector (designated pFB1-G). The enhanced green fluorescent protein (EGFP)-IP_3R1/pcDNA3.1 zeo vector encoding full-length mouse IP_3R1 [44] was used as a template to amplify the deletion mutants of IP_3R1 by polymerase chain reaction (PCR) with the following oligonucleotides as primers. IP_3R1 (1–343): sense,

5′-GCGGAATTCATGTCTGACAAAATGTCGAG-3′ and antisense, 5′-CGCGAATTCCCTACTCCGAGATGCATC CT-3′; IP_3R1 (344–545): sense, 5′-GCGGAATTCTTGA GAAACGCGCAAGAAAA-3′ and antisense, 5′-CGCGAA TTCGCGCTGATCCCCCAGCTCCT-3′; IP_3R1 (546–750): sense, 5′-GCGGAATTCCATGCTCCTTTCAGACATAT-3′ and antisense, 5′-CGCGAATTCCAGGTACTGGCGGTC CAGAC-3′; IP_3R1 (751–915): sense, 5′-GCGGAATTCGC CATCAATGAAATCTCCGG-3′ and antisense, 5′-CGCG AATTCAGACCTCATCACGTTACTGC-3′; IP_3R1 (1,593–1,800): sense, 5′-GCGGAATTCTCCAGAGACTACCGAA ATAT-3′ and antisense, 5′-CGCGAATTCTCACTTGTC GAGGTGACACTGAA-3′; IP_3R1 (1,801–2,000): sense, 5′-GCGGAATTCGAGGGGGCCTCCAACCTGGT-3′ and antisense, 5′-CGCGAATTCTCAACACACCAAATTGT AGTTGG-3′; and IP_3R1 (2,001–2,217): sense, 5′-GCG GAATTCGAGACACTGCAGTTTCTGGA-3′ and antisense, 5′-CGCGAATTCTCAGAATTCACAGATGCTGG GCA-3′. The fragments of IP_3R1 (916–1,581) and IP_3R1 (1,593–2,217) were obtained by digesting vectors GST-IIIab/pGEX4T-1 or GST-IV-Va/pGEX4T-1 [17] with *Eco*RI/*Sal*I or *Eco*RI, respectively. These fragments were finally subcloned into pFB1-G.

The cDNA encoding mouse Syx1B (NCBI Accession ID; NM_024414) was amplified by PCR from a mouse hippocampus cDNA library using the following primers: sense, 5′-GCGGAATTCGATGAAGGATCGGACTCAG-3′ and antisense, 5′-CGCGAATTCCTACAAGCCCAGTGT CCC-3′. The resulting fragment was subcloned into mammalian expression vector pEGFP-C1 (Invitrogen, Tokyo, Japan) (designated EGFP-Syx1B). To generate the *Escherichia coli* expression vectors encoding the deletion mutants of Syx1B fused with maltose-binding protein (MBP) (designated MBP-Syx1B-Habc, MBP-Syx1B-SNARE, and MBP-Syx1B-dTMD), the Syx1B fragments were amplified by PCR using EGFP-Syx1B as a template, and the resulting fragments of Syx1B were subcloned into the *Eco*RI site of the pMAL-c plasmid. The following primers were used to amplify each Syx1B fragment. Syx1B-Habc: sense, 5′-CGGAATTCAAGGATCGGACT CAGGAGCT-3′ and antisense, 5′-CGGAATTCTCAGCG GTCCCGGTACTTGGAC-3′; Syx1B-SNARE: sense, 5′-C GGAATTCGATATCAAAATGGACTCGCA-3′ and antisense, 5′-GCGGAATTCTCAAATTTTCTTCCTCCTGG CCT-3′; and Syx1B-dTMD: sense, 5′-CGGAATTCAAG GATCGGACTCAGGAGCT-3′ and antisense, 5′-GCGG AATTCTCAAATTTTCTTCCTCCTGGCCT-3′.

Recombinant protein purification

GST-fused IP_3R1 fragments were produced using the Sf9-Baculovirus overexpression system [17]. First, recombinant baculoviruses were generated using the pFB1-G plasmids described above with the Bac-to-Bac Baculovirus Expression System (Invitrogen). Sf9 cells were infected with the resultant baculoviruses, and the proteins produced were purified using glutathione–Sepharose 4B (GE Healthcare). MBP-fused Syx1B deletion mutants were expressed in *E. coli* JM109 and purified using amylose resin (New England Biolabs). Purified proteins were dialyzed using a cytosol-like medium [CLM; 50 mM HEPES–KOH (pH 7.2), 110 mM KCl, 10 mM NaCl, 5 mM KH_2PO_4, and 1 mM 2-mercaptoethanol]. Concentrations of purified recombinant proteins were measured with a Protein Assay Kit (BioRad, Tokyo, Japan) using bovine serum albumin (BSA) as a standard.

Subcellular fractionation

Adult brain tissues were isolated from postnatal day 56 ICR mice and were homogenized in homogenization buffer [0.32 M sucrose, 5 mM HEPES–KOH (pH 7.4), 100 μM PMSF, and a Complete Protease Inhibitor Cocktail Tablet (EDTA-free) (Roche, Tokyo, Japan)] by a glass-Teflon homogenizer. Homogenates were centrifuged at 800*g* for 10 min at 4°C, and the resulting supernatants were centrifuged at 100,000*g* for 1 h at 4°C. The pellet was collected as the crude microsomal fraction.

Binding experiments

For the pull-down assays, microsomal fractions were solubilized in pull-down buffer [20 mM Tris–HCl (pH 7.4), 100 mM NaCl, 2 mM Na_3VO_4, 10 mM NaF, 2 mM EGTA, 0.5% Triton-X100, and a Complete Protease Inhibitor Cocktail Tablet (EDTA-free) (Roche)] for 2 h at 4°C. The insoluble fraction was precipitated by centrifugation at 20,400*g* for 15 min at 4°C. The supernatant was incubated with 40 μl of 50% glutathione–Sepharose 4B bead slurry for 2 h at 4°C. The mixture was centrifuged at 1,500*g* for 20 s at 4°C, and the supernatant was used as the precleared microsomal lysate. Recombinant GST-fused IP_3R1 fragments or MBP-Syx1B fragments (3.8×10^{-10} mol, an equal amount to 10 μg GST) were diluted in 1 ml of CLM and incubated with glutathione–Sepharose 4B or amylose resin beads for 1–2 h at 4°C. Beads were washed three times with pull-down buffer and mixed with the precleared microsomal lysate, and this mixture was agitated by a rotary shaker for 1–2 h at 4°C. The beads were washed 3 times with pull-down buffer, and the bound proteins were analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting.

In the direct binding assay, 3.8×10^{-10} mol of GST-fused IP_3R1 fragments and MBP-fused Syx1B fragments were incubated in CLM containing 1% TritonX-100 for 12 h at 4°C, followed by incubation for another 2 h at 4°C

after the addition of 20 μL of a 50% suspension of amylose resin beads (New England Biolabs). The bound proteins were analyzed by SDS-PAGE and immunoblotting as described above.

For immunoprecipitation experiments, crude microsomal fractions were solubilized in lysis buffer A [20 mM Tris–HCl (pH 7.0), 100 mM NaCl, 2 mM Na_3VO_4, 10 mM NaF, 1% TritonX-100, and a Complete Protease Inhibitor Cocktail Tablet (EDTA-free) (Roche)] for 1–2 h at 4°C. The homogenate was centrifuged at 20,400*g* for 15 min at 4°C to remove the insoluble fraction. The supernatant was diluted using a dilution buffer [20 mM Tris–HCl (pH 7.0), 100 mM NaCl, 2 mM Na_3VO_4, 10 mM NaF, and a Complete Protease Inhibitor Cocktail Tablet (EDTA-free) (Roche)] to lower the TritonX-100 concentration to 0.5% and was then precleared with Protein G-Sepharose 4 Fast Flow (GE Healthcare) for 1–2 h at 4°C.

For immunoprecipitation using whole-cell lysate, the cells were first suspended in Lysis buffer B [20 mM HEPES–NaOH (pH 7.4), 100 mM NaCl, 2 mM EGTA, 0.1% TritonX-100, and a Complete Protease Inhibitor Cocktail Tablet (EDTA-free)] and solubilized by mild agitation for 1–2 h at 4°C. The cell lysates were then homogenized by 10 passages through a 27-gauge needle and centrifuged at 20,000*g* for 30 min at 4°C to remove the insoluble fraction. The supernatant was precleared by the addition of Protein G-Sepharose 4 Fast Flow (GE Healthcare), incubated with rabbit normal IgG (Chemicon, Temecula, CA, USA) or polyclonal anti-Syx1B antibody for 1–2 h at 4°C, and incubated with Protein G-Sepharose 4 Fast Flow beads for 1 h at 4°C. After washing the beads 3 times with IP buffer [20 mM Tris–HCl (pH 7.0), 100 mM NaCl, 2 mM Na_3VO_4, 10 mM NaF, 0.5% TritonX-100, a Complete Protease Inhibitor Cocktail Tablet (EDTA-free)

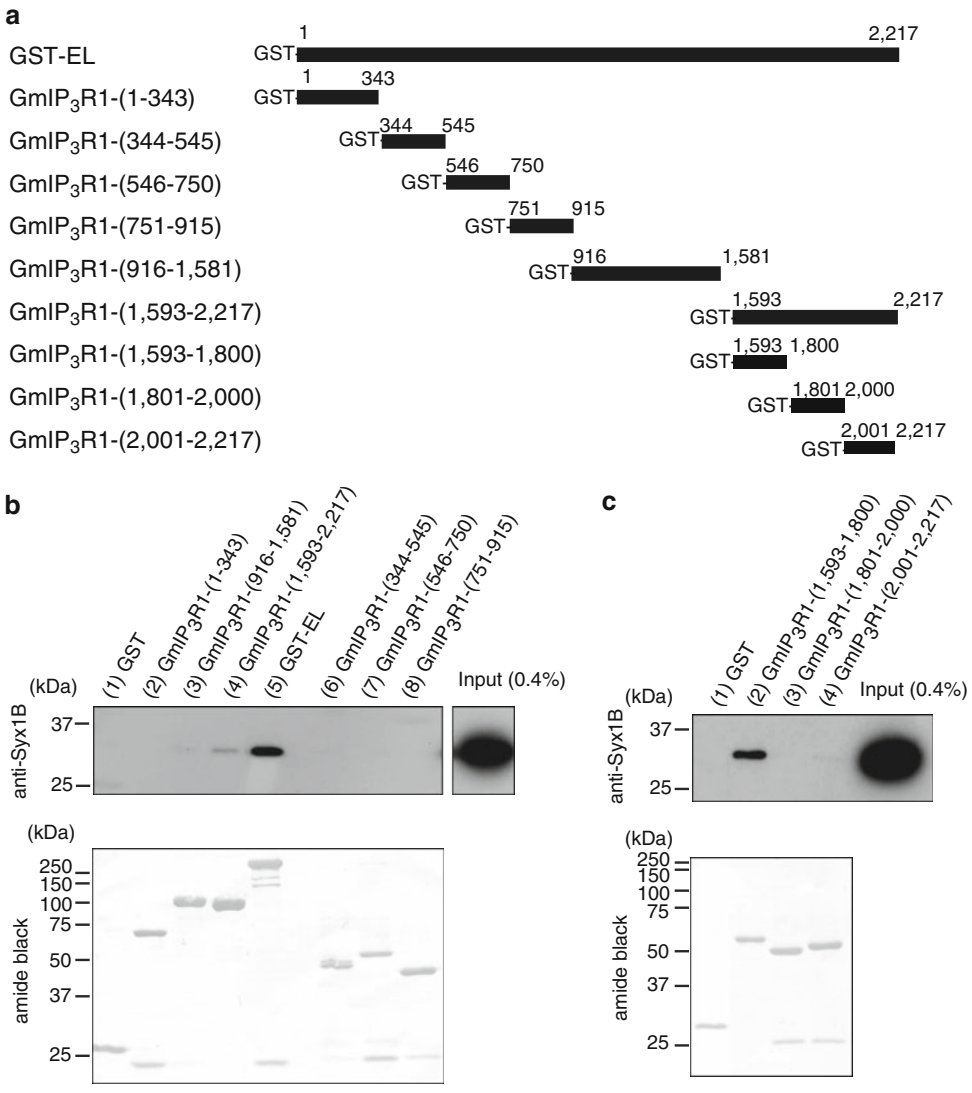

Fig. 2 Identification of the Syx1B-binding region in IP_3R1. **a** Schematic representation of GST-fused IP_3R1 deletion mutants. The various regions of the IP_3R1 were prepared as GST fusion proteins. The *numbers* refer to amino acids. **b**, **c** GST pull-down assays with mouse brain microsomal lysate. The microsomal lysate was incubated with each GST-fusion proteins immobilized on Glutathione–Sepharose 4B beads, and the bound proteins were analyzed by immunoblotting with anti-Syx1B. *Upper panels* in (**b**, **c**) immunoblot using anti-Syx1B; *lower panels* in (**b**, **c**) amido black staining indicating GST and GST-fused IP_3R1 deletion mutants

(Roche)] or lysis buffer B, the bound proteins were analyzed by SDS-PAGE and immunoblotting.

Immunoblotting

Proteins were separated by SDS-PAGE and transferred to polyvinylidene fluoride (PVDF) membrane (Millipore, Bedford, MA, USA) by electroblotting. The membrane was blocked with 5% skim milk for 1 h at room temperature and then incubated with primary antibody diluted in 5% skim milk at the appropriate dilution (anti-Syx1B, 1: 2000; KM1112, 1:1,000; KM1083, 1:1,000; KM1082, 1:1,000; anti-GST (GE Healthcare), 1:2,000; and anti-MBP (New England Biolabs), 1:3,000) for 2–3 h at room temperature. After washing 3 times with PBS containing 0.1% Tween 20 (PBS-T), the membrane was incubated with secondary antibody conjugated with horseradish peroxidase (Jackson ImmunoResearch, West Grove, PA, USA) for 1 h at room temperature or overnight at 4°C. After washing 3 times with PBS-T, immunoreactive protein bands were detected with chemiluminescence using ECL or ECL Plus (GE Healthcare).

Coiled-coil prediction

Amino acid sequences were analyzed using the second structure prediction program which was developed by Lupas et al. [45]. The window size chosen was 21.

Results

IP_3Rs consist of an N-terminal IP_3-binding domain, an internal coupling domain, a C-terminal domain including a six-membrane spanning channel pore-forming subdomain, and a C-terminal gatekeeper subdomain [11, 12] (Fig. 1a). To investigate the interaction of IP_3Rs with Syx1B, we first performed a GST pull-down assay using mouse brain microsomal lysate and recombinant GST-EL containing the long N-terminal cytosolic region (amino acid residues 1–2,217) of IP_3R1 [16] (Fig. 1a). Syx1B co-precipitated with GST-EL but not with GST alone (Fig. 1b). We further explored the Syx1B-binding site in the N-terminal region of IP_3R1 using various GST-fused IP_3R1 deletion mutants (Fig. 2) and found that Syx1B co-precipitated with GST-fused mutants containing amino acid residues 1,593–2,217 of IP_3R1 (Fig. 2b, lanes 4, 5). We next used three GST-fused IP_3R1 mutants containing amino acid residues 1,593–1,800, 1,801–2,000, or 2,001–2,217 and found that Syx1B specifically co-precipitated with GST-fused mutant 1,593–1,800 (Fig. 2c, lane 2). Syx1 possesses a single C-terminal transmembrane domain (TMD, amino acid residues 266–288), a SNARE domain (known as H3, amino acid residues 189–265), an N-terminal regulatory domain (Habc, amino acid residues 1–143), and a linker region (amino acid residues 144–188) connecting the SNARE and Habc domains [46] (Fig. 3a). We prepared three MBP-fused Syx1B mutants containing amino acid residues 1–143 (Habc), 179–265 (SNARE), or 1–265 (dTMD). To investigate the IP_3R1-binding region in Syx1B, we performed the MBP pull-down assay using mouse brain lysate and MBP-fused Syx1B deletion mutants (Fig. 3a). IP_3R1 co-precipitated with MBP-Syx1B-SNARE but not with MBP alone (Fig. 3b). IP_3R1 that co-precipitated with MBP-Syx1B-dTMD was weakly detected by immunoblotting after a long exposure (Fig. 3b). To test if this interaction occurs between purified proteins, we performed the MBP pull-down assay with purified GST-IP_3R (1,593–2,217) and MBP-fused Syx1B-deletion mutants. GST-IP_3R (1,593–2,217) co-precipitated with MBP-Syx1B-SNARE and -dTMD, but not

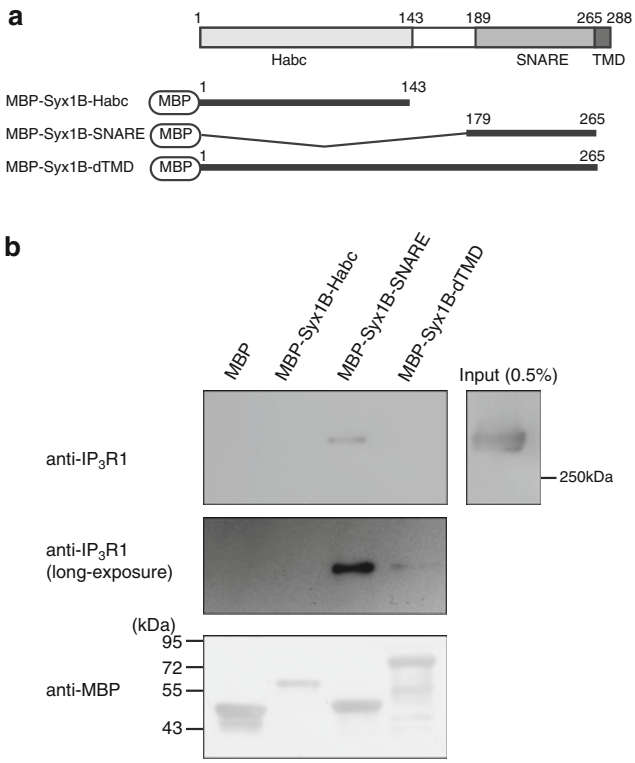

Fig. 3 Identification of IP_3R1-binding region in Syx1B. **a** Schematic representation of the domain structure of Syx1B and its deletion mutants fused to MBP. N-terminal regulatory domain (Habc, amino acids 1–143), the linker region (amino acids 144–188), the SNARE domain (amino acids 189–265), and the single C-terminal transmembrane domain (TMD, amino acid residues 266–288). The *numbers* refer to amino acids. **b** MBP pull-down assays with mouse brain microsomal lysate. The microsomal lysate was incubated with MBP or MBP-fused Syx1B deletion mutants, and the eluates from the amylose resin beads were analyzed by immunoblotting with anti-IP_3R1. Immunoblots with short exposure (*upper panel*) and long exposure (*middle panel*) using anti-IP_3R1, *lower panel* immunoblot using anti-MBP to detect MBP and MBP-fused Syx1B deletion mutants

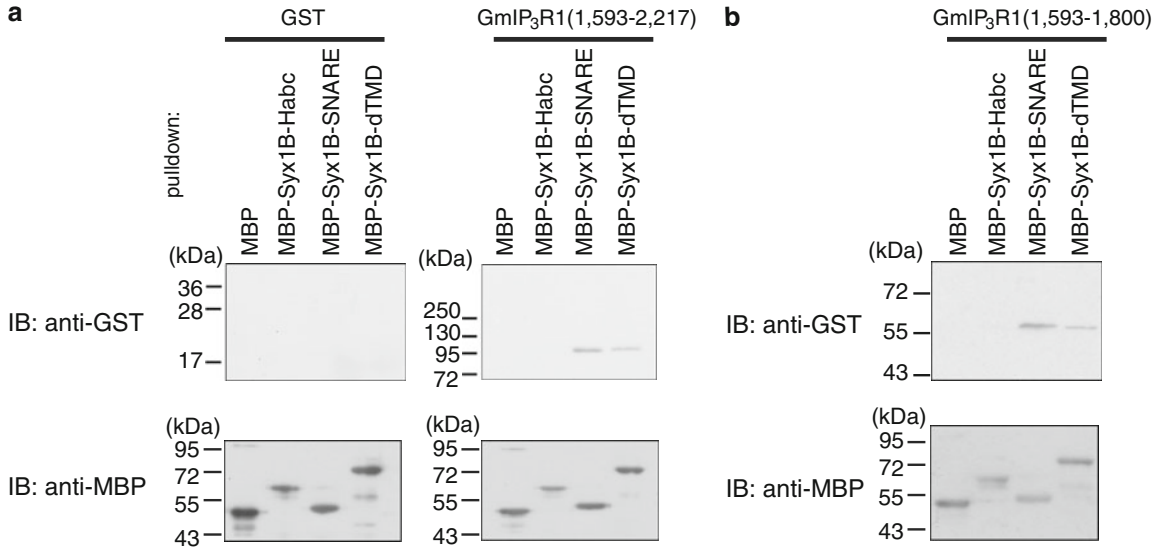

Fig. 4 Direct binding of IP_3R1 with Syx1B. **a**, **b** Indicated recombinant GST-IP_3R1 mutants were incubated with the indicated MBP-Syx1B mutants, and the eluates from the amylose resin beads were analyzed by immunoblotting with anti-GST antibody (*upper panel*) or with anti-MBP antibody (*lower panel*)

with MBP alone (Fig. 4a). We next tested the interaction between GST-IP_3R (1,593–1,800) and MBP-fused Syx1B mutants. As a result, GST-IP_3R (1,593–1,800) was also co-precipitated with MBP-Syx1B-SNARE and -dTMD (Fig. 4b). These results indicated that Syx1B directly interacts with IP_3R1. Next, we examined whether Syx1B binds to IP_3R1 in vivo. Immunoprecipitation experiments using anti-Syx1B demonstrated that brain IP_3R1 co-immunoprecipitated with Syx1B (Fig. 5). The protein sequence of mouse IP_3R1 is highly homologous to that of mouse IP_3R2 (approximately 70.9% identity) and IP_3R3 (approximately 65.2% identity) [4]. As expected, we found that IP_3R2 and IP_3R3 co-immunoprecipitated with Syx1B (Fig. 5), suggesting that all IP_3R isoforms form a complex with Syx1B. PC12 cells express IP_3R1, IP_3R3 [47–49], and Syx1B [50]. We performed an immunoprecipitation assay to examine whether endogenous IP_3Rs interact with Syx1B in PC12 cells. Both IP_3R1 and IP_3R3 co-immunoprecipitated with Syx1B (Fig. 6), indicating that Syx1B interacts with IP_3R1 and IP_3R3 in PC12 cells.

These results suggested the existence of common structural feature of Syx1B-binding region among all IP_3Rs. We found the predicted coiled-coil domain in the first 28 amino acids of Syx1B-binding region of IP_3R1 (Fig. 7). In addition, this predicted coiled coil domain in this region was conserved both in IP_3R2 and in IP_3R3 (Fig. 7).

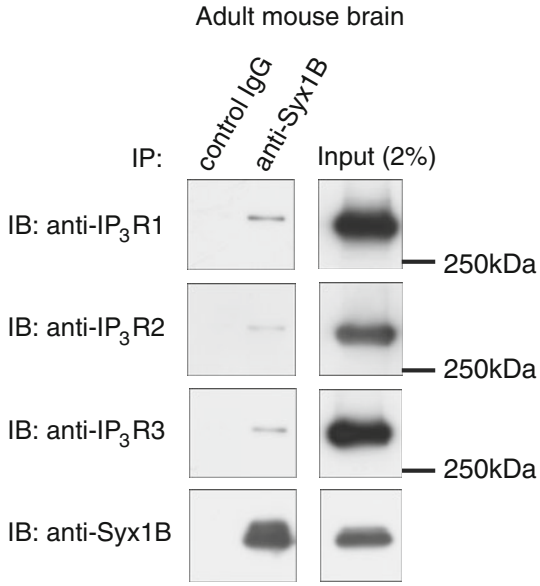

Fig. 5 IP_3R1 binds with Syx1B in vivo. Immunoprecipitation of endogenous proteins from mouse brain microsomal lysate using anti-Syx1B. The precipitates were analyzed by western blotting using anti-Syx1B, anti-IP_3R1 (KM1112), IP_3R2 (KM1083), and IP_3R3 (KM1082) isoform specific antibodies

Discussion

Syx1 binds to plasma membrane ion channels directly and regulates their channel activity [34–43]. In the present study, we demonstrated for the first time that Syx1B binds to internal Ca^{2+} channel IP_3Rs. The Syx1B-binding site was located within amino acid residues 1,593–1,800 of the internal coupling domain of IP_3R1 (Fig. 3). Many IP_3R-binding proteins have been isolated [16–18, 20, 51], but no reports have described binding proteins that demonstrate specific interaction with amino acid residues 1,593–1,800 of IP_3R1. Although this region of IP_3R1 is not highly

conserved in IP_3R2 (approximately 41.8% identity) and IP_3R3 (approximately 39.9% identity), the first 28 amino acid residues of this region in all IP_3Rs were predicted to form the coiled-coil structure (Fig. 7), which has been generally recognized as an important domain for protein–protein interaction [45]. In addition, the first 28 amino acid residues of IP_3R1 are highly conserved in IP_3R2 (approximately 57.1% identity) and IP_3R3 (approximately 71.4% identity) [4]. Because Syx1B can interact with all IP_3Rs (Fig. 5), these results suggest that the amino acid residues (1,593–1,620) of IP_3R1 are important for specific binding with Syx1B. We previously showed that inhibition of IP_3R1 channel opening by a high concentration of Ca^{2+} is mediated by calmodulin (CaM) in vitro [14, 15]. CaM associates with amino acid residues 1,564–1,585 of IP_3R1

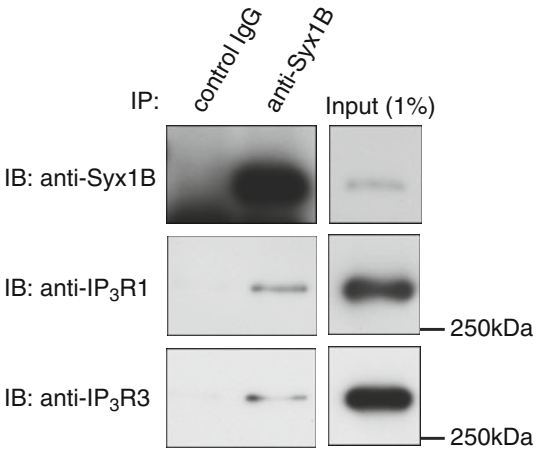

Fig. 6 IP_3R1 binds to Syx1B in neuroendocrine cell line PC12. Whole cell lysates were subjected to immunoprecipitation by anti-Syx1B. The precipitates were analyzed by western blotting using anti-Syx1B, anti-IP_3R1 (KM1112), and anti-IP_3R3 (KM1082) antibodies

[52, 53], which are located just upstream of the region of the Syx1B-binding site that we found in this study. Thus, the further investigation of the relationship between Ca^{2+}/CaM-dependent channel inhibition and Syx1B-binding is of interest.

The SNARE domain of Syx1 reportedly binds to both SNAP25 and VAMP2 to form the SNARE complex, which is believed to drive the fusion of the vesicle and plasma membranes [54]. In addition, the SNARE domain of Syx1 interacts with VDCCs, resulting in the formation of the "Ca^{2+} microdomain" required for coupling depolarization-dependent Ca^{2+} influx to trigger exocytosis [55]. Previous studies reported that anti-Syx1 antibody, which reacted against Syx1A and Syx1B, raised immunoreactive signals mainly on the plasma membrane and synaptic vesicles of presynaptic terminals around the dendrites of Purkinje cells [56, 57]. In addition, another study suggested that Syx1B, not Syx1A is predominant isoforms expressed in cerebellum [58] These reports suggested that Syx1B is localized predominantly on the plasma membrane and synaptic vesicles, although a small amount is localized on ER, nuclear membranes in granule cells [56]. We showed that the SNARE domain of Syx1B interacts with IP_3Rs (Figs. 3, 4). These findings raise the possibility that the Stx1B-IP_3Rs complex makes a bridge between the ER and plasma membranes, resulting in the formation of the Ca^{2+} microdomain required for coupling IP_3-induced Ca^{2+} release to trigger exocytosis. In fact, IP_3-induced Ca^{2+} release from IP_3R1 is known to trigger dopamine secretion in response to bradykinin in neuroendocrine PC12 cells [49], where IP_3R1 and IP_3R3 were found to interact with Syx1B (Fig. 6). Whether IP_3Rs interacts with the SNARE complex to form the Ca^{2+} micro-domain remains to be determined in a future study. We have previously shown

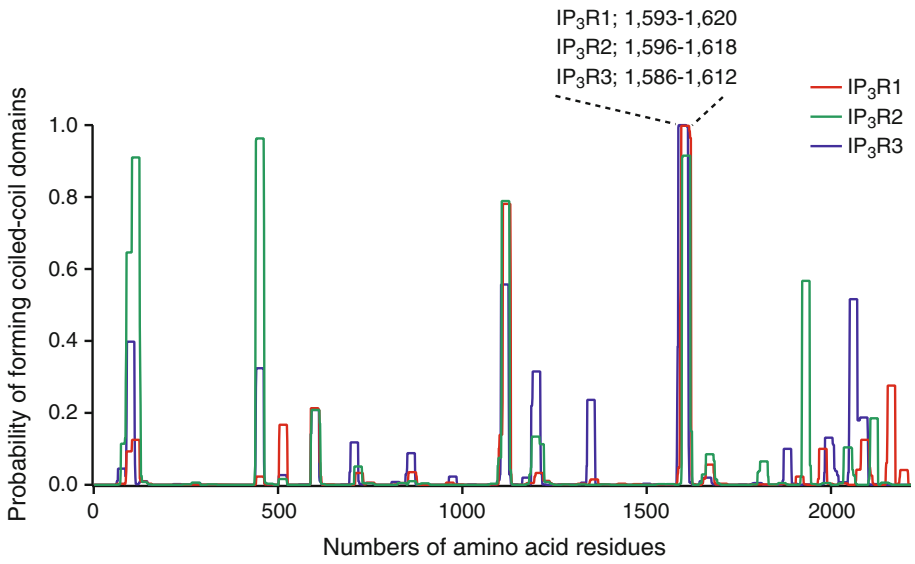

Fig. 7 Predicted coiled coil domain in Syx1B-binding region of IP_3Rs. Probability of coiled-coil domains for the whole length of the N-terminal cytoplasmic region of each IP_3Rs was predicted using the method developed by Lupas et al. [45]. The *numbers* indicated are amino acid residues of each IP_3Rs

that enhanced paired-pulse facilitation and enhanced post-tetanic potentiation are observed in the hippocampus of IP_3R1-knockout mice [7, 59], suggesting that the presynaptic neurotransmitter mechanism is altered by the absence of IP_3R1. Further studies that focus on the IP_3Rs, Syx1B, and the SNARE complex may accelerate our understanding of the molecular mechanism underlying IP_3R-mediated synaptic plasticity.

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Conflict of interest The authors declare that they have no conflict of interest.

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