Carbonic anhydrase-related protein is a novel binding protein for inositol 1,4,5-trisphosphate receptor type 1

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INTRODUCTION

The inositol 1,4,5-trisphosphate (IP3) receptor (IP3R) is an intracellular IP3-gated Ca2+ channel that is located on intracellular Ca2+ stores and modulates Ca2+ signalling. Using the yeast two-hybrid system, we screened a mouse brain cDNA library with bait constructs for mouse IP3R type 1 (IP3R1) to identify IP3R1-associated proteins. In this way, we found that carbonic anhydrase-related protein (CARP) is a novel IP3R1-binding protein. Western blot analysis revealed that CARP is expressed exclusively in Purkinje cells of the cerebellum, in which IP3R1 is abundantly expressed. Immunohistochemical analysis showed that the subcellular localization of CARP in Purkinje cells is coincident with that of IP3R1. Biochemical analysis also showed that CARP is co-precipitated with IP3R1. Using deletion mutagenesis, we established that amino acids 45–291 of CARP are essential for its association with IP3R1, and that the CARP-binding site is located within the modulatory domain of IP3R1 amino acids 1387–1647. CARP inhibits IP3 binding to IP3R1 by reducing the affinity of the receptor for IP3. As reported previously, sensitivity to IP3 for IP3-induced Ca2+ release in Purkinje cells is low compared with that in other tissues. This could be due to co-expression of CARP with IP3R in Purkinje cells and its inhibitory effects on IP3 binding.

Key words: carbonic anhydrase-related protein (CARP), cerebellum, inositol 1,4,5-trisphosphate receptor, ion channel, Purkinje cell, yeast two-hybrid system.

EXPERIMENTAL

Yeast two-hybrid assays

Screening of the mouse brain cDNA library by the yeast two-hybrid method was performed using the MATCHMAKER™ Two-Hybrid System according to the manufacturer’s protocol (Clontech Laboratories, Inc., Palo Alto, CA, U.S.A.).

Abbreviations used: FKBP, FK506-binding protein; IP3, d-myo-inositol 1,4,5-trisphosphate; IP3R, IP3 receptor; (m)IP3R1, (mouse) IP3R type 1; CARP, carbonic anhydrase-related protein; GST, glutathione S-transferase; GST-EL, GST–IP3R1 type 1–2217 construct.

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fractionation using Sepharose CL-2B (Amersham Biosciences, with sense, 5'-GCAACCAGCAGAATC-

1464): sense strand, 5'-GCAACCAGCAGAATC-\n
TGTACACGGAGA-

TGGTAATGGTCA-

ACTAGATCCAGGACC-

′

or

Ia l one, then ligated to pGBT9 (Clontech). All of the appropriate cDNAs were translationally in-frame and devoid of PCR-induced errors.

The resulting cDNAs were digested with either EcoRI plus Sall or Sall alone, then ligated to pGBT9 (Clontech). All of the plasmid constructs were sequenced to confirm that the cloning of the appropriate cDNAs was translationally in-frame and devoid of PCR-induced errors.

Design and synthesis of the bait constructs
We used part of the IP3R1 modulatory domain as bait. cDNAs of six bait constructs (referred to MD1–MD6; Figure 1) were generated by PCR using the following sets of primers (the sequence from IP3R1 is underlined), which map to amino acid residues 1245–2264 and are overlapping. MD1 (mIP3R1 aa 1245–1464): sense strand, 5'-GGGAAATTCGGTGAGGAGGTGTGAGGAGGCAAGAATGTGTACACGGAGA-

AATATCAT-

AGAGAAG-

GGGGGTCGACGC-

ATTTCCTCCTGGCATC-

MD 2 (mIP3R1 aa 1387–1647): sense, 5'-GGGAAATTCGGTGAGGAGGTGTGAGGAGGCAAGAATGTGTACACGGAGA-

AATATCAT-

AGAGAAG-

GGGGGTCGACGC-

ATTTCCTCCTGGCATC-

MD 3 (mIP3R1 aa 1593–1785): sense, 5'-GGGAAATTCGGTGAGGAGGTGTGAGGAGGCAAGAATGTGTACACGGAGA-

AATATCAT-

AGAGAAG-

GGGGGTCGACGC-

ATTTCCTCCTGGCATC-

MD 4 (mIP3R1 aa 1685–1943): sense, 5'-GGGAAATTCGGTGAGGAGGTGTGAGGAGGCAAGAATGTGTACACGGAGA-

AATATCAT-

AGAGAAG-

GGGGGTCGACGC-

ATTTCCTCCTGGCATC-

MD 5 (mIP3R1 aa 1865–2160): sense, 5'-GGGAAATTCGGTGAGGAGGTGTGAGGAGGCAAGAATGTGTACACGGAGA-

AATATCAT-

AGAGAAG-

GGGGGTCGACGC-

ATTTCCTCCTGGCATC-

MD 6 (mIP3R1 aa 1953–2264): sense, 5'-GGGAAATTCGGTGAGGAGGTGTGAGGAGGCAAGAATGTGTACACGGAGA-

AATATCAT-

AGAGAAG-

GGGGGTCGACGC-

ATTTCCTCCTGGCATC-

The resulting cDNAs were digested with either EcoRI plus Sall or Sall alone, then ligated to pGBT9 (Clontech). All of the plasmid constructs were sequenced to confirm that the cloning of the appropriate cDNAs was translationally in-frame and devoid of PCR-induced errors.

Construction of cDNA library
A mouse brain cDNA library was constructed in pGAD-GL (Clontech). Briefly, total RNA from mouse brains (6-week-old ddY mice; Nippon SLC, Hamamatsu, Japan) was enriched in poly(A)+ mRNA using oligo(dT)–cellulose chromatography. Double-stranded cDNAs were generated using random hexamers as primers and were ligated to EcoRI adapters. After digestion with EcoRI, cDNAs over 400 bp in size were collected by size fractionation using Sepharose CL-2B (Amersham Biosciences, Piscataway, NJ, U.S.A.), and inserted into the EcoRI site of pGAD GL. Approx. 5 × 10⁶ independent clones were generated in Escherichia coli XLI1-Blue MRF’ (Stratagene, La Jolla, CA, U.S.A.), and plasmid DNA was isolated after one amplification.

Preparation of antibodies
Monoclonal antibodies against mIP3R1, i.e. 4C11, 18A10 and KM1112, were prepared as described elsewhere [25–27]. A peptide corresponding to amino acid residues 267–279 (CDGILGDNFRPTQ) of mouse CARP, a region that shows marked variation among members of the carbonic anhydrase family, was custom-synthesized. The peptide was conjugated to keyhole-limpet haemocyanin via the N-terminal Cys residue, using m-maleimidobenzoyl-N-hydroxysuccinimide ester. A polyclonal antibody was raised to this peptide in rabbits (New England White; Hokudo Tohya Immunity Laboratory, Hokkaido, Japan). The antibody was purified from antisera using antigenic peptide-conjugated beads according to a standard protocol.

Western blot analysis
Expression of CARP in various organs was analysed by Western blotting. Each organ dissected from mice was homogenized in homogenizing buffer [0.3 M sucrose, 1 mM EDTA, 1 mM 2-mercaptoethanol, protease inhibitors (0.1 mM PMSF, 10 μM leupeptin, 10 μM pepstatin A, 10 μM E-64) and 10 mM Tris/HCl, pH 7.4] with a glass/Teflon homogenizer. For preparations from heart, liver, kidney, adrenal gland, testis and muscle, these organs were minced with scissors into small pieces before homogenization. Homogenates were centrifuged at 100 000 g for 20 min at 4 °C. The resultant supernatant (10 μg) was separated on SDS/5%-PAGE, transferred to nitrocellulose, and immunodetected with anti-CARP antibody.

Immunohistochemistry
Preparation and immunohistochemical analyses of primary cultured Purkinje cells from the cerebellum were according to methods described elsewhere [28]. The following combinations

Figure 1  Schematic diagram of the baits of IP3R1
Shown is the structure of mIP3R1, which consists of three functional domains: a ligand-binding domain, a modulatory domain and a channel domain. The modulatory domain contains binding sites for FKBP12 (aa 1400–1401) and Ca2+-calmodulin (CaM; aa 1564–1585), putative ATP-binding sites (aa 1773–1778, 1775–1780 and 2016–2021) and Ser residues for phosphorylation by protein kinase A (P; residues 1588 and 1755). Binding sites for chromogranins A and B (CGA/B) are located in channel domain (between the 5th and 6th transmembrane regions). In the present study, we used a part of the modulatory domain as bait. cDNAs of six bait constructs (designated MD1–MD6) were prepared as described in the Experimental section. These constructs cover the part of modulatory domain comprising amino acids 1245–2264.
Expression in S19 cells of recombinant IP₃R1 lacking the channel domain

DNA encoding the N-terminal region of mIP₃R1 (residues 1–225) was inserted into the glutathione S-transferase (GST) fusion vector pGEX-KG. The GST–IP₃R1-(1–225) fragment was subcloned into the baculovirus transfer vector pBlueBac4.5 (Invitrogen). The 3′-region downstream from the Smal site of GST–IP₃R1-(1–225) was replaced with the Smal/EcoRI fragment of mIP₃R1 (corresponding to residues 79–2217) to generate the construct GST–IP₃R1-(1–2217) (termed GST-EL). Recombinant baculovirus carrying GST-EL was generated with a Bac-N-Blue™ Transfection Kit (Invitrogen) according to the manufacturer’s protocols. GST-EL was expressed in 2 × 10⁶ S19 cells by infection with recombinant baculoviruses at a multiplicity of infection of 5, and incubation for 48 h. Cells expressing GST-EL were homogenized in 10 mM Hepes (pH 7.4), 100 mM NaCl, 2 mM EDTA, 1 mM 2-mercaptoethanol, 0.1% Triton X-100 and protease inhibitors with a glass/Teflon homogenizer. The homogenate was centrifuged at 20,000 g for 30 min. GST-EL was purified from the supernatant using glutathione–Sepharose 4B (Amersham Biosciences) according to the recommendations of the vendor.

Identification of binding sites using the two-hybrid assay

To determine binding sites, truncated constructs of IP₃R1 and CARP were prepared using pGBT9 and pGAD-GL respectively. IP₃R1 truncated constructs contained the following amino acids: pGBT9-Δ1, aa 1387–1464; pGBT9-Δ2, aa 1387–1520; pGBT9-Δ3, aa 1387–1598; pGBT9-Δ4, aa 1513–1598; pGBT9-Δ5, aa 1513–1647. CARP truncated constructs contained the following amino acids: pGAD-GL-ΔC1, aa 1–127; pGAD-GL-ΔC2, aa 1–147; pGAD-GL-ΔC3, aa 1–170; pGAD-GL-ΔC4, aa 1–180; pGAD-GL-ΔC5, aa 1–217; pGAD-GL-ΔC6, aa 1–234; pGAD-GL-ΔC7, aa 45–291; pGAD-GL-ΔC8, aa 121–291; pGAD-GL-ΔC9, aa 184–291. All plasmid constructs were sequenced to confirm that cloning of the appropriate cDNAs was in-frame for transcription.

[^H]IP₃ binding assay

[^H]IP₃ binding to IP₃R1 was assayed by poly(ethylene glycol) precipitation in the presence or absence of CARP, as reported previously [26]. A 0.5 µg portion of purified IP₃R1 [21,29] was incubated with or without 10 µg of purified CARP–His, in 50 µl of a solution containing 50 mM Tris/HCl, pH 8.0, 1 mM EDTA, 1 mM 2-mercaptoethanol, 9.6 nM[^H]IP₃ and various concentrations of IP₃ for 10 min at 4 °C. Non-specific binding was measured in the presence of 10 µM IP₃.

RESULTS AND DISCUSSION

IP₃R is modulated by various agents, including Ca²⁺ [12,13], Ca²⁺—calmodulin [14,15,30–32], FKB12 [16,17], ATP [14,18,33,34] and protein kinases [19–24]. Most of these bind or phosphorylate the central portion of IP₃R; this domain is therefore considered to modulate IP₃R function. It is relatively diverse among IP₃R family members, and is modified differently by different modulators, presumably to produce unique channel properties appropriate to distinct circumstances. In the present study, we screened a mouse brain cDNA library using the yeast two-hybrid method, with the modulatory domain of IP₃R as bait. We divided amino acids 1245–2264 of mIP₃R1 into six overlapping fragments (Figure 1).
Figure 2  Tissue distribution of CARP

(A) The tissue distribution of CARP was determined by Western blot of protein from the indicated mouse tissues. The soluble fraction (10 µg/lane) was subjected to SDS/5 %-PAGE, transferred to nitrocellulose, and immunodetected with 2 µg/ml anti-CARP polyclonal antibody. (B) Co-expression of CARP and IP₃R₁ in cultured Purkinje cells. Cultured Purkinje cells were immunostained with anti-CARP antibody (left; green) and anti-IP₃R₁ antibody (middle; red). Arrowheads show examples of co-localization of CARP and IP₃R₁.

The primary screen with the bait constructs MD₁, MD₃, MD₄, MD₅ and MD₆ yielded some tens of candidates that were unlikely to be IP₃R₁-binding proteins, because of translational frame shifting or the presence of regions known to be untranslated sequences. We did, however, obtain 13 positive clones using the MD₂ construct. DNA sequence analysis revealed that all encoded CARP; the clones contained different lengths of 5′ untranslated region of CARP cDNA followed by the full-length coding region in-frame.

Although the MD₁ and MD₂ constructs included a putative FKBP₁₂-binding site [16,17], we did not find FKBP in positive clones in this yeast two-hybrid screening. Our observation may support a recent report that FKBP₁₂ does not bind to IP₃R₁ [35]. Alternatively, it may be due to amplitude of the cDNA library (i.e. FKBP may not be in our cDNA library) or the requirement for additional protein(s) for binding of FKBP to IP₃R₁. The MD₂ construct also has a calmodulin-binding site. However, we did not detect binding of calmodulin in this screening, because calmodulin binding to IP₃R₁ is Ca²⁺-dependent.

CARP was originally identified by the screening of Purkinje cell-specific genes [36]. CARP is predicted to be composed of 291 amino acids, and has an acidic amino acid cluster of 16 Glu and four Asp residues within the N-terminal 50 amino acids [36]. CARP has a central carbonic anhydrase motif, but lacks carbonic anhydrase activity due to the absence of catalytic zinc coordinating residues. Indeed, it has been reported that CARP has no carbonic anhydrase activity [36,37]. So far, the function of CARP is unknown. The human CARP gene has also been cloned [38], and shown to be evolutionarily highly conserved with its mouse orthologue (98% identical in amino acid residues), suggestive of one or more key roles in cellular function.

Tissue distribution and subcellular localization of CARP

CARP is known to be highly expressed in Purkinje cells of the cerebellum, but its tissue distribution has not been extensively studied. We first examined the tissue distribution of CARP by Western blot analysis. Figure 2(A) shows the expression of CARP in the soluble fraction from various organs. CARP was expressed predominantly in the cerebellum as reported previously, where IP₃R₁ is also expressed abundantly. Low-level expression was observed in the cerebrum, olfactory bulb, olfactory epithelium,
vomeronasal organ, lung, submandibular gland, liver, adrenal gland, stomach, small intestine and large intestine. No signal was observed in the heart, thymus, spleen, pancreas, ovary, uterus, testis or muscle.

Immunohistochemistry of the cerebellum showed, as reported previously, that CARP is expressed predominantly in the cytoplasm of cerebellar Purkinje cells, coincident with IP₃R1 (results not shown) [3,36,39,40]. Expression of IP₃R1 in Purkinje cells is abundant and widespread, but not homogeneous, especially in dendrites, due to the formation of clusters [41]. If CARP binds to IP₃R1, the distribution of CARP also would not be homogeneous, and could co-localize with IP₃R1 clusters, although CARP is a cytosolic soluble protein. To define the subcellular localization of CARP and IP₃R1, we prepared primary cultured Purkinje cells for immunohistochemical analysis. Figure 2(B) showed double staining of CARP (green) and IP₃R1 (red) expression in Purkinje cells. Both proteins were expressed in cytoplasm, dendrites and axons (Figure 2B). Subcellular localization revealed that CARP co-localized with IP₃R1 clusters (Figure 2B, arrowheads). Abundant and highly specialized co-expression of CARP and IP₃R1 and their co-localization in Purkinje cells suggests physiological coupling of these proteins through binding.

**Biochemical analysis of the interaction of CARP with IP₃R1**

We next examined the interaction between CARP and IP₃R1 by biochemical methods to obtain further evidence for the interaction, using pull-down experiments. We first developed an expression system in Sf9 cells to make soluble IP₃R1 by removing the channel domain. The soluble IP₃R1, designated GST-EL, comprises both the ligand-binding domain and the modulatory domain (amino acids 1–2217 of mIP₃R1), and has GST attached to its N-terminus. A mouse cerebellar cytosolic fraction was incubated with GST-EL or GST, and binding of CARP to the recombinant proteins was analysed by immunoblotting with anti-CARP antibody. Figure 3(A) shows that CARP bound specifically to GST-EL, and not to GST alone. In the reciprocal experiment, the detergent extract of mouse cerebellar microsomes was processed for pull-down assays with GST–CARP, and binding of IP₃R1 was analysed using anti-IP₃R1 antibody. IP₃R1 interacted with GST–CARP, but not with GST (Figure 3B). To determine whether the binding of CARP to IP₃R1 is direct, purified His-tagged CARP was pulled down with GST-EL. As shown in Figure 3(C), CARP–His₆ bound specifically to GST-EL, indicating that the interaction between CARP and IP₃R1 is direct. Together with the results of the yeast two-hybrid screening, these findings show that CARP is a novel IP₃R1-binding protein.

**Determination of mutual interaction domains in CARP and IP₃R1**

To determine the respective interaction domains of CARP and IP₃R1, truncated mutants of both genes were prepared and analysed using the yeast two-hybrid system. Figure 4 shows a schematic representation of the deletion mutants and the results of β-galactosidase (β-gal) assay in the yeast two-hybrid system (n=3). The β-gal assay was evaluated by the time of blue colony appearance: +++, 30 min; ++, 2 h; +, 8 h.
found that CARP is a novel IP3R1-binding protein, and is expressed in Purkinje cells abundantly. CARP is co-localized with IP3R1 in Purkinje cells. CARP binds to IP3R1 and reduces the affinity of the receptor for its ligand, IP3. This could be a cause of the low IP3 sensitivity of IP3-induced Ca2+ release in Purkinje cells.

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REFERENCES

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