IRBIT, a Novel Inositol 1,4,5-Trisphosphate (IP$_3$) Receptor Binding Protein, Is Released from the IP$_3$ Receptor upon IP$_3$ Binding to the Receptor

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1 The abbreviations used are: IP$_3$, inositol 1,4,5-trisphosphate; IP$_3$R, inositol 1,4,5-trisphosphate receptor; IP$_3$R1, type 1 inositol 1,4,5-trisphosphate receptor; mGluRs, group 1 metabotropic glutamate receptors; B$_2$Rs, B$_2$ bradykinin receptors; IRBIT, IP$_3$R binding protein released with inositol 1,4,5-trisphosphate; 2-ME, 2-mercaptoethanol; GST, glutathione S-transferase; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; His, hexahistidine; GFP, green fluorescent protein; IP$_4$, inositol 4,5-bisphosphate; IP$_4'$, inositol 1,3,4,5-tetrakisphosphate; IP$_6'$, inositol 1,2,3,4,5,6-hexakisphosphate; PBS, phosphate-buffered saline; FRET, fluorescence resonance energy transfer
SUMMARY

The inositol 1,4,5-trisphosphate (IP$_3$) receptors (IP$_3$Rs) are IP$_3$-gated Ca$^{2+}$ channels on intracellular Ca$^{2+}$ stores. Herein, we report a novel protein, termed IRBIT (IP$_3$R binding protein released with inositol 1,4,5-trisphosphate), which interacts with type 1 IP$_3$R (IP$_3$R1) and was released upon IP$_3$ binding to IP$_3$R1. IRBIT was purified from a high salt extract of crude rat brain microsomes with IP$_3$ elution using an affinity column with the huge immobilized N-terminal cytoplasmic region of IP$_3$R1 (residues 1-2217). IRBIT, consisting of 530 amino acids, had a domain homologous to S-adenosylhomocysteine hydrolase in the C-terminal and, in the N-terminal, a 104 amino acid appendage containing multiple potential phosphorylation sites. In vitro binding experiments showed the N-terminal region of IRBIT to be essential for interaction and the IRBIT binding region of IP$_3$R1 was mapped to the IP$_3$-binding core. IP$_3$ dissociated IRBIT from IP$_3$R1 with an EC$_{50}$ of ~0.5 µM, i.e. it was 50 times more potent than other inositol polyphosphates. Moreover, alkaline phosphatase treatment abolished the interaction, suggesting that the interaction was dualistically regulated by IP$_3$ and phosphorylation. Immunohistochemical studies and co-immunoprecipitation assays showed the relevance of the interaction in a physiological context. These results suggest that IRBIT is released from activated IP$_3$R, raising the possibility that IRBIT acts as a signaling molecule downstream from IP$_3$R.
INTRODUCTION

The hydrolysis of phosphatidylinositol 4,5-bisphosphate in response to cell surface receptor activation leads to the production of an intracellular second messenger, inositol 1,4,5-trisphosphate (IP$_3$). IP$_3$ mediates the release of Ca$^{2+}$ from intracellular Ca$^{2+}$ storage organelles, mainly the endoplasmic reticulum, by binding to its receptor (IP$_3$R). In these IP$_3$/Ca$^{2+}$ signaling cascades, IP$_3$R works as a signal converter from IP$_3$ to Ca$^{2+}$ (1-3).

IP$_3$R is a tetrameric intracellular IP$_3$-gated Ca$^{2+}$ release channel (3, 4). There are three distinct types of IP$_3$R in mammals (5-7). Type 1 IP$_3$R (IP$_3$R1) is highly expressed in the central nervous system, particularly in the cerebellum (8, 9). Mouse IP$_3$R1 is composed of 2749 amino acids (5), and is divided into three functionally distinct regions: the IP$_3$-binding domain near the N terminus, the channel-forming domain with six membrane-spanning regions close to the C terminus, and the regulatory domain separating the two regions (10, 11). Deletion mutagenesis analysis of the IP$_3$-binding domain has shown that residues 226-578 of IP$_3$R1 are close to the minimum for specific and high affinity ligand binding, thus assigned to the IP$_3$ binding core (12). The precise gating mechanism of IP$_3$R triggered by IP$_3$ remains unclear, but IP$_3$ binding induces a substantial but as yet undefined conformational change, which may cause channel opening (10). Besides this channel opening, such IP$_3$-induced conformational change has been assumed to be responsible for degradation of IP$_3$R (13, 14).

The increase in the cytoplasmic Ca$^{2+}$ concentration resulting from IP$_3$R activation regulates the activities of thousands of downstream targets that play key roles in many aspects of cellular processes, including fertilization, development, proliferation, secretion and synaptic plasticity (1, 2, 15). To control such a vast array of cell functions, Ca$^{2+}$ signals need to be precisely regulated in terms of space, time
and amplitude (2, 15). Such a complex regulation of Ca\textsuperscript{2+} signals has been partly attributed to the diversity of IP\textsubscript{3}R isoform expression, assembly of heterotetrameric complexes of IP\textsubscript{3}R isoforms, subcellular distributions of IP\textsubscript{3}R, and regulation of IP\textsubscript{3}R by Ca\textsuperscript{2+} itself, ATP, and phosphorylation (3, 4, 16). IP\textsubscript{3}R channels are also regulated by their interacting proteins (4, 17), including calmodulin (18, 19), FKBP12 (20-22, but also see 23, 24), calcineurin (21, 25, but also see 23, 24), ankyrin (26-28), sigma-1 receptor (28), chromogranins A and B (29-31), IRAG (32), Fyn (33), and BANK (34). Moreover, a family termed CaBP has been shown to interact with IP\textsubscript{3}R in a Ca\textsuperscript{2+}-dependent manner, and to directly activate IP\textsubscript{3}R in the absence of IP\textsubscript{3} (35). IP\textsubscript{3}R has also been demonstrated to be physically coupled to its upstream or downstream signaling molecules by protein-protein interactions. For example, IP\textsubscript{3}R is coupled with group 1 metabotropic glutamate receptors (mGluRs) via the Homer family of proteins (36), and with B\textsubscript{2} bradykinin receptors (B\textsubscript{2}Rs) by an unknown mechanism (37). Activations of mGluRs and B\textsubscript{2}Rs lead to the production of IP\textsubscript{3} in proximity to IP\textsubscript{3}R, the result being efficient and specific signal propagation. Another example is Trp3, a candidate for plasma membrane Ca\textsuperscript{2+} channels regulated by intracellular Ca\textsuperscript{2+} store depletion (capacitative calcium entry channels). IP\textsubscript{3}R has been shown to interact with Trp3 directly, and to activate it via a conformational coupling mechanism (38, 39). These protein-protein interactions are supposed to regulate the IP\textsubscript{3}/Ca\textsuperscript{2+} signaling pathway and contribute to the specificity of intracellular Ca\textsuperscript{2+} dynamics.

To gain further insights into regulation of the IP\textsubscript{3}/Ca\textsuperscript{2+} signaling pathway, we searched for IP\textsubscript{3}R-binding proteins. In particular, we focused on molecules that interact with IP\textsubscript{3}R in a manner regulated by IP\textsubscript{3}, because such molecules may recognize the conformational change in IP\textsubscript{3}R induced by IP\textsubscript{3} binding, and/or may function as novel upstream or downstream signaling molecules of IP\textsubscript{3}R. For this
purpose, we used an affinity column conjugated with the N-terminal 2217 amino acid residues of IP₃R1 containing most of the large cytoplasmic region of the receptor molecule. By eluting bound proteins with IP₃ from this affinity column, we identified a novel IP₃R-binding protein, IRBIT (IP₃R binding protein released with inositol 1,4,5-trisphosphate). IRBIT bound to IP₃R1 in vitro and in vivo, and co-localized intensively with IP₃R1. Moreover, IRBIT was released from IP₃R1 at a physiological concentration of IP₃. On the basis of these results, we consider herein the role of IRBIT in IP₃/Ca²⁺ signaling.

EXPERIMENTAL PROCEDURES

Preparation of IP₃R1 Affinity Column

The cDNA encoding the N-terminal region of mouse IP₃R1 (residues 1-225) was inserted into glutathione S-transferase (GST) fusion vector pGEX-KG (40). The GST-IP₃R1(1-225) fragment was subcloned into the baculovirus transfer vector pBlueBac4.5 (Invitrogen). The 3'-region downstream from Sma I site of GST-IP₃R1(1-225) was replaced with the Sma I-EcoRI fragment of mouse IP₃R1 (corresponding to residues 79-2217) to generate GST-IP₃R1(1-2217) (termed GST-EL, for the EcoRI Large fragment) construct. GST alone was subcloned into pBlueBac4.5 as a control. Sf9 cells were cultured in TNM-FH medium supplemented with 10% fetal bovine serum at 27 °C. Recombinant baculoviruses carrying GST-EL or GST were generated with Bac-N-Blue™ Transfection Kit (Invitrogen) according to the manufacturer’s protocols. GST-EL and GST were expressed in 2 x 10⁸ Sf9 cells by infecting recombinant baculoviruses at a multiplicity of infection of 5, and incubating for 48 h. Cells were harvested and stored at -80 °C. Frozen cells were suspended in 10 ml of 10 mM Hepes (pH 7.4), 100 mM NaCl, 2 mM EDTA, 1 mM 2-mercaptoethanol (2-ME), 0.1% Triton X-100, and protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 10
µM leupeptin, 2 µM pepstatin A and 10 µM E-64), and were homogenized with a glass-Teflon homogenizer (1000 rpm, 10 strokes). The homogenate was centrifuged at 20,000 x g for 30 min. The supernatant was incubated with 3 ml of glutathione Sepharose 4B (Amersham Pharmacia Biotech) for 3 h at 4 °C. After washing eight times with 40 ml of 10 mM Hepes (pH 7.4), 250 mM NaCl, 2 mM EDTA, 1 mM 2-ME, and 0.1% Triton X-100, GST-EL or GST coupled with glutathione-Sepharose was packed into columns and equilibrated with 10 mM Hepes (pH 7.4), 100 mM NaCl, 2 mM EDTA, 1 mM 2-ME, and 0.1% Triton X-100. About five milligram of GST-EL was immobilized.

Purification and Partial Amino Acids Sequencing of IRBIT

Adult rat cerebella (~5 g) were homogenized in 45 ml of Homogenize buffer (10 mM Hepes (pH 7.4), 320 mM Sucrose, 2 mM EDTA, 1 mM 2-ME, and protease inhibitors) with a glass-Teflon homogenizer (950 rpm, 10 strokes), and the homogenate was centrifuged at 1,000 x g for 10 min. The supernatant (S1 fraction) was centrifuged at 100,000 x g for 60 min to obtain the cytosolic fraction (the supernatant) and the crude microsome (the pellet). The crude microsome was homogenized in 25 ml of homogenize buffer containing 500 mM NaCl with a glass-Teflon homogenizer (1,200 rpm, 10 strokes), incubated on ice for 15 min, and centrifuged at 100,000 x g for 60 min to obtain the high salt extract (the supernatant) and the stripped-crude microsome (the pellet). The high salt extract was diluted five times with 10 mM Hepes (pH 7.4), 2 mM EDTA, 1 mM 2-ME, 0.01% Brij 35, and protease inhibitors. The diluted high salt extract was pre-cleared with glutathione-Sepharose and loaded onto GST-EL affinity column equilibrated with binding buffer (10 mM Hepes (pH 7.4), 100 mM NaCl, 2 mM EDTA, and 1 mM 2-ME). GST column was used as a control. The columns were washed with 20 column volume of binding
buffer and bound proteins were eluted with binding buffer containing 50 µM IP₃ (Dojindo) and 0.05% Brij 35. The eluted material was concentrated, separated by SDS-polyacrylamide gel electrophoresis (PAGE) on 10% gel and stained with Coomassie Brilliant Blue. The 60-kDa protein band was excised from the gel and digested with lysyl endopeptidase (Wako) essentially according to the method as described (41). The polypeptides were separated by C-18 reversed-phase column (µRPC C2/C18 SC 2.1/10, Amersham Pharmacia Biotech) connected on a SMART system (Amersham Pharmacia Biotech). Amino acids sequence of each peptide was determined by 494 procise protein sequencer (Applied Biosystems). Two peptide sequences, N-YSFMATVTK-C and N-QIQFADDMQEFTK-C were obtained.

**cDNA cloning of IRBIT**

BLAST searches of two peptide sequences derived from the 60-kDa protein against the non-redundant database revealed that these sequences are matched the sequence of a human cDNA deposited in a patent (accession number CAC09285). Based on the databases on mouse expressed sequence tags (accession number AW229870 and BE282170) homologous to this cDNA, primers (5’-ATGTGATGCTGCACGTACGTGC-3’ and 5’-GGTTGTTTCATGTGGACTGCTGTC-3’) were synthesized. cDNA of IRBIT was amplified by polymerase chain reaction (PCR) using mouse cerebellum oligo dT-primed, first-strand cDNA as a template. PCR product was cloned into pBluescript II KS(+) (Stratagene) and sequenced. Sequences of three independent clones were confirmed.

**Preparation of Recombinant Proteins**

The cDNA encoding N-terminal region (residues 1-104) of IRBIT was subcloned into the bacterial hexahistidine (His) fusion vector pET-23a(+) (Novagen) to generate
IRBIT(1-104)-His construct. The same cDNA was subcloned into the GST fusion vector pGEX-4T-1 (Amersham Pharmacia Biotech) to generate GST-IRBIT(1-104) construct. The cDNA fragments corresponding to the amino acid residues 1-225, 1-343, 341-923, 600-1248, 916-1581, and 1553-1943 of mouse IP$_3$R1 were inserted into pGEX-KG to generate GST-Ia, GST-Iab, GST-IIa, GST-IIbIIIa, GST-IIIab, and GST-IV construct, respectively. Residues 1593-2217 of mouse IP$_3$R1 were inserted into pGEX-4T-1 to generate GST-IV-Va construct. These fusion proteins were expressed in Escherichia coli. GST-EL was expressed in Sf9 cells as described above. Expressed IRBIT(1-104)-His was purified using ProBond resin (Invitrogen). GST fusion proteins were purified using glutathione-Sepharose. GST-IbIIa (residues 224-604 of mouse IP$_3$R1) and its site-directed mutants K508A and R441Q were described previously (Ref. 42, GST-IbIIa was termed G224 therein).

Production of Affinity Purified Anti-IRBIT Antibody

A Japanese White rabbit was immunized with purified IRBIT(1-104)-His by subcutaneous injection with the complete Freund’s adjuvant at a 14-day interval. The anti-IRBIT antisera was affinity-purified by passing serum from the immunized rabbit over a GST-IRBIT(1-104) covalently coupled with cyanogen bromide-activated Sepharose 4B (Amersham Pharmacia Biotech), and specific antibodies bound to the column were eluted with 100 mM glycine-HCl (pH 2.5).

Subcellular Fractionation and immunoblotting

Cerebrum, cerebellum, heart, lung, liver, kidney, thymus, spleen, testis, and ovary were dissected from adult mouse and S1 fraction were obtained essentially as described above. The cytosol, the crude microsome, the high salt extract, and the stripped-crude microsome of mouse cerebellum were obtained essentially as
described above. Proteins with the amount indicated were subjected to 10% SDS-PAGE and transferred onto polyvinylidene fluoride membrane by electroblotting. After blocking, membranes were immunoblotted with anti-IRBIT antibody (1 µg/ml) for 1 h at room temperature, followed by horseradish peroxidase-conjugated donkey anti-rabbit IgG (Amersham Pharmacia Biotech). Immunoreactive bands were visualized with enhanced chemiluminescence detection system (Amersham Pharmacia Biotech).

Generation and Transfection of Expression Constructs

The cDNA encoding full-length IRBIT was subcloned into the pcDNA3 (Invitrogen). The cDNA encoding full-length of IRBIT or its deletion mutants (residues 1-277, 1-104, and 105-530) were subcloned into the pEGFP-C1 (Clontech) to generate green fluorescent protein (GFP) fusion proteins constructs. Mouse IP$_3$R1 expression vector pBact-STneoB-C1 was described previously (43). Cos-7 cells were cultured in DMEM with 10% fetal bovine serum, penicillin, and streptomycin at 37 °C. Transient transfections were performed using TransIT transfection reagents (Mirus) according to the manufacturer’s instruction. Transfected cells were processed for immunoblotting, pulldown experiments, or immunostaining two days after transfection.

In Vitro Binding Experiments

Mouse cerebellar cytosolic fraction was diluted two times with 10 mM Hepes (pH 7.4), 200 mM NaCl, 2 mM EDTA, 1 mM 2-ME, and 0.02% Triton X-100. The high salt extract was diluted five times with 10 mM Hepes (pH 7.4), 2 mM EDTA, 1 mM 2-ME, and 0.01% Triton X-100. Diluted fractions (the final NaCl concentration of both fractions was 100 mM) were incubated with 20 µg of GST-EL or GST for 2 h at 4 °C.
After adding 10 µl of glutathione-Sepharose and another 2-h incubation, the resins were washed five times with wash buffer (10 mM Hepes (pH 7.4), 100 mM NaCl, 2 mM EDTA, 1 mM 2-ME, and 0.01% Triton X-100), and bound proteins were eluted with 20 mM glutathione. Eluted proteins were analyzed by Western blotting with anti-IRBIT antibody.

For dephosphorylation, the diluted high salt extract was incubated with or without bacterial alkaline phosphatase (Toyobo) in the presence of 2 mM MgCl₂ for 30 min at 37 °C, added by 5 mM EDTA, and processed for pulldown assay as described above.

For the dissociation experiments, IRBIT in the diluted high salt extract was pulled down with GST-EL and washed as described above, and resins were added by 100 µl of wash buffer containing IP₃, inositol 4,5-bisphosphate (IP₂) (Dojindo), inositol 1,3,4,5-tetrakisphosphate (IP₄) (Calbiochem), inositol 1,2,3,4,5,6-hexakisphosphate (IP₆) (Calbiochem), or ATP (Amersham Pharmacia Biotech) (0.1, 0.3, 1, 3, 10 µM, each). After incubation on ice for 10 min, samples were centrifuged at 10,000 rpm for 1 min, and supernatant was subjected to immunoblot analysis with anti-IRBIT antibody or goat anti-GST antibody (Amersham Pharmacia Biotech). For quantitation, Alexa 680-conjugated goat anti-rabbit IgG (Molecular Probes) was used as a secondary antibody. Intensity of fluorescence of immunoreactive bands of IRBIT was measured using Odyssey infrared imaging system (Aloka). Quantitative data (the mean ± SD from at least three independent experiments) are expressed as percentage of the amount of IRBIT in 10 µM-IP₃ eluate.

For the determination of the IRBIT binding region and the critical amino acid of IP₃RI, the diluted high salt extract were processed for pulldown assay with 100 pmol of GST, GST-EL, GST-Ia, GST-Iab, GST-IbIIa, GST-IIab, GST-IIbIIIa, GST-IIIab, GST-IV, GST-IV-Va, K508A, or R441Q as described above, and analyzed by Western blotting
with anti-IRBIT antibody.

For the determination of the IP$_3$R1-interacting region of IRBIT, Cos-7 cells expressing GFP-tagged full-length IRBIT or its truncated mutants were lysed in lysis buffer (10 mM Hepes (pH7.4), 100 mM NaCl, 2 mM EDTA, 1 mM 2-ME, 0.5% Nonidet P-40, and protease inhibitors) for 30 min at 4 °C, followed by centrifugation (100,000 x g, 30 min). The supernatants were processed for pulldown assay with GST-EL or GST as described above, and bound proteins were subjected to immunoblot analysis with anti-GFP antibody (Medical & Biological Laboratories).

**Indirect Immunofluorescence and Confocal Microscopy**

Transfected Cos-7 cells grown on glass coverslips were washed once in phosphate-buffered saline (PBS), fixed in 4% formaldehyde in PBS for 15 min, permeabilized in 0.1% Triton X-100 in PBS for 5 min, and blocked in PBS containing 2% normal goat serum for 60 min at room temperature. For washing out cytosolic proteins, transfected cells were washed once in PBS, permeabilized in ice-cold permeabilization buffer (80 mM PIPES (pH 7.2), 1 mM MgCl$_2$, 1 mM EGTA, and 4% polyethylene glycol) containing 0.1% saponin for 10 min on ice, and washed twice with ice-cold permeabilization buffer before fixation. Cells were then stained with rabbit anti-IRBIT antibody (1 µg/ml, for 60 min at room temperature) and rat anti-IP$_3$R1 antibody 18A10 (44) (for overnight at 4 °C). Following four 5-min PBS washes, Alexa 488-conjugated goat anti-rabbit IgG and Alexa 594-conjugated goat anti-rat IgG (Molecular Probes) were applied for 45 min at 37 °C. Following four 5-min PBS washes, the coverslips were mounted with Vectashield (Vector Laboratories) and observed under the IX-70 confocal fluorescence microscopy (Olympus) with a 60x objective.
Immunoprecipitation

Immunoprecipitation was performed as described (45) with modifications. Adult mouse cerebellum was homogenized in 10 volumes of 4 mM Hepes (pH 7.4), 320 mM Sucrose, and protease inhibitors with a glass-Teflon homogenizer. The homogenate was centrifuged at 800 x g for 10 min, and the supernatant was subjected to another centrifugation at 9000 x g for 15 min. The supernatant from the second centrifugation was solubilized in 1% sodium deoxycholate at 36 °C for 30 min, followed by adding 0.1 volume of 1% Triton X-100 in 50 mM Tris-HCl (pH 9.0), and the preparation was centrifuged at 100000 x g for 10 min. The supernatant was incubated with 5 µl of Protein G Sepharose 4 fast flow (Amersham Pharmacia Biotech) for 2 h at 4 °C to clarify nonspecific binding to protein G beads. At the same time, 3 µg of rabbit anti-IRBIT antibody, control rabbit IgG, rat anti-IP3R1 antibody 10A6 (46), control rat IgG, mouse anti-IP3R2 antibody KM1083 (47), or control mouse IgG was preincubated with 5 µl of Protein G beads for 2 h, and the protein G-antibody complex was spun down at 3000 rpm for 2 min. The clarified supernatant was then added to the antibody-bound protein G beads, and the mixture was incubated for 2 h at 4 °C. Beads were washed five times with 10 mM Hepes (pH7.4), 100 mM NaCl, and 0.5% Triton X-100 and analyzed by Western blotting with anti-IRBIT antibody, mouse anti-IP3R1 antibody KM1112 (47), KM1083, or mouse anti-IP3R3 antibody KM1082 (47).

RESULTS

Purification and cDNA Cloning of a Novel IP3R-interacting Protein

To identify IP3R-interacting molecules, we used a GST fusion protein of the N-terminal 2217 amino acids of mouse IP3R1 (GST-EL). This region is the large cytoplasmic portion of IP3R1 containing the IP3 binding domain and regulatory
domain (10, 11). GST-EL or GST was expressed using a baculovirus/Sf9 cell system and conjugated to glutathione-Sepharose. The extract with a high salt buffer (containing 500 mM NaCl) from crude rat cerebellar microsomes, which was thought to be enriched with peripherally membrane-bound proteins, was loaded onto a glutathione-Sepharose affinity column on which GST-EL or GST was immobilized. To detect proteins which were dissociated from IP$_3$R in the presence of IP$_3$, the proteins bound to the affinity columns were eluted by addition of 50 µM IP$_3$. A protein with a mass of about 60 kDa was detected in the 50 µM IP$_3$-eluate from the GST-EL column (Fig. 1A), but not from the GST column (data not shown). Two peptide sequences derived from the 60-kDa protein were determined. BLAST searches of non-redundant databases revealed that these two sequences matched the sequence of a human cDNA deposited in a patent. On the basis of sequence information on mouse expressed sequence tags homologous to this cDNA, the cDNA of the 60-kDa protein was obtained by reverse transcriptase-PCR from the mouse cerebellum. The predicted amino acid sequence of the cloned cDNA revealed a protein composed of 530 amino acid residues (Fig. 1B), with a calculated molecular mass of 58.9 kDa, which was close to its apparent molecular mass of 60 kDa estimated by SDS-PAGE (Fig. 1A). We designated the 60-kDa protein IRBIT (IP$_3$R binding protein released with inositol 1,4,5-trisphosphate).

Homology analysis of the deduced amino acid sequence of IRBIT revealed the C-terminal region (residues 105-530) to be homologous (51% identical, 74% similar) to the methylation pathway enzyme S-adenosylhomocysteine hydrolase (EC 3.3.1.1.) (48) (Fig. 1, C and D). An appendage of the N-terminal region (residues 1-104) of IRBIT had no homology with reported proteins and contained a serine-rich region (residues 62-103) (Fig. 1, B and D). Motif searches of the IRBIT sequence revealed the presence of a putative coiled-coil motif (residues 111-138) and a putative NAD$^+$
binding region (residues 314-344) (Fig. 1, B and D). There were 17 potential phosphorylation sites for protein kinases such as casein kinase II, PKC, PKA/PKG and tyrosine kinases, out of which seven sites were concentrated in the N-terminal region (Fig. 1B). Neither putative membrane-spanning regions nor signal sequences were found. Recently, a mRNA expressed in dendritic cells was cloned from a human cDNA library, and it was named DCAL (49), but its physiological function was not addressed. The 100% identical amino acid sequences of IRBIT and DCAL indicate that IRBIT is a mouse homologue of human DCAL.

Although IRBIT was homologous with S-adenosylhomocysteine hydrolase, which catalyzes the reversible hydrolysis of S-adenosylhomocysteine to adenosine and homocysteine, recombinant IRBIT expressed in bacteria had no enzyme activities in the hydrolysis direction, nor had any effects on the enzyme activity of S-adenosylhomocysteine hydrolase (data not shown).

**Tissue Distribution and Subcellular Localization of IRBIT**

We generated an affinity-purified antibody against the N-terminal region of the IRBIT (Fig. 1B, boxed). To confirm the specificity of this antibody, we transfected the cDNA of IRBIT into Cos-7 cells and the whole cells lysates obtained were analyzed by immunoblotting with the anti-IRBIT antibody. As shown in Fig. 2A, the anti-IRBIT antibody recognized only a single protein with a size of ~60 kDa. The molecular mass of the exogenously expressed IRBIT (Fig. 2A, lane 1) was the same as that of the endogenous protein in Cos-7 (Fig. 2A, lane 3), confirming that the cDNA clone encodes the full-length IRBIT protein. We examined the expression of IRBIT in several mouse tissues by immunoblot analysis with this anti-IRBIT antibody. IRBIT was detected ubiquitously, with the highest expressions in the cerebrum and cerebellum (Fig. 2B).
Next, we investigated the subcellular distribution of IRBIT by fractionation of the mouse cerebellum. IRBIT was present in both the cytosolic and the crude microsome fraction (Fig. 2C, lanes 2 and 3, respectively). The crude microsome fraction was further separated into a peripherally membrane-bound fraction (the fraction from which IRBIT was originally purified) and a stripped-membrane fraction, with the aforementioned high salt buffer. As shown in Fig. 2C, IRBIT in the crude microsome fraction was partially extracted with the high salt buffer (Fig. 2C, lane 4). In contrast, IP₃R1, which is an integral membrane protein of the endoplasmic reticulum, was not extracted (Fig. 2C, lower panel). These results indicate IRBIT to be both a cytosolic and a peripherally membrane-bound protein.

*IRBIT in the high salt extract interacted with IP₃R1 and the N-terminal region of IRBIT was essential for the interaction*

IRBIT was present in both the cytosolic and the peripherally membrane-bound fraction of the mouse cerebellum (Fig. 2C). We investigated whether IRBIT in these fractions interacted with IP₃R1 in vitro employing GST pulldown techniques. The cytosol and high salt extracts from crude mouse cerebellar microsomes were incubated with GST-EL or GST and binding of IRBIT to the recombinant proteins was analyzed by immunoblotting with anti-IRBIT antibody. As shown in Fig. 3A, IRBIT in the high salt extract interacted with GST-EL (Fig. 3A, lane 6), but not with GST (Fig. 3A, lane 5). In contrast, IRBIT in the cytosolic fraction did not interact with GST-EL (Fig. 3A, lane 3). The same result was obtained when both fractions were dialyzed against the same buffer, indicating that the difference was due neither to a difference in buffer composition nor to excluded small molecules (data not shown). We speculated that the difference might be attributable to a post-translational modification of IRBIT such as phosphorylation. To test this possibility, we treated the
high salt extract with alkaline phosphatase, a nonspecific phosphatase, followed by incubation with GST-EL or GST. As shown in Fig. 3B, IRBIT in the high salt extract no longer interacted with GST-EL after phosphatase treatment (Fig. 3B, lane 6). This result raises the possibility that phosphorylation of IRBIT may be necessary for the association with IP$_3$R1, although the possibility that phosphorylation of other proteins may regulate the interaction between IRBIT and IP$_3$R1 cannot be excluded.

To determine the region of IRBIT responsible for the interaction with IP$_3$R1, GST pulldown experiments were carried out using GFP-tagged deletion mutants of IRBIT (Fig. 4A). As shown in Fig. 4B, both GFP-IRBIT and GFP-IRBIT(1-277) bound to GST-EL efficiently (Fig. 4B, lanes 3 and 6, respectively). Although GFP-IRBIT(1-104) interacted with GST-EL, the interaction was much weaker than those of GFP-IRBIT and GFP-IRBIT(1-277) (Fig. 4B, compare lanes 7 and 9 with lanes 1 and 3, and lanes 4 and 6). In contrast, GFP-IRBIT(105-530), which lacked the N-terminal region, and GFP alone did not interact with GST-EL (Fig. 4B, lanes 12 and 15, respectively). These results demonstrate the N-terminal region of IRBIT to be essential for the interaction with IP$_3$R1, and the following ~170 amino acids containing a coiled-coil structure might be important for stabilizing the interaction.

**IRBIT Co-localized with IP$_3$R1 on the Endoplasmic Reticulum in Transfected Cos-7 Cells**

To test whether IRBIT interacts with IP$_3$R1 in intact cells, IRBIT and IP$_3$R1 were co-expressed in Cos-7 cells, and their distribution were analyzed by confocal immunofluorescence microscopy. IRBIT was diffusely distributed in the cytoplasm, with no immunoreactivity in the nucleus (Fig. 5A). Because IRBIT was shown to be present in both the cytosolic and the crude microsome fraction by biochemical fractionation (Fig. 2C), we attempted to visualize only the membrane-bound population of IRBIT. For this purpose, we permeabilized plasma membranes of
transfected Cos-7 cells with saponin and washed out cytosolic IRBIT prior to fixation. As shown in Fig. 5B, in cells treated with saponin, localization of IRBIT on the reticular structure was revealed (Fig. 5B, left panels). The immunoreactivity of IRBIT extensively overlapped with that of IP₃R1 (Fig. 5B, middle panels, and merged image right panels). The staining pattern of IP₃R1 was not altered by permeabilization with saponin (data not shown). Since IRBIT expressed alone showed a coarse distribution instead (data not shown), these results indicate that IRBIT co-expressed with IP₃R1 localized on the endoplasmic reticulum via the interaction with IP₃R1. IP₃R1 was expressed in Cos-7 cells to a trace level, whereas type 2 IP₃R (IP₃R2) and type 3 IP₃R (IP₃R3) were predominantly expressed (50, 51). These endogenous IP₃Rs showed again a coarse, not a reticular, distribution in Cos-7 cells both in a previous report and in our hands (ref. 52 and data not shown, respectively). Furthermore, complex of IRBIT and endogenous IP₃R2/IP₃R3 were revealed by co-immunoprecipitation assay (data not shown). Taken together, these findings support our idea that IRBIT interacted not only with IP₃R1 but also with IP₃R2 and IP₃R3 (see below).

When we transfected IP₃R1 and GFP-IRBIT instead of IRBIT and observed the fluorescence of GFP, essentially the same results were obtained (Fig. 5, C and D). To confirm the specificity of co-localization, we transfected GFP-IRBIT(105-530), which did not interact with GST-EL due to lack of the N-terminal region (Fig. 4), with IP₃R1 into Cos-7 cells. In contrast to GFP-IRBIT, GFP-IRBIT (105-530) was distributed in the nucleus as well as the cytosol (Fig. 5E). IRBIT does not harbor predicted nuclear localization signals, and the reason GFP-IRBIT(105-530) localized in the nucleus is unclear at present. When the cytosolic population was washed out by permeabilization, GFP-IRBIT(105-530) localized only in the nucleus and did not co-localize with IP₃R1 (Fig. 5F). This observation is consistent with biochemical results indicating the N-terminal region of IRBIT to be necessary for binding to IP₃R1 (Fig.
IRBIT Interacted with IP₃R In Vivo

To demonstrate an in vivo association between IRBIT and IP₃R in native tissues, we performed co-immunoprecipitation experiments using mouse cerebellum. Cerebellar lysates were immunoprecipitated with anti-IRBIT antibody and the immunoprecipitates were analyzed by immunoblotting with anti–IP₃R1, anti–IP₃R2, or anti–IP₃R3 antibody. All three IP₃R isoforms were co-immunoprecipitated by anti-IRBIT antibody, but not control IgG (Fig. 6A). In the reciprocal experiments, immunoprecipitation of IP₃R1 or IP₃R2 resulted in the co-precipitation of IRBIT (Fig. 6, B and C). IRBIT was not detected in the anti-IP₃R3 precipitates, probably due to the inefficiency of immunoprecipitation with anti-IP₃R3 antibody (data not shown). When we performed immunoprecipitation assay using lysates of Cos-7 cells transfected with IRBIT and IP₃R3, in which most IP₃R3 forms homotetramer (51), IRBIT was shown to interact with IP₃R3 (data not shown). As for IP₃R2, essentially the same result was obtained (data not shown). These results confirm IRBIT interacted with all IP₃R isoforms in vivo.

Physiological Concentration of IP₃ Selectively Dissociated IRBIT from IP₃R1

IRBIT was originally identified in the GST-EL column eluate with 50 μM IP₃ (Fig. 1A), suggesting that IP₃ disrupted the interaction between IRBIT and IP₃R1. However, 50 μM is a relatively high concentration compared to the physiological range of IP₃, which was estimated to be a few micromolar after stimulation (53). Thus, we examined the dose-dependency of IP₃ with which IRBIT was dissociated from GST-EL, and its selectivity against other related inositol polyphosphates. IRBIT in the high salt extract of crude mouse cerebellar microsomes was pulled down with GST-
EL, and eluted with 0.1 – 10 µM IP₃, IP₂, IP₄, IP₆ or ATP. As shown in Fig. 7A, IP₃ dissociated IRBIT from GST-EL most efficiently in a dose-dependent manner (Fig. 7Aa, lower panel). We confirmed GST-EL to be undetectable in the IP₃ eluates (Fig. 7Aa, upper panel), even with longer exposure (data not shown). The EC₅₀ (the concentration required for half-maximal dissociation of IRBIT from GST-EL) was ~0.5 µM, which was within the physiological IP₃ concentration range (53) (Fig. 7B). IP₃ dissociated IRBIT from GST-EL about 50 times more efficiently than other inositol polyphosphates (Fig. 7, Ab-d and B). ATP, which has three phosphate groups like IP₃, did not dissociate IRBIT from GST-EL even at 10 µM (Fig. 7, Ae and B). These results indicate that IRBIT was dissociated from IP₃R1 selectively within the physiological concentration range of IP₃.

IRBIT Interacted with the IP₃-Binding Region of IP₃R1 and Lys-508 of IP₃R1 Was Essential for Interactions with Both IRBIT and IP₃

We investigated which region, the IP₃-binding region or the regulatory region, of IP₃R1 was necessary for the interaction with IRBIT, using eight deletion mutants of IP₃R1 constructed as GST fusion proteins based on the domain structure of IP₃R1 (54) (Fig. 8A). As shown in Fig. 8B, GST-IbIIa (residues 224-604), which contains the IP₃ binding core region (residues 226-578) (12) bound to IRBIT to the same extent as GST-EL. In contrast, other GST fusion proteins, including GST-Iab and GST-IIab, did not interact with IRBIT. Next, we performed a site-directed mutagenesis analysis to determine the IP₃R1 amino acids important for the interaction with IRBIT. Lys-508 of IP₃R1 was a critical amino acid residue for IP₃ binding (12), and substitution of Lys-508 of GST-IbIIa with alanine (K508A) resulted in an enormous loss of IP₃ binding affinity (42). Conversely, R441Q, in which Arg-441 of GST-IbIIa was substituted for Gln, had higher IP₃ affinity than GST-IbIIa (42). GST pulldown assays using these
recombinant proteins showed that IRBIT bound to GST-IbIIa and R441Q to the same extent, but not to K508A (Fig. 8C). Taken together, these results indicate that IRBIT binds to the IP3-binding region of IP3R1 and that Lys-508 of IP3R1 is required for the interaction with IRBIT as well as IP3, supporting the observation that IP3 disrupts the interaction between IRBIT and IP3R1.

DISCUSSION

We screened IP3R1 binding proteins released from IP3R1 in the presence of IP3, and identified a novel protein, IRBIT, from a high salt extract of crude cerebellar microsomes. IRBIT interacted with IP3R1 in vitro and in vivo, and co-localized extensively with IP3R1 on endoplasmic reticulum in transfected cells. These results strongly suggest that IRBIT associates with IP3R1 in basal states. Moreover, the physiological concentration of IP3, but not of other inositol polyphosphates, dissociated IRBIT from IP3R1. IRBIT bound to the IP3 binding region of IP3R1, and Lys-508 of IP3R1 was essential for the interactions with both IP3 and IRBIT. These results suggest that IRBIT is released from IP3R1 with IP3 produced in response to extracellular stimuli. Although many IP3R-binding proteins have been reported (18-39), IRBIT is the first molecule for which the interaction with IP3R was shown to be regulated by IP3.

IRBIT is composed of two regions, the N-terminal region (residues 1-104) essential for the interaction with IP3R1, and the C-terminal region (residues 105-530) homologous to S-adenosylhomocysteine hydrolase (48). Crystallographic studies (55, 56) and site-directed mutagenesis studies (57-60) have determined amino acid residues of S-adenosylhomocysteine hydrolase involved in substrate binding or NAD+ binding (Fig. 1C). Although most of these residues were well conserved in IRBIT, we did not detect enzyme activity of recombinant IRBIT expressed in bacteria.
We concluded that IRBIT dose not have S-adenosylhomocysteine hydrolase activity, probably due to substitution of amino acids important for substrate binding, such as Leu-54, Phe-302 and His-353 of S-adenosylhomocysteine hydrolase (Fig. 1C), as discussed by another group (49). Domains that are homologous to certain enzymes, but are catalytically inactive, such as the esterase domain of the neuroligin family (61) and the carbonic anhydrase domain of receptor tyrosine phosphatase β (62), are reportedly involved in protein-protein interactions. The C-terminal region of IRBIT may be one such domain. However, the possibility that IRBIT has enzyme activity with a different substrate specificity can not be excluded.

\textit{In vitro} binding experiments and immunostaining studies showed the N-terminal region of IRBIT to be essential, though not sufficient, for the interaction with IP$_3$R1. The IRBIT-binding region of IP$_3$R1 was shown to be its IP$_3$-binding region, and Lys-508 of IP$_3$R1, the critical amino acid for IP$_3$ binding, was required for this interaction. Based on mutagenesis analysis, Yoshikawa \textit{et al.} proposed that basic amino acid residues, including Lys-508, contribute to form a positively charged pocket for binding to the three negatively charged phosphate groups of IP$_3$ (12). This model leads us to speculate that acidic or phosphorylated amino acid residues in the N-terminal region of IRBIT may be involved in interaction with the positively charged IP$_3$-binding pocket of IP$_3$R1. This hypothesis is supported by the following findings; (1) although IRBIT is a neutral protein (calculated pI of 6.48), its N-terminal region is relatively acidic (calculated pI of 4.98), (2) seven potential phosphorylation sites are concentrated in the N-terminal region of IRBIT, and phosphorylation was supposed to be required for the interaction, (3) Lys-508 of IP$_3$R1 was essential for the interaction with IRBIT, (4) IP$_3$ disrupted the interaction, and (5) a high salt buffer disrupted the interaction between IRBIT and GST-EL$^2$ and extracted IRBIT from crude microsomes, indicating that the interaction is dependent on an electrostatic
bond. Deletion mutagenesis results also indicate that residues 105-277 of IRBIT, which contain a coiled-coil region, contribute to the interaction. The crystal structure of the IP₃ binding region of mouse IP₃R1 in the complex with IP₃ was recently resolved (63). IP₃ bound to the positively charged cleft of the IP₃ binding region, and the side chain of Lys-508 formed the hydrogen bond with the phosphate group at the 5-position of IP₃ (63). Remarkably, the C-terminal region of the IP₃ binding domain containing Lys-508 (residues 437-604) formed an ‘armadillo repeat’-like fold (63), which generally acts as a protein-protein interaction motif (64). IRBIT may interact with IP₃R1 via this motif. However, the armadillo repeat-like fold is not sufficient for interaction, since GST-IIab (residues 341-923 of IP₃,R1) did not interact with IRBIT.

IRBIT was dissociated from IP₃R1 selectively with IP₃ at an EC₅₀ of ~0.5 μM. This EC₅₀ value is higher than the Kd of purified IP₃,R1 for IP₃ (Kd = 83-100 nM) determined by conventional IP₃ binding assays (46, 65). This difference may be attributable to different buffer conditions because the IP₃ binding affinity of IP₃R depends strongly on pH and ionic strength (66-68). Conventional IP₃ binding assays were performed under optimal binding conditions, with a higher pH (8.0-8.3) and a lower ionic strength (salt free). Surface plasmon resonance biosensor studies using the N-terminal region of IP₃R1 (residues 1-604) demonstrated the Kd value determined under near physiological conditions (pH 7.4 and 150 mM NaCl) to be 336 nM (68), i.e. ~7.5-fold lower than the affinity determined by the conventional IP₃ binding assay (69), and close to the EC₅₀ (~0.5 μM) required for the dissociation of IRBIT from GST-EL determined at pH 7.4 and 100 mM NaCl. Therefore, taken together with the findings that IRBIT bound to the IP₃ binding region of IP₃R1 and that both IRBIT and IP₃ were dependent on Lys-508 of IP₃R1 for the interaction, these results indicate that IRBIT is released from IP₃R1 upon IP₃ binding to IP₃R1, probably via a competitive mechanism.
Phosphorylation, as well as IP₃, is considered to regulate the interaction between IRBIT and IP₃R. *In vitro* binding experiments showed IRBIT extracted from the membrane fraction with a high salt buffer to interact with IP₃R1, whereas IRBIT in the cytosolic fraction did not. The difference in the phosphorylation state of IRBIT may account for this discrepancy, because alkaline phosphatase treatment of the high salt extract disrupted the interaction between IRBIT and IP₃R1. IRBIT has 17 potential phosphorylation sites, and seven of these sites are concentrated in the N-terminal region, which is necessary for the interaction with IP₃R1. These findings raise the possibility that the dephosphorylated form of IRBIT is free in the cytosol, whereas the phosphorylated form is membrane-bound via the interaction with IP₃R1, although we could not rule out the possibility that phosphorylation of other proteins may regulate the interaction. We propose that the interaction between IRBIT and IP₃R1 is dualistically regulated by IP₃ and, either directly or indirectly, by phosphorylation. Further studies are needed to determine whether or not the interaction is regulated by direct phosphorylation of IRBIT.

Using the detector cell/capillary electrophoresis system, Luzzi *et al.* estimated intracellular IP₃ concentrations before and after stimulation to be tens of nanomolar and a few micromolar, respectively (53). Because the EC₅₀ of IP₃ (~0.5 µM) required for the dissociation of IRBIT from IP₃R1 was between these concentrations, IRBIT is assumed to be released from IP₃R1 after IP₃ production has been induced by extracellular stimuli. What is the physiological significance of the dissociation of IRBIT from IP₃R1 after stimulation and what is the function of IRBIT? We propose four possible roles of IRBIT. First, IRBIT may modulate the channel activity of IP₃R1. Recently, Yang *et al.* showed that CaBP family members can act as direct ligands of IP₃R (35). Interestingly, the CaBP-binding region of IP₃R was within its 600 N-terminal residues (35), which also contain the IRBIT-binding region. Considering our
preliminary data showing that IRBIT does not directly modulate the channel activity of IP₃R, IRBIT may block the binding of CaBP to IP₃R1 and inhibit IP₃-independent activation of IP₃R1. Second, IRBIT may regulate the stability of IP₃R. IP₃-generating stimuli cause degradation of IP₃R (13, 14, 50, 70-72). Zhu et al. proposed that the conformational change in IP₃R induced by IP₃ binding unmasks the putative sites that facilitate ubiquitin conjugation (13, 14), resulting in degradation of IP₃R by the ubiquitin/proteasome pathway (71, 72). Alternatively, dissociation of IRBIT induced by IP₃ binding may reveal the putative degradation signals or protease attack sites of IP₃R. Third, IRBIT may play the role of a linker molecule coupling IP₃R and other proteins to allow efficient signal propagation. Proteins possibly linked with IP₃R include proteins whose activities are regulated by Ca²⁺ released from IP₃R, or plasma membrane receptors, analogous with mGluR (36) and B₂R (37). Indeed, substantial amounts of IRBIT were present in the stripped microsome fraction (Fig. 2C), which might represent IRBIT tightly bound to membrane proteins other than IP₃R. IP₃ may disrupt these complexes, resulting in desensitization of signals and/or translocation of linked proteins. To identify molecules possibly coupled with IP₃R, we are now searching for IRBIT-interacting proteins. Fourth, IRBIT may be a direct downstream signal transducer of IP₃R1. It has been thought that the only direct downstream molecule of IP₃R1 is the calcium ion, which acts on a wide variety of target molecules. Besides a multifunctional and universal second messenger like Ca²⁺, IP₃R1 may utilize IRBIT as a downstream signaling molecule with more restricted target molecules than Ca²⁺. In this model, IRBIT released from IP₃-bound IP₃R1 must be different (for example, in terms of phosphorylation state) from IRBIT originally present in the cytosol, because significant amounts of IRBIT already exist in the cytosol in the basal state. In this respect, the model in which only phosphorylated IRBIT binds to IP₃R1 appears to be reasonable. Screening of IRBIT-binding proteins
may reveal the target molecules of IRBIT.

Finally, the dissociation of IRBIT from IP₃R in the presence of IP₃ is a feature which may be utilized for the development of a new IP₃ indicator based on fluorescence resonance energy transfer (FRET). FRET occurs when two fluorophores are in proximity and in the right orientation such that an excited donor fluorophore can transfer its energy to a second, acceptor fluorophore (73). Based on the cAMP-dependent dissociation of catalytic and regulatory subunits of cAMP-dependent protein kinase, Adams et al. developed a fluorescent indicator for cAMP (74). Similarly, Miyawaki et al. reported a genetically encoded Ca²⁺ indicator based on the Ca²⁺-dependent interaction between calmodulin and calmodulin-binding peptide (75). Although IP₃ concentration changes could be detected by monitoring translocation of the GFP-tagged pleckstrin homology domain (76), a FRET-based IP₃ indicator has yet to be developed due to lack of suitable molecules. IP₃-dependent dissociation of IRBIT and IP₃R1 is a characteristic which can provide a new tool allowing real-time imaging of the spatiotemporal dynamics of IP₃ concentrations in living cells, although further studies focusing on the regulation of this interaction by phosphorylation are needed.

In summary, we identified IRBIT, a novel IP₃R1-interacting protein, which was released from IP₃R1 in the presence of IP₃. Further studies aimed at elucidating the function of IRBIT, including the screening of IRBIT-interacting proteins, are anticipated to provide important insights into IP₃/Ca²⁺ signaling.

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**FOOTNOTES**

2 H. Ando, unpublished observation

The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI/Data Bank with accession number(s) AB092504.

**FIGURE REAGENTS**

Fig. 1. Purification and cDNA cloning of IRBIT.

(A) SDS-PAGE of IRBIT (indicated by *arrow*) purified from the high salt extract of crude rat cerebellar microsomes with the GST-EL column. IRBIT was eluted from the column with 50 μM IP₃. The eluted material was concentrated, separated by SDS-
PAGE on 10% gel and stained with Coomassie Brilliant Blue. The bands above 200 kDa and at 100-200 kDa were mostly leaked GST-EL and its degradates, respectively since these bands were recognized by various anti-IP3RI antibodies.

(B) The deduced amino acid sequences of IRBIT. Two digested peptides obtained from the purified IRBIT are bold-underlined. The serine-rich region is dashed-underlined. The putative coiled-coil region is double-underlined. The putative NAD+ binding site is underlined. Putative phosphorylation sites for Casein kinase II (closed circles), PKC (open circles), PKA/PKG (a closed square), and tyrosine kinases (open squares) are indicated above the sequences. The N-terminal region is boxed.

(C) Sequence alignment of the C-terminal region of IRBIT and rat S-adenosylhomocysteine hydrolase (AHCY) (48). Identical residues (*) and similar residues (:) are indicated. Residues involved in substrate binding and NAD+ binding of S-adenosylhomocysteine hydrolase are indicated by closed circles and open circles, respectively.

(D) Schematic representation of the structure of IRBIT. NTR and CTR indicate the N-terminal region and the C-terminal region, respectively. Serine-rich region (SER), coiled-coil region (CC) and NAD+ binding site (NAD) are indicated.

**Fig. 2. Tissue distribution and subcellular fractionation of IRBIT.**

(A) Western blot analysis of exogenously expressed and endogenous IRBIT. Cos-7 cells were transiently transfected with IRBIT (lane 1) or mock control (lanes 2 and 3), and the whole cell lysates were analyzed by Western blotting with anti-IRBIT antibody. In lane 3, 10-times amounts of the lysate were loaded as compared with in lanes 1 and 2.

(B) Tissue distribution of IRBIT. S1 fractions (2 µg of total protein) of adult mouse tissues were analyzed by Western blotting with anti-IRBIT antibody.
Subcellular fractionation of the mouse cerebellum. S1 fraction (lane 1) of mouse cerebella was centrifuged at 100,000 x g to obtain the cytosolic fraction (lane 2) and the crude microsomes (lane 3). The crude microsomes were extracted with the high salt buffer containing 500 mM NaCl and centrifuged at 100,000 x g to obtain the peripherally membrane-bound fraction (lane 4) and the stripped-crude microsomes (lane 5). Upper, each fraction (1 µg of total protein) was analyzed by Western blotting with anti-IRBIT antibody. Lower, each fraction (0.2 µg of total protein) was analyzed by Western blotting with anti-IP₃R1 antibody.

Fig. 3. IRBIT in the high salt extract but not in the cytosol interacted with IP₃R1 in vitro.

(A) Mouse cerebellar cytosolic fraction (lanes 1-3) and the high salt extract of crude microsomes (lanes 4-6) were incubated with GST-EL (lanes 3 and 6) or GST (lanes 2 and 5). Bound proteins were pulled down with glutathione-Sepharose, eluted with glutathione, and analyzed by Western blotting using anti-IRBIT antibody (upper panel). GST-EL and GST pulled down with glutathione-Sepharose were visualized by staining with Coomassie Brilliant Blue (lower panel).

(B) The high salt extract of crude mouse cerebellar microsomes was incubated without (lanes 1-3) or with (lanes 4-6) alkaline phosphatase prior to pulldown with GST-EL (lanes 3 and 6) or GST (lanes 2 and 5). IRBIT binding was analyzed as in (A).

Fig. 4. The N-terminal region of IRBIT was essential for interaction with IP₃R1.

(A) Schematic representation of the structure of IRBIT and its GFP-tagged truncated mutants.

(B) GST pulldown assay from the lysates of Cos-7 cells expressing GFP-IRBIT (lanes 1-3), GFP-IRBIT(1-277) (lanes 4-6), GFP-IRBIT(1-104) (lanes 7-9), GFP-IRBIT(105-530)
The lysates of Cos-7 cells expressing each construct (input; I) were incubated with GST-EL (E) or GST (G). Bound proteins were pulled down with glutathione-Sepharose, eluted with glutathione, and subjected to immunoblot analysis with anti-GFP antibody.

**Fig. 5. IRBIT co-localized with IP$_3$R1 in transfected Cos-7 cells.**

IP$_3$R1 was transiently transfected into Cos-7 cells with IRBIT (A and B), GFP-IRBIT (C and D), and GFP-IRBIT(105-530) (E and F). The localization of the corresponding proteins was analyzed by indirect immunofluorescence (IP$_3$R1 and IRBIT) and fluorescence (GFP-IRBIT and GFP-IRBIT(105-530)) confocal microscopy. B, D, and F, cells were permeabilized in saponin and cytosolic proteins were washed out prior to fixation. *Left panels* show IRBIT (B), GFP-IRBIT (D), and GFP-IRBIT(105-530) (F). *Middle panels* show IP$_3$R1. *Right panels* show merged images of fluorescence from left and middle panels. B, *Lower panels* are higher magnification images of upper panels. *Scale bars*, 10 μm.

**Fig. 6. IRBIT associated with IP$_3$R in vivo.**

(A) Cerebellar lysates were immunoprecipitated with anti-IRBIT or control antibody. The immunoprecipitates were subjected to SDS-PAGE followed by Western blotting with anti-IP$_3$R1, anti-IP$_3$R2, anti-IP$_3$R3, or anti-IRBIT antibody. (B) Cerebellar lysates were immunoprecipitated with anti-IP$_3$R1 or control antibody. The immunoprecipitates were subjected to Western blotting with anti-IRBIT or anti-IP$_3$R1 antibody. (C) Cerebellar lysates were immunoprecipitated with anti-IP$_3$R2 or control antibody. The immunoprecipitates were subjected to Western blotting with anti-IRBIT or anti-IP$_3$R2 antibody. *Arrowheads* indicate immunoglobulin heavy chains.
Fig. 7. Physiological concentration of IP₃ selectively dissociated IRBIT from IP₃R1.

(A) The high salt extract of crude mouse cerebellar microsomes was incubated with GST-EL. Bound proteins were pulled down with glutathione-Sepharose, and eluted with glutathione (Glu) (a) or 0.1 – 10 µM IP₃ (a), IP₂ (b), IP₄ (c), IP₆ (d), or ATP (e). IRBIT in the eluates were analyzed with anti-IRBIT antibody and Alexa 680-conjugated secondary antibody (a, lower, and b-e). GST-EL in the glutathione and 0.1 – 10 µM IP₃ eluate was analyzed with anti-GST antibody (a, upper).

(B) The intensity of the immunoreactive bands of IRBIT was quantified by infrared imaging system, and relative intensity was plotted against concentration. Results are shown as the mean ± SD from at least three independent experiments.

Fig. 8. IRBIT Interacted with the IP₃-binding region of IP₃R1 and Lys-508 was critical for this interaction.

(A) Schematic representation of the structure of mouse IP₃R1 and the recombinant GST fusion proteins used in this study. The IP₃ binding core region is indicated with a gray box. Putative membrane-spanning regions are indicated by solid vertical bars. Roman numbers below IP₃R1 indicate the domain structure determined by the limited trypsin digestion (54). Numbers above the lines represent corresponding amino acid numbers.

(B) Determination of the IRBIT binding region of IP₃R1. The high salt extract of crude mouse cerebellar microsomes was incubated with GST fusion proteins described in (A). Bound proteins were pulled down with glutathione-Sepharose, eluted with glutathione, and analyzed by Western blotting using anti-IRBIT antibody.

(C) Site-directed mutagenesis analysis. The high salt extract was processed for pulldown assay with GST-IbIIa, R441Q and K508A as described in (B). Bound proteins were analyzed by Western blotting using anti-IRBIT antibody (upper panel).
GST fusion proteins pulled down with glutathione-Sepharose were visualized by staining with Coomassie Brilliant Blue *(lower panel).*
Figure 1
Figure 2
Figure 3
Figure 4
Figure 5
Figure 6
Figure 7
Figure 8
IRBIT, a novel inositol 1,4,5-trisphosphate (IP3) receptor binding protein, is released from the IP3 receptor upon IP3 binding to the receptor

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