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

Junji HIROTA*[1,2,3], Hideaki ANDO*†[1], Kozo HAMADA† and Katsuhiko MIKOSHIBA*†‡[2]

*Division of Molecular Neurobiology, Department of Basic Medical Sciences, Institute of Medical Science, The University of Tokyo, 4-6-1 Shirokanedai, Minato-ku, Tokyo 108-8639, Japan, †The Laboratory for Developmental Neurobiology, Brain Science Institute, RIKEN (The Institute of Physical and Chemical Research), 2-1 Hirosawa, Wako, Saitama, 351-0198, Japan, and ‡Calcium Oscillation Project, International Cooperative Research Project (ICORP), Japan Science and Technology Corporation (JST), 3-14-4-Shirokanedai, Minato-ku, Tokyo 108-0071, Japan

The inositol 1,4,5-trisphosphate (IP_3) receptor (IP_3R) is an intracellular IP_3-gated Ca^{2+} channel that is located on intracellular Ca^{2+} stores and modulates Ca^{2+} signalling. Using the yeast two-hybrid system, we screened a mouse brain cDNA library with bait constructs for mouse IP_3R type 1 (IP_3R1) to identify IP_3R1-associated proteins. In this way, we found that carbonic anhydrase-related protein (CARP) is a novel IP_3R1-binding protein. Western blot analysis revealed that CARP is expressed exclusively in Purkinje cells of the cerebellum, in which IP_3R1 is abundantly expressed. Immunohistochemical analysis showed that the subcellular localization of CARP in Purkinje cells is coincident with that of IP_3R1. Biochemical analysis also showed that CARP is co-precipitated with IP_3R1. Using deletion mutagenesis, we established that amino acids 45–291 of CARP are essential for its association with IP_3R1, and that the CARP-binding site is located within the modulatory domain of IP_3R1 amino acids 1387–1647. CARP inhibits IP_3 binding to IP_3R1 by reducing the affinity of the receptor for IP_3. As reported previously, sensitivity to IP_3 for IP_3-induced Ca^{2+} release in Purkinje cells is low compared with that in other tissues. This could be due to co-expression of CARP with IP_3R in Purkinje cells and its inhibitory effects on IP_3 binding.

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

INTRODUCTION

The inositol 1,4,5-trisphosphate (IP_3) receptor (IP_3R), an IP_3-gated Ca^{2+} channel located on intracellular Ca^{2+} stores, plays a crucial role in a variety of cell functions by converting IP_3 signalling into Ca^{2+} signalling [1,2]. Molecular cloning studies have revealed that the IP_3R family consists of at least three subtypes [3–5]. Structural and functional studies have shown that IP_3Rs are composed of three functional domains: a ligand-binding domain, a modulatory domain and a channel domain [6]. An N-terminal stretch of approx. 600 amino acids in the large cytoplasmic domain is responsible for IP_3 binding [7–10]. Mutational analysis has revealed that three amino acid residues in this region [Arg-265, Lys-508 and Arg-511 of mouse IP_3R type 1 (mIP_3R1)] are critical for IP_3 binding, and that Arg-658 is a determinant of binding specificity for various inositol phosphates [10]. The six membrane-spanning segments clustered near the C-terminus correspond to the Ca^{2+} channel [11]. With the exception of the region between membrane-spanning regions 5 and 6, high sequence identity is maintained between channel domains of the IP_3R family.

The modulatory domain of IP_3R1 is located between the N-terminal IP_3-binding domain and the C-terminal Ca^{2+} channel domain. This domain contains binding sites for various modulators, such as Ca^{2+} [12,13], Ca^{2+}–calmodulin [14,15], FK506-binding protein 12K (FKBP12) [16,17] and ATP [14,18], and also sites for phosphorylation by cAMP-dependent protein kinase [19–21], cGMP-dependent protein kinase [22] and protein kinase C [23,24]. This modulatory domain may transduce IP_3 binding to channel opening. IP_3-induced Ca^{2+} release may be regulated by various modifications to the modulatory domain. Interestingly, the identity of this region among members of the IP_3R family is low in comparison with that of other regions, and this results in different modifications in diffeent subtypes, which suggests that each type of IP_3R/Ca^{2+} channel function may be modulated differently to produce unique channel properties.

In the present study, we have screened a mouse brain cDNA library using the yeast two-hybrid system to identify novel IP_3R1-binding proteins. We used six overlapping fragments within amino acids 1245–2264 of the modulatory domain of IP_3R1 as bait constructs. We have thus identified a novel interaction between IP_3R1 and carbonic anhydrase-related protein (CARP), a novel IP_3R1-binding protein. Here we report the properties of CARP as an IP_3R1-binding protein and its effects on IP_3R1 function.

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; IP_3, D-*myo*-inositol 1,4,5-trisphosphate; IP_3R, IP_3 receptor; (m)IP_3R1, (mouse) IP_3R type 1; CARP, carbonic anhydrase-related protein; GST, glutathione S-transferase; GST-EL, GST–IP_3R1-(1–2217) construct.

[1] These authors contributed equally to this work.

[2] To whom correspondence should be addressed (e-mails hirotaj@mail.rockefeller.edu and mikosiba@ims.u-tokyo.ac.jp).

[3] Present address: Department of Developmental Biology and Neurogenetics, The Rockefeller University, 1230 York Ave. New York, NY 10021, U.S.A. (e-mail hirotaj@mail.rockefeller.edu).

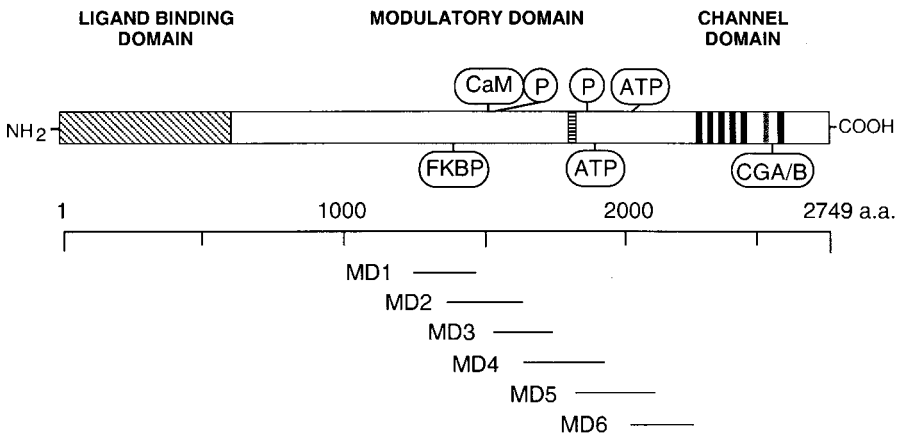

Figure 1 Schematic diagram of the baits of IP_3R1

Shown is the structure of mIP_3R1, 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 Ca^{2+}–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.

Design and synthesis of the bait constructs

We used part of the IP_3R1 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 IP_3R1 is underlined), which map to amino acid residues 1245–2264 and are overlapping. MD1 (mIP_3R1 aa 1245–1464): sense strand, 5′-GGGAATTCGGTGGAGGTTGTGCAGGCAACCAGCAGAATC-3′; antisense strand, 5′-GGGGGTCGACGTGTTGTTACAGGCCCTGCAGA-3′; MD2 (mIP_3R1 aa 1387–1647): sense, 5′-GGGAATTCGGTGGAGGCAAGAATGTGTACACGGAGA-3′; antisense, 5′-GGGGGTCGACCTCACATTTCCTCCTGGCATC-3′; MD3 (mIP_3R1 aa 1593–1785): sense, 5′-GGGAATTCGGAGGAGGATCCAGAGACTACCGAAATATCAT-3′; antisense, 5′-GGGGGTCGACGCTTGTGGAACTAGATCCAGGACC-3′; MD4 (mIP_3R1 aa 1685–1943): sense, 5′-GGGAATTCGGTGGAGGAGACAGAGGCTATGGAGAGAAG-3′; antisense, 5′-GGGGGTCGACTCCCCAGACTGGTAATGGTCA-3′; MD5 (mIP_3R1 aa 1865–2160): sense, 5′-GGGAATTCGGTGGAGGAGTGGCCCAGCAGGAAATCAA-3′; antisense, 5′-GGGGGTCGACGGTGAGCGAGGATGTAGATG-3′; MD6 (mIP_3R1 aa 2015–2264): sense, 5′-GGGGGTCGACCCGGAGGAGGCCTTGGTCTTCTTGG-3′; antisense, 5′-GGGGGTCGACAGACAGGCTGGGCTCGAAGT-3′. The resulting cDNAs were digested with either *Eco*RI plus *Sal*I or *Sal*I 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 *Eco*RI adapters. After digestion with *Eco*RI, 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 *Eco*RI site of pGAD GL. Approx. 5×10^5 independent clones were generated in *Escherichia coli* XL1-Blue MRF' (Stratagene, La Jolla, CA, U.S.A.), and plasmid DNA was isolated after one amplification.

Preparation of antibodies

Monoclonal antibodies against mIP_3R1, 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 [0.32 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

of primary and secondary antibodies were used for double staining: rabbit anti-CARP polyclonal antibody and FITC-conjugated anti-rabbit IgG antibody, anti-IP_3R1 monoclonal antibody, 18A10 and Texas Red-conjugated anti-rat IgG antibody.

Expression in Sf9 cells of recombinant IP_3R1 lacking the channel domain

DNA encoding the N-terminal region of mIP_3R1 (residues 1–225) was inserted into the glutathione S-transferase (GST) fusion vector pGEX-KG. The GST–IP_3R1-(1–225) fragment was subcloned into the baculovirus transfer vector pBlueBac4.5 (Invitrogen). The 3′-region downstream from the *Sma*I site of GST–IP_3R1-(1–225) was replaced with the *Sma*I/*Eco*RI fragment of mIP_3R1 (corresponding to residues 79–2217) to generate the construct GST–IP_3R1-(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 2×10^8 Sf9 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.

Expression of recombinant CARP in *E. coli*

Full-length CARP cDNA was cloned into the *Nde*I and *Hin*dIII sites of the pET23a vector (Stratagene) in-frame for transcription, and the His tag was introduced into the C-terminus of recombinant CARP. A single colony of *E. coli* BL21 (DE3) transformed with the CARP expression vector was selected into 1.5 ml of Luria–Bertani medium containing 100 μg/ml ampicillin and incubated at 37 °C for 10 h. Then 1 ml of the culture was inoculated into 1 litre of Luria–Bertani medium containing 100 μg/ml ampicillin and incubated at 25 °C until it reached a D_{600} of 0.7, when the culture was supplemented with isopropyl β-D-thiogalactoside (final concentration 0.5 mM). Incubation was continued at 25 °C for 8 h, and cells were harvested by centrifugation, washed with 10 ml of PBS and sonicated in 50 ml of PBS at 4 °C. After centrifugation to remove insoluble material, the supernatant was applied to a High-Trap Chelating column (Amersham Biosciences). Recombinant CARP was purified first according to the manufacturer's protocol and then by Mono Q anion-exchange chromatography (Amersham Biosciences), and finally dialysed against the buffer used in the pull-down experiment or the IP_3-binding experiment to exchange buffer.

For preparation of a GST fusion protein with recombinant CARP, full-length CARP cDNA was cloned into the *Bam*HI and *Xho*I sites of pGEX-KG to generate the GST–CARP construct. GST–CARP was expressed in *E. coli* and purified using glutathione–Sepharose as described above.

Biochemical analysis of CARP binding to IP_3R1

Adult mouse cerebella were homogenized in 10 mM Hepes (pH 7.4), 320 mM sucrose, 2 mM EDTA, 1 mM 2-mercaptoethanol and protease inhibitors, and the homogenate was centrifuged at 1000 ***g*** for 10 min. The supernatant was centrifuged at 100 000 ***g*** for 60 min to obtain the cytosolic fraction (the supernatant) and the crude microsome (the pellet). The cytosolic fraction was added to 100 mM NaCl and incubated with 20 μg of GST-EL or GST for 2 h at 4 °C. After addition of 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-mercaptoethanol and 0.01 % Triton X-100), and bound proteins were eluted with 20 mM glutathione. Eluted proteins were analysed by Western blotting with anti-CARP antibody.

The crude microsomes were solubilized with 1 % Triton X-100 in 50 mM Hepes (pH 7.4), 2 mM EDTA, 1 mM 2-mercaptoethanol and protease inhibitors for 30 min at 4 °C, and centrifuged at 20 000 ***g*** for 30 min. The supernatants were processed for pull-down assays with 10 μg of GST–CARP or GST as described above, and bound proteins were subjected to immunoblot analysis with the anti-IP_3R1 antibody KM1112.

For the direct binding assay, purified CARP–His_6 (5 μg) in wash buffer was pulled down with 20 μg of GST-EL or GST as described above, and bound proteins were subjected to immunoblot analysis with anti-CARP antibody. Experiments were repeated at least three times to confirm results.

Identification of binding sites using the two-hybrid assay

To determine binding sites, truncated constructs of IP_3R1 and CARP were prepared using pGBT9 and pGAD-GL respectively. IP_3R1 truncated constructs contained the following amino acids: pGBT9-ΔI1, aa 1387–1464; pGBT9-ΔI2, aa 1387–1520; pGBT9-ΔI3, aa 1387–1598; pGBT9-ΔI4, aa 1513–1598; pGBT9-ΔI5, 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.

$[^3H]IP_3$ binding assay

$[^3H]IP_3$ binding to IP_3R1 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 IP_3R1 [21,29] was incubated with or without 10 μg of purified CARP–His_6 in 50 μl of a solution containing 50 mM Tris/HCl, pH 8.0, 1 mM EDTA, 1 mM 2-mercaptoethanol, 9.6 nM $[^3H]IP_3$ and various concentrations of IP_3 for 10 min at 4 °C. Non-specific binding was measured in the presence of 10 μM IP_3.

RESULTS AND DISCUSSION

IP_3R is modulated by various agents, including Ca^{2+} [12,13], Ca^{2+}–calmodulin [14,15,30–32], FKBP12 [16,17], ATP [14,18,33,34] and protein kinases [19–24]. Most of these bind or phosphorylate the central portion of IP_3R; this domain is therefore considered to modulate IP_3R function. It is relatively diverse among IP_3R 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 IP_3R1 as bait. We divided amino acids 1245–2264 of mIP_3R1 into six overlapping fragments (Figure 1).

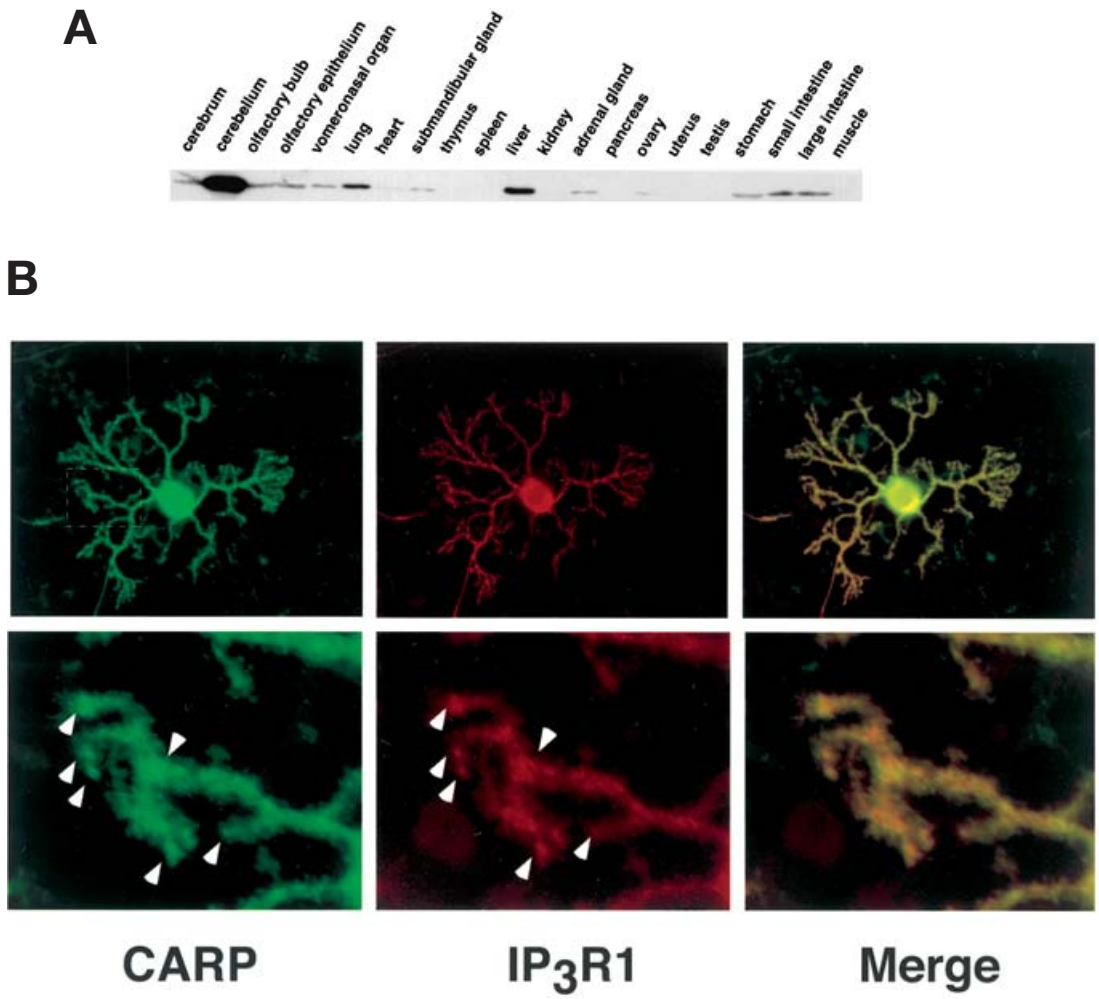

Figure 2 Tissue distribution of CARP

(**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 IP_3R1 in cultured Purkinje cells. Cultured Purkinje cells were immunostained with anti-CARP antibody (left; green) and anti-IP_3R1 antibody (middle; red). Arrowheads show examples of co-localization of CARP and IP_3R1.

The primary screen with the bait constructs MD1, MD3, MD4, MD5 and MD6 yielded some tens of candidates that were unlikely to be IP_3R1-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 IP_3R1 [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 IP_3R1. The MD2 construct also has a calmodulin-binding site. However, we did not detect binding of calmodulin in this screening, because calmodulin binding to IP_3R1 is Ca^{2+}-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 co-ordinating 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 IP_3R1 is also expressed abundantly. Low-level expression was observed in the cerebrum, olfactory bulb, olfactory epithelium,

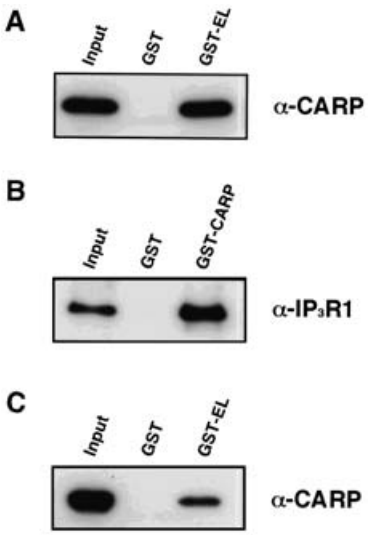

Figure 3 Biochemical analysis of the interaction of CARP with IP_3R1

(**A**) The mouse cerebellar cytosolic fraction was incubated with GST-EL or GST. Bound proteins were pulled down with glutathione–Sepharose, eluted with glutathione and analysed by Western blotting using anti-CARP antibody. (**B**) The detergent extract of mouse cerebellar microsomes was processed for a pull-down assay with GST–CARP or GST as described in (**A**), and bound proteins were subjected to immunoblot analysis with anti-IP_3R1 antibody KM1112. (**C**) Purified CARP–His_6 was pulled down with GST-EL or GST, and bound proteins were subjected to immunoblot analysis with anti-CARP antibody.

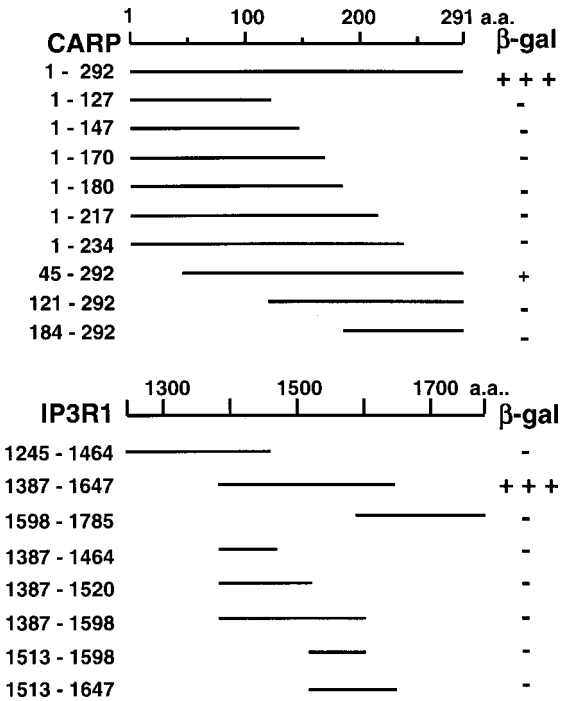

Figure 4 Determination of mutual interaction domains in CARP and IP_3R1

The structures of the N-terminal and C-terminal deletion mutants of CARP and IP_3R1 are shown. Interactions between CARP mutants and MD2, CARP and IP_3R1 mutants were analysed using the yeast two-hybrid system. The figure shows a schematic representation of the prepared deletion mutants and 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.

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 IP_3R1 (results not shown) [3,36,39,40]. Expression of IP_3R1 in Purkinje cells is abundant and widespread, but not homogeneous, especially in dendrites, due to the formation of clusters [41]. If CARP binds to IP_3R1, the distribution of CARP also would not be homogeneous, and could co-localize with IP_3R1 clusters, although CARP is a cytosolic soluble protein. To define the subcellular localization of CARP and IP_3R1 in Purkinje cells, we prepared primary cultured Purkinje cells for immunohistochemical analysis. Figure 2(B) showed double staining of CARP (green) and IP_3R1 (red) expression in Purkinje cells. Both proteins were expressed in cytoplasm, dendrites and axons (Figure 2B). Subcellular localization revealed that CARP co-localized with IP_3R1 clusters (Figure 2B, arrowheads). Abundant and highly specialized co-expression of CARP and IP_3R1 and their co-localization in Purkinje cells suggests physiological coupling of these proteins through binding.

Biochemical analysis of the interaction of CARP with IP_3R1

We next examined the interaction between CARP and IP_3R1 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 IP_3R1 by removing the channel domain. The soluble IP_3R1, designated GST-EL, comprises both the ligand-binding domain and the modulatory domain (amino acids 1–2217 of mIP_3R1), 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 IP_3R1 was analysed using anti-IP_3R1 antibody. IP_3R1 interacted with GST–CARP, but not with GST (Figure 3B). To determine whether the binding of CARP to IP_3R1 is direct, purified His-tagged CARP was pulled down with GST-EL. As shown in Figure 3(C), CARP–His_6 bound specifically to GST-EL, indicating that the interaction between CARP and IP_3R1 is direct. Together with the results of the yeast two-hybrid screening, these findings show that CARP is a novel IP_3R1-binding protein.

Determination of mutual interaction domains in CARP and IP_3R1

To determine the respective interaction domains of CARP and IP_3R1, 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 assays. We detected weak binding activity following deletion of the N-terminal 44 amino acids of CARP, and any further deletions abolished binding to IP_3R1. This indicates that the minimum binding site of CARP to IP_3R1 comprises amino acids 45–291. In the case of deletion of IP_3R1, we found that amino acids 1387–1647 are necessary for binding to CARP.

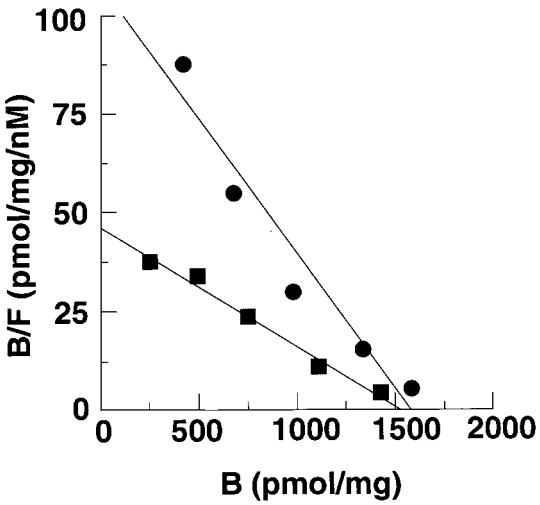

Figure 5 Inhibitory effects of CARP on IP_3 binding to IP_3R1

Scatchard analysis of the inhibition of specific [^{3}H]IP_3 binding by IP_3 to the purified IP_3R1 in the presence (■) or absence (●) of CARP. The result of a typical experiment performed in duplicate is shown. The K_d values (mean ± S.D.) from three separate experiments performed in duplicate in the presence and absence of CARP were 33.5 ± 2.07 nM and 18.2 ± 4.58 nM respectively.

Effect of CARP on binding of IP_3 to IP_3R1

IP_3-induced Ca^{2+} release in intact Purkinje cells is known to require a much higher concentration of IP_3 (EC_{50} 10 μM) [42] than in other tissues or with isolated IP_3R1 (EC_{50} 100 nM–1 μM) [14,21,29,42]. Since CARP is highly expressed with IP_3R1 in Purkinje cells and binds IP_3R1, we speculated that the lower sensitivity of IP_3R1 to IP_3 in Purkinje cells might be due to an inhibitory effect of CARP on IP_3 binding, and accordingly analysed the effects of CARP on IP_3R1 in terms of IP_3 binding.

We used purified IP_3R1 in binding assays to evaluate the effects of CARP on the affinity of binding of IP_3 to IP_3R1. Purified IP_3R1 was incubated with various concentration of IP_3 in the presence or absence of purified recombinant CARP. A Scatchard plot is shown in Figure 5. Scatchard analysis indicated that the dissociation constant (K_d) of the binding of IP_3 to purified IP_3R1 in the presence and absence of CARP was 33.5 ± 2.07 nM and 18.2 ± 4.58 nM respectively, but that the maximal number of IP_3-binding sites was unaltered by CARP (B_{max} values in the presence and absence of CARP were 1630 ± 108 and 1720 ± 234 pmol/mg respectively). These results suggest that CARP inhibits IP_3 binding to IP_3R1 by lowering the affinity of the receptor for IP_3. Given that the CARP-binding site of IP_3R1 is distinct from the IP_3-binding site, it is likely that CARP changes the conformation of IP_3R1 by binding and reduces sensitivity to IP_3, rather than causing competitive inhibition between the CARP- and IP_3-binding sites. A high IP_3R1 density has been proposed to be one of the factors contributing to a low sensitivity to IP_3 for IP_3-induced Ca^{2+} release in Purkinje cells [43]. In addition to this, we now provide an additional explanation, i.e. that an inhibitory effect of CARP on IP_3 binding to IP_3R1 results in low sensitivity to IP_3 in Purkinje cells.

Conclusions

In conclusion, we screened a mouse brain cDNA library to identify IP_3R1-binding proteins using the yeast two-hybrid system. We found that CARP is a novel IP_3R1-binding protein, and is expressed in Purkinje cells abundantly. CARP is co-localized with IP_3R1 in Purkinje cells. CARP binds to IP_3R1 and reduces the affinity of the receptor for its ligand, IP_3. This could be a cause of the low IP_3 sensitivity of IP_3-induced Ca^{2+} release in Purkinje cells.

We thank Ms M. Iwai for technical assistance, Dr M. Oguro-Okano, Dr T. Michikawa and Dr T. Furuichi for fruitful discussions, and Dr A. C. F. Perry for critical reading of the manuscript.

REFERENCES

1 Berridge, M. J. (1993) Inositol trisphosphate and calcium signalling. Nature (London) **361**, 315–325
2 Mikoshiba, K. (1997) The InsP3 receptor and intracellular Ca^{2+} signaling. Curr. Opin. Neurobiol. **7**, 339–345
3 Furuichi, T., Yoshikawa, S., Miyawaki, A., Wada, K., Maeda, N. and Mikoshiba, K. (1989) Primary structure and functional expression of the inositol 1,4,5-trisphosphate-binding protein P400. Nature (London) **342**, 32–38
4 Blondel, O., Takeda, J., Janssen, H., Seino, S. and Bell, G. I. (1993) Sequence and functional characterization of a third inositol trisphosphate receptor subtype, IP3R-3, expressed in pancreatic islets, kidney, gastrointestinal tract, and other tissues. J. Biol. Chem. **268**, 11356–11363
5 Yamamoto-Hino, M., Sugiyama, T., Hikichi, K., Mattei, M. G., Hasegawa, K., Sekine, S., Sakurada, K., Miyawaki, A., Furuichi, T., Hasegawa, M. and Mikoshiba K. (1994) Cloning and characterization of human type 2 and type 3 inositol 1,4,5-trisphosphate receptors. Receptors Channels **2**, 9–22
6 Furuichi, T., Kohda, K., Miyawaki, A. and Mikoshiba, K. (1994) Intracellular channels. Curr. Opin. Neurobiol. **4**, 294–303
7 Mignery, G. A. and Sudhof, T. C. (1990) The ligand binding site and transduction mechanism in the inositol-1,4,5-triphosphate receptor, EMBO J. **9**, 3893–3898
8 Miyawaki, A., Furuichi, T., Ryou, Y., Yoshikawa, S., Nakagawa, T., Saitoh, T. and Mikoshiba, K. (1991) Structure-function relationships of the mouse inositol 1,4,5-trisphosphate receptor. Proc. Natl. Acad. Sci. U.S.A. **88**, 4911–4915
9 Newton, C. L., Mignery, G. A. and Sudhof, T. C. (1994) Co-expression in vertebrate tissues and cell lines of multiple inositol 1,4,5-trisphosphate (InsP3) receptors with distinct affinities for InsP3. J. Biol. Chem. **269**, 28613–28619
10 Yoshikawa, F., Morita, M., Monkawa, T., Michikawa, T., Furuichi, T. and Mikoshiba, K. (1996) Mutational analysis of the ligand binding site of the inositol 1,4,5-trisphosphate receptor. J. Biol. Chem. **271**, 18277–18284
11 Michikawa, T., Hamanaka, H., Otsu, H., Yamamoto, A., Miyawaki, A., Furuichi, T., Tashiro, Y. and Mikoshiba, K. (1994) Transmembrane topology and sites of N-glycosylation of inositol 1,4,5-trisphosphate receptor. J. Biol. Chem. **269**, 9184–9189
12 Mignery, G. A., Johnston, P. A. and Sudhof, T. C. (1992) Mechanism of Ca^{2+} inhibition of inositol 1,4,5-trisphosphate (InsP3) binding to the cerebellar InsP3 receptor. J. Biol. Chem. **267**, 7450–7455
13 Sienaert, I., Missiaen, L., De Smedt, H., Parys, J. B., Sipma, H. and Casteels, R. (1997) Molecular and functional evidence for multiple Ca^{2+}-binding domains in the type 1 inositol 1,4,5-trisphosphate receptor. J. Biol. Chem. **272**, 25899–25906
14 Maeda, N., Kawasaki, T., Nakade, S., Yokota, N., Taguchi, T., Kasai, M. and Mikoshiba, K. (1991) Structural and functional characterization of inositol 1,4,5-trisphosphate receptor channel from mouse cerebellum. J. Biol. Chem. **266**, 1109–1116
15 Yamada, M., Miyawaki, A., Saito, K., Nakajima, T., Yamamoto-Hino, M., Ryo, Y., Furuichi, T. and Mikoshiba, K. (1995) The calmodulin-binding domain in the mouse type 1 inositol 1,4,5-trisphosphate receptor. Biochem. J. **308**, 83–88
16 Cameron, A. M., Steiner, J. P., Sabatini, D. M., Kaplin, A. I., Walensky, L. D. and Snyder, S. H. (1995) Immunophilin FK506 binding protein associated with inositol 1,4,5-trisphosphate receptor modulates calcium flux. Proc. Natl. Acad. Sci. U.S.A. **92**, 1784–1788
17 Cameron, A. M., Nucifora, Jr, F. C., Fung, E. T., Livingston, D. J., Aldape, R. A., Ross, C. A. and Snyder, S. H. (1997) FKBP12 binds the inositol 1,4,5-trisphosphate receptor at leucine-proline (1400–1401) and anchors calcineurin to this FK506-like domain. J. Biol. Chem. **272**, 27582–27588
18 Maes, K., Missiaen, L., Parys, J. B., Sienaert, I., Bultynck, G., Zizi, M., De Smet, P., Casteels, R. and De Smedt, H. (1999) Adenine-nucleotide binding sites on the inositol 1,4,5-trisphosphate receptor bind caffeine, but not adenophostin A or cyclic ADP-ribose. Cell Calcium **25**, 143–152

19 Supattapone, S., Danoff, S. K., Theibert, A., Joseph, S. K., Steiner, J. and Snyder, S. H. (1988) Cyclic AMP-dependent phosphorylation of a brain inositol trisphosphate receptor decreases its release of calcium. Proc. Natl. Acad. Sci. U. S. A. **85**, 8747–8750
20 Hajnoczky, G., Gao, E., Nomura, T., Hoek, J. B. and Thomas, A. P. (1993) Multiple mechanisms by which protein kinase A potentiates inositol 1,4,5-trisphosphate-induced Ca^{2+} mobilization in permeabilized hepatocytes. Biochem. J. **293**, 413–422
21 Nakade, S., Rhee, S. K., Hamanaka, H. and Mikoshiba, K. (1994) Cyclic AMP-dependent phosphorylation of an immunoaffinity-purfied homotetrameric inositol 1,4,5-trisphosphate receptor (type I) increases Ca^{2+} flux in reconstituted lipid vesicles. J. Biol. Chem. **269**, 6735–6742
22 Komalavilas, P. and Lincoln, T. M. (1996) Phosphorylation of the inositol 1,4,5-trisphosphate receptor. Cyclic GMP-dependent protein kinase mediates cAMP and cGMP dependent phosphorylation in the intact rat aorta. J. Biol. Chem. **271**, 21933–21938
23 Ferris, C. D., Huganir, R. L., Bredt, D. S., Cameron, A. M. and Snyder, S. H. (1991) Inositol trisphosphate receptor: phosphorylation by protein kinase C and calcium calmodulin-dependent protein kinases in reconstituted lipid vesicles. Proc. Natl. Acad. Sci. U.S.A. **88**, 2232–2235
24 Cameron, A. M., Steiner, J. P., Roskams, A. J., Ali, S. M., Ronnett, G. V. and Snyder, S. H. (1995) Calcineurin associated with the inositol 1,4,5-trisphosphate receptor-FKBP12 complex modulates Ca^{2+} flux. Cell **83**, 463–472
25 Maeda, N., Niinobe, M., Nakahira, K. and Mikoshiba, K. (1988) Purification and characterization of P400 protein, a glycoprotein characteristic of Purkinje cell, from mouse cerebellum. J. Neurochem. **51**, 1724–1730
26 Maeda, N., Niinobe, M. and Mikoshiba, K. (1990) A cerebellar Purkinje cell marker P400 protein is an inositol 1,4,5-trisphosphate (InsP3) receptor protein. Purification and characterization of InsP3 receptor complex. EMBO J. **9**, 61–67
27 Sugiyama, T., Furuya, A., Monkawa, T., Yamamoto-Hino, M., Satoh, S., Ohmori, K., Miyawaki, A., Hanai, N., Mikoshiba, K. and Hasegawa, M. (1994) Monoclonal antibodies recognizing the subtypes of inositol 1,4,5-trisphosphate receptor: Application to the studies on inflammatory cells. FEBS Lett. **354**, 149–154
28 Yuzaki, M. and Mikoshiba, K. (1992) Pharmacological and immunocytochemical characterization of metabotropic glutamate receptors in cultured Purkinje cells. J. Neurosci. **12**, 4253–4263
29 Hirota, J., Michikawa, T., Miyawaki, A., Furuichi, T., Okura, I. and Mikoshiba, K. (1995) Kinetics of calcium release by immunoaffinity-purified inositol 1,4,5-trisphosphate receptor in reconstituted lipid vesicles. J. Biol. Chem. **270**, 19046–19051
30 Hirota, J., Michikawa, T., Natsume, T., Furuichi, T. and Mikoshiba, K. (1999) Calmodulin inhibits inositol 1,4,5-trisphosphate-induced calcium release through the purified and reconstituted inositol 1,4,5-trisphosphate receptor type 1. FEBS Lett. **456**, 322–326
31 Michikawa, T., Hirota, J., Kawano, S., Hiraoka, M., Yamada, M., Furuichi, T. and Mikoshiba, K. (1999) Calmodulin mediates calcium-dependent inactivation of the cerebellar type 1 inositol 1,4,5-trisphosphate receptor. Neuron **23**, 799–808
32 Patel, S., Morris, S. A., Adkins, C. E., O'Beirne, G. and Taylor, C. W. (1997) Ca^{2+}-independent inhibition of inositol trisphosphate receptors by calmodulin: redistribution of calmodulin as a possible means of regulating Ca^{2+} mobilization. Proc. Natl. Acad. Sci. U.S.A. **94**, 11627–11632
33 Mak, D. O., McBride, S. and Foskett, J. K. (1999) ATP regulation of type 1 inositol 1,4,5-trisphosphate receptor channel gating by allosteric tuning of Ca^{2+} activation. J. Biol. Chem. **274**, 22231–22237
34 Maes, K., Missiaen, L., De Smet, P., Vanlingen, S., Callewaert, G., Parys, J. B. and De Smedt, H. (2000) Differential modulation of inositol 1,4,5-trisphosphate receptor type 1 and type 3 by ATP. Cell Calcium **27**, 257–267
35 Bultynck, G., De Smet, P., Rossi, D., Callewaert, G., Missiaen, L., Sorrentino, V., De Smedt, H. and Parys, J. B. (2001) Characterization and mapping of the 12 kDa FK506-binding protein (FKBP12)-binding site on different isoforms of the ryanodine receptor and of the inositol 1,4,5-trisphosphate receptor. Biochem. J. **354**, 413–422
36 Kato, K. (1990) Sequence of a novel carbonic anhydrase-related polypeptide and its exclusive presence in Purkinje cells. FEBS Lett. **271**, 137–140
37 Sjoblom, B., Elleby, B., Wallgren, K., Jonsson, B. H. and Lindskog, S. (1996) Two point mutations convert a catalytically inactive carbonic anhydrase-related protein (CARP) to an active enzyme. FEBS Lett. **398**, 322–325
38 Skaggs, L. A., Bergenhem, N. C., Venta, P. J. and Tashian, R. E. (1993) The deduced amino acid sequence of human carbonic anhydrase-related protein (CARP) is 98 % identical to the mouse homologue. Gene **126**, 291–292
39 Nogradi, A., Jonsson, N., Walker, R., Caddy, K., Carter, N. and Kelly, C. (1997) Carbonic anhydrase II and carbonic anhydrase-related protein in the cerebellar cortex of normal and lurcher mice. Brain Res. Dev. Brain Res. **98**, 91–101
40 Furuichi, T., Simon-Chazottes, D., Fujino, I., Yamada, N., Hasegawa, M., Miyawaki, A., Yoshikawa, S., Guenet, J.-L. and Mikoshiba, K. (1993) Widespread expression of inositol 1,4,5-trisphosphate receptor type 1 gene (insp3rl) in the mouse central nervous system. Receptors Channels **1**, 11–24
41 Takei, K., Stukenbrok, H., Metcalf, A., Mignery, G. A., Sudhof, T. C., Volpe, P. and De, C. P. (1992) Ca^{2+} stores in Purkinje neurons: endoplasmic reticulum subcompartments demonstrated by the heterogeneous distribution of the InsP3 receptor, Ca^{2+}-ATPase, and calsequestrin. J. Neurosci. **12**, 489–505
42 Khodakhah, K. and Ogden, D. (1993) Functional heterogeneity of calcium release by inositol trisphosphate in single Purkinje neurones, cultured cerebellar astrocytes, and peripheral tissues. Proc. Natl. Acad. Sci. U. S. A. **90**, 4976–4980
43 Ogden, D. and Capiod, T. (1997) Regulation of Ca^{2+} release by InsP3 in single guinea pig hepatocytes and rat Purkinje neurons. J. Gen. Physiol. **109**, 741–756

Received 14 January 2003/11 February 2003; accepted 3 March 2003
Published as BJ Immediate Publication 3 March 2003, DOI 10.1042/BJ20030110