A new inositol 1,4,5-trisphosphate binding protein similar to phospholipase C-δ_1

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We have reported that two inositol 1,4,5-trisphosphate binding proteins, with molecular masses of 85 and 130 kDa, were purified from rat brain; the former protein was found to be the δ_1-isoenzyme of phospholipase C (PLC-δ_1) and the latter was an unidentified novel protein [Kanematsu, Takeya, Watanabe, Ozaki, Yoshida, Koga, Iwanaga and Hirata (1992) J. Biol. Chem. **267**, 6518–6525]. Here we describe the isolation of the full-length cDNA for the 130 kDa Ins(1,4,5)P_3 binding protein, which encodes 1096 amino acids. The predicted sequence of the 130 kDa protein had 38.2 % homology to that of PLC-δ_1. Three known domains of PLC-δ_1 (pleckstrin homology and putative catalytic X and Y domains) were located at residues 110–222, 377–544 and 585–804 with 35.2 %, 48.2 % and 45.8 % homologies respectively. However, the protein showed no PLC activity to phosphatidylinositol 4,5-bisphosphate and phosphatidylinositol. The 130 kDa protein expressed by transfection in COS-1 cells bound Ins(1,4,5)P_3 in the same way as the molecule purified from brain. Thus the 130 kDa protein is a novel Ins(1,4,5)P_3 binding protein homologous to PLC-δ_1, but with no catalytic activity. The functional significance of the 130 kDa protein is discussed.

INTRODUCTION

D-*myo*-Inositol 1,4,5-trisphosphate, a product of the receptor-activated hydrolysis of phosphatidylinositol 4,5-bisphosphate, plays an important role as an intracellular second messenger by mobilizing Ca^{2+} from non-mitochondrial store sites [1]. Ins(1,4,5)P_3 is metabolized by two known routes. One is dephosphorylation, catalysed by Ins(1,4,5)P_3 5-phosphatase present in both cytosol and membrane fractions of cells, the result being formation of Ins(1,4)P_2, which is subsequently degraded to free inositol by other phosphatase activities [2]. Alternatively, phosphorylation of the 3-hydroxyl group of Ins(1,4,5)P_3 by an ATP-dependent kinase present in the cell cytosol produces Ins(1,3,4,5)P_4 [3]. Three types of proteins previously identified are Ins(1,4,5)P_3-interacting macromolecules: Ins(1,4,5)P_3 receptors on the endoplasmic reticulum involved in Ca^{2+} release [4,5] and two types of enzyme related to Ins(1,4,5)P_3 metabolism. In 1985 we described for the first time the chemical modification of Ins(1,4,5)P_3 [6]. This analogue has the azidobenzoyl group at the C-2 position for photoaffinity labelling and causes irreversible inactivation of the receptor protein for Ca^{2+} release, after photolysis. On the basis of these findings and the report [7] that biological activities of Ins(1,4,5)P_3 are related to two adjacent phosphates at C-4 and C-5 and the phosphate at C-1 increases the affinity for its recognition by the receptor site, we attempted further chemical modifications of Ins(1,4,5)P_3 at the C-2 position and we examined their biological effects on the above-mentioned Ins(1,4,5)P_3-recognizable proteins [8,9]. The analogues were designed to enable further functionalization for Ins(1,4,5)P_3-immobilized matrices, which proved to be useful for purifying the known Ins(1,4,5)P_3-interacting proteins [10].

In this laboratory, two proteins with molecular masses of 130 and 85 kDa have been isolated as novel Ins(1,4,5)P_3 binding proteins from rat brain, by using the Ins(1,4,5)P_3-immobilized matrices [11,12]. Partial amino acid sequence determination revealed that the 85 kDa molecule is phospholipase C-δ_1 (PLC-δ_1). We thus asked whether the recombinant molecule of PLC-δ_1 prepared with an *Escherichia coli* expression system could bind Ins(1,4,5)P_3 and we further carried out truncation experiments to determine the region of PLC-δ_1 responsible for Ins(1,4,5)P_3 binding [13]. The data obtained clearly indicate that the first 60 amino acids from the N-terminus of the molecule are required for Ins(1,4,5)P_3 binding but not for enzymic activity. The synthetic peptide, consisting of 14 amino acids corresponding to residues 30–43 of PLC-δ_1 and containing six basic amino acids, was investigated for its ability to bind Ins(1,4,5)P_3; antibody raised against the peptide was also examined for its ability to block Ins(1,4,5)P_3 binding to the peptide and to the native form of PLC-δ_1, revealing that the region of residues 30–43 of PLC-δ_1 could be a minimal domain examined so far for the binding of Ins(1,4,5)P_3 by PLC-δ_1 [14].

On the other hand, little is known about the 130 kDa molecule, except for the fact that it could be a novel Ins(1,4,5)P_3 binding protein [11,12]. In the present study, we isolated the full-length cDNA encoding the 130 kDa protein, expressed the product in COS-1 cells, and further characterized this intriguing protein.

EXPERIMENTAL

Materials

[α-^{32}P]dCTP (specific radioactivity 110 TBq/mmol), [γ-^{32}P]ATP (specific radioactivity 110 TBq/mmol) and deoxyadenosine

Abbreviations uses: PH domain, pleckstrin homology domain; PLC, phospholipase C.

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5′-[[α-^{35}S]-thio]-triphosphate (specific radioactivity > 37 TBq/mmol) were obtained from Amersham (U.K.). [^{3}H]Ins(1,4,5)P_3 (specific radioactivity 777 GBq/mmol), [^{3}H]PtdIns (specific radioactivity 458.8 GBq/mmol) and [^{3}H]PtdIns(4,5)P_2 (specific radioactivity 222 GBq/mmol) were from Du Pont-New England Nuclear (Boston, MA, U.S.A.). cDNA synthesis and sequencing kits were obtained from Stratagene (La Jolla, CA, U.S.A.) and United States Biochemicals (Cleveland, OH, U.S.A.). DNA labelling kits and a site-directed mutagenesis kit were from TaKaRa (Kyoto, Japan). Various DNA-modifying enzymes and restriction endonucleases were purchased from Nippon Gene (Toyama, Japan) and TaKaRa. pT7BlueT vector was from Novagen (Madison, WI, U.S.A.). LipofectAMINE reagent was from GIBCOBRL (Gaithersburg, MD, U.S.A.). Biotinylated goat anti-(mouse IgG) antibodies was from Amersham. An alkaline phosphatase staining kit was obtained from PIERCE (Rockford, IL, U.S.A.). A prestained molecular mass marker set for SDS/PAGE and sheets for Western blotting were obtained from Bio-Rad (Richmond, CA, U.S.A.). All other reagents were of the highest grade available.

Poly(A)$^+$ RNA extraction and fractionation

Total poly(A)$^+$ RNA was prepared from rat brains and fractionated by centrifugation on a sucrose density gradient (5–20 %) as described previously [15]. Identification of fractions containing the 130 kDa protein mRNA was performed by PCR [16] with the following mixed oligonucleotides as primers based on the 130 kDa protein partial amino acid sequences [11] (KNTETFXNNGLADQITED; P-22); TK-1 5′-AA(A/G)AA-(T/C)AC(A/G/C/T)GA(A/G)AC(T/C/A/G)TT-3′ and an antisense TK-2 5′-TC(T/C)TC(A/G/C/T)GT(A/G/T)AT-(T/C)TG(A/G)TC-3′. A 53 bp DNA fragment was obtained in fractions 10–12. The 53 bp DNA fragment was inserted into the pT7 blue (R) vector and the nucleotide was sequenced by the dideoxynucleotide chain termination method [17] using the Sequenase DNA-sequencing kit.

Construction and screening of the cDNA library

The poly(A)$^+$ RNA fraction (fraction 10) enriched for the 130 kDa protein mRNA was used for construction of the following cDNA library. The cDNA library was synthesized with Uni-ZAP XR vector and was screened with ^{32}P-labelled 53-bp DNA fragment, prepared by PCR and labelled with a polynucleotide 5′ end labelling kit. Plaques of positive clones (pcMT1–3) were isolated, and bacteriophage DNAs were digested with restriction enzymes and characterized by restriction endonuclease mapping.

DNA sequencing

Restriction fragments of isolated 130 kDa protein cDNA (pcMT3) were subcloned into pBluescript, pUC118 and pUC119 [18]. Both strands of all regions were sequenced by the dideoxynucleotide chain termination method [17] with the Sequenase DNA-sequencing kit.

Expression of cDNA constructs in COS-1 cells

A single *Xho*I site in the coding region of pcMT3 was destroyed by a silent mutation [Arg^{24} (CGA^{538}) to (CGT^{538})] directed with a synthetic oligonucleotide 5′-CGACCCTCGTGCCGGCGC-CGAT-3′. To generate a new *Xho*I site [(C^{390},T^{391}) to (T^{390},C^{391})] in the 5′-non-coding region of pcMT3, synthetic oligonucleotide 5′-CCTTCTGGTGCTCGAGCGCACCCTTA-3′ was used as a primer. The designed cDNA was digested with *Xho*I and inserted into the *Xho*I site of the mammalian expression vector *Xho*I/pSG5, which was modified by introducing *Eco*RV-*Xho*I-*Not*I (GATATCTCGAGAGCGGCCGC) sites between *Eco*RI and *Bgl*II sites of pSG5 (Stratagene). The orientation was confirmed by restriction endonuclease mapping. The plasmid (pcMT31, 20 μg) was transfected into 5×10^6 COS-1 cells by using liposomes (LipofectAMINE reagent) in accordance with the manufacturer's protocol. The transfected cells were cultured in Dulbecco's modified Eagle's medium containing 10 % (w/v) fetal calf serum in 10 cm dishes for 2 days.

Polyacrylamide-gel electrophoresis and immunoblotting

The proteins were separated in SDS/PAGE (8 % gel) and transferred to a nitrocellulose membrane. The membrane was incubated for 1 h with monoclonal antibody (2F9) against the 130 kDa protein [12] after blocking with 5 % (w/v) dried milk solution, and then bound antibodies were revealed by the alkaline phosphatase reaction using biotinylated goat anti-(mouse IgG) and an avidin–biotin complex kit.

Assay of [^{3}H]Ins(1,4,5)P_3 binding

Transfected COS-1 cells were harvested in a buffer solution containing 50 mM NaCl, 10 mM Hepes buffer (pH 8.0), 1 mM EDTA, 1 mM NaN_3, 10 mM 2-mercaptoethanol and several protease inhibitors, and lysed by passing cells through a 26-gauge needle 15 times, followed by a centrifugation at 100000 ***g*** for 60 min to obtain the cytosol fraction. The fraction was assayed for the binding of [^{3}H]Ins(1,4,5)P_3 as described [10].

RESULTS

Molecular cloning and the structure of the 130 kDa Ins(1,4,5)P_3 binding protein

Screening of a rat-brain cDNA library enriched for encoding the 130 kDa protein yielded three positive clones (pcMT1–3). Figure 1 shows the restriction map and sequencing strategy of pcMT3 with the longest cDNA insert. As shown in Figure 2(a), the sequence thus determined consisted of 5233 nucleotides containing a single open reading frame, beginning at nucleotide 467 and ending at 3754. The protein is therefore predicted to comprise 1096 amino acids and to have a molecular mass of 122.8 kDa, almost identical to the apparent size of the isolated molecule [11,12]. The amino acid sequences of three lysylendopeptidase-cleaved peptides from the purified protein are identified in the deduced amino acid sequence (P-22, NTETFXNNGLADQIC-EDXXF, residues 172–191; P-14, XPLXFMEGNQNTPXF, 228–242; and P-13, AIESFAXNIXV, 1024–1034), indicating that the clone we obtained is genuine for encoding the 130 kDa protein. The hydropathy profile of the deduced protein sequence does not show any significant hydrophobic segments shared with other transmembrane proteins (results not shown). The determined nucleotide and amino acid sequences were subjected to a homology search on the EMBL, GenBank, PIR and SWISS-PROT databases. The 130 kDa protein has an amino acid sequence (95–850) that has 38.2 % homology to that of PLC-δ_1 (1–756). PLC-δ_1 carries three known domains, pleckstrin homology (PH), and putative catalytic X and Y domains, in its sequence [19,20]. When sequence homology was examined with special reference to these domains, it was found that a PH domain and catalytic X and Y domains were localized in regions 110–222, 377–544 and 585–844 of the 130 kDa protein amino

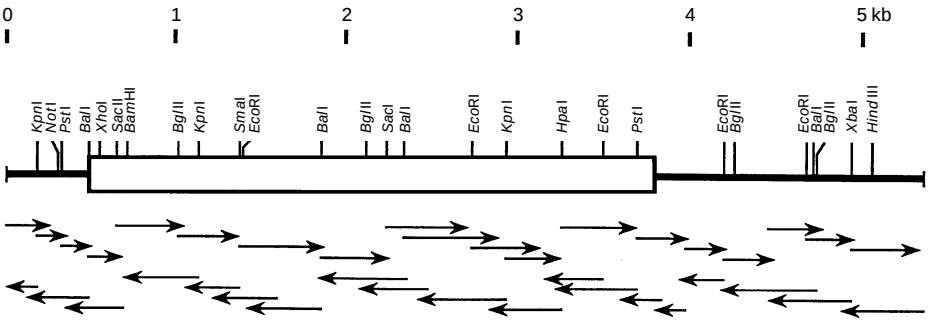

Figure 1 Restriction map and sequencing strategy for cDNA clones of 130 kDa protein

Arrows indicate the direction and extent of each sequence determination. The protein-coding region is indicated by a thick open bar.

acid sequence, with 35.2 %, 48.2 % and 45.8 % scores respectively (Figure 2b). However, the 130 kDa protein has no PLC activities ([11,12], and see below).

Expression of 130 kDa Ins(1,4,5)P_3 binding protein

The expression plasmid pcMT31, carrying the entire protein-coding sequence, was constructed and transfected into 5×10^6 COS-1 cells. The vector alone was also transfected into COS-1 cells as a control. Transient expression of the 130 kDa protein was detected by Western blotting analysis probed with anti-(130 kDa protein) antibodies (2F9) [12]. As shown in Figure 3(a), one band with an apparent molecular mass of 130 kDa was recognized by 2F9 antibody in the extract from COS-1 cells transfected with pcMT31, but not in the control extract. The positive band with the same molecular mass was observed in a cytosol fraction from rat brain.

The expressed protein in the cytosol fraction was examined for its capacity for binding Ins(1,4,5)P_3. As shown in Figure 3(b), the cytosol fraction of COS-1 cells transfected with pcMT31 exhibited a specific binding of [^{3}H]Ins(1,4,5)P_3, whereas no significant [^{3}H]Ins(1,4,5)P_3 binding was observed with the same amount of control cytosol, which is free from Ins(1,4,5)P_3 receptor located in the membrane fraction. Binding specificity to recombinant 130 kDa protein was examined by using D-enantiomers of several inositol polyphosphates and PtdIns(4,5)P_2 as inhibitors (Figure 3c). The binding specificity of the expressed protein was virtually identical with that of the 130 kDa protein isolated from brain [11,12]. PtdIns(4,5)P_2 could also act as an inhibitor, albeit with a lower affinity, for the binding of [^{3}H]Ins(1,4,5)P_3. The 130 kDa protein seems to be capable of binding Ins(1,4,5,6)P_4 with the same affinity as for Ins(1,4,5)P_3. On the other hand, PLC-δ_1 showed about 10-fold less affinity for Ins(1,4,5,6)P_4 than for Ins(1,4,5)P_3 [12]. The affinity of Ins(3,4,5,6)P_4 for both 130 kDa protein and PLC-δ_1 was almost the same; i.e. 15–30-fold less affinity than Ins(1,4,5)P_3 [12].

Because the 130 kDa protein was found to be similar to PLC-δ_1, one would expect that the 130 kDa protein has an intrinsic PLC activity. However, the 130 kDa protein isolated from rat brain did not show any PLC activity towards PtdIns(4,5)P_2 [11,12]. Recombinant 130 kDa protein was therefore assayed for PLC activity. The cell extracts were assayed for PLC activity at pH 7.2 at a free Ca^{2+} concentration of 10 μM, with [^{3}H]PtdIns(4,5)P_2 at 50 μM {containing 22000 d.p.m. in [^{3}H]PtdIns(4,5)P_2 per tube} as a substrate, by the method of Smrcka et al. [21]. The overexpressed and control cell extracts exhibited intrinsic PLC activities of 2215 ± 105 and 2492 ± 185 d.p.m. in [^{3}H]Ins(1,4,5)P_3 formed per 0.5 μg of protein extract for 5 min ($n = 7$) respectively. The enzyme assays were also performed at different free Ca^{2+} concentrations ranging from 0.1 to 100 μM. However, there was no difference in the activities between transfected and control cell extracts. Furthermore [^{3}H]PtdIns at 100 μM (containing 44000 d.p.m. in [^{3}H]PtdIns per tube) was also used as a substrate for the assay of PLC activity at various free Ca^{2+} concentrations. For instance, at a free Ca^{2+} concentration of 100 μM and pH of 7.2, the radioactivity released was 798 ± 68 and 830 ± 62 d.p.m. per μg of protein extract per 5 min ($n = 4$) from the transfected and control extracts respectively. These results indicate that recombinant 130 kDa protein has no intrinsic PLC activity towards PtdIns(4,5)P_2 and PtdIns. Because the deduced sequence of the 130 kDa protein contains several possible phosphorylatable sites for cyclic-AMP-dependent kinase and protein kinase C, the cell extract was also assayed for PLC activity after the preparation had been treated with the catalytic subunit of cyclic-AMP-dependent kinase and/or protein kinase C plus phorbol ester together with Mg-ATP. However, there was no change in the activities. The enzyme activities of cell extracts were next examined together with recombinant PLC-δ_1 to determine whether the extract had an inhibitory action on exogenous PLC-δ_1, but there was no difference in the activities between experimental and control extracts.

These results clearly indicate that the 130 kDa protein is a new Ins(1,4,5)P_3/Ins(1,4,5,6)P_4 binding protein homologous to PLC-δ_1, but without PLC catalytic activity. Furthermore the 130 kDa protein showed no dephosphorylating activities towards several inositol polyphosphates [11] and no activities of Ins(1,4,5,6)P_4 3-kinase and Ins(1,2,4,5,6)P_5 3-kinase (M. Hirata and S. B. Shears, unpublished work).

DISCUSSION

In this study we isolated cDNA encoding the 130 kDa protein from an enriched λZAPII cDNA library of rat brain by using the oligonucleotides complementary to peptide sequences determined previously [11,12] and found that the protein comprises 1096 amino acids and has a molecular mass of 122.8 kDa. The cDNA we isolated here is a genuine one for 130 kDa protein for the following reasons: (i) the amino acid sequence deduced from an open reading frame of cDNA contained three peptide sequences that we had determined with peptides from a purified sample of the 130 kDa protein; (ii) the recombinant protein molecule expressed in COS-1 cells was recognized with monoclonal

(a)

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AGGCAGCCGCCGCCGCACTTCCTGAGGCCGCCGCTCCGCGTCGGCGGGCAGGTGGCTGGTGGCCGGTCCGCAGTCGCCGCTCGGGCCGCGCGCGTCCAGGCTTCGGAGGGTTCGGAGGCT 120
CCGGGCTCCGGCGCCCGCTGTCTTTTGTCTGCGCAGAGCCGGCGCTCGCCTCCCCTCGCCGGTACCTCCCGCTGCTTTTCGCCTGCTTCTCCCTCCCGCTCACATCTTCTCCCCGCCCCC 240
GCCGCCGGTCCGCGCCGGCCGCTGACCTCCTGGACCACAGCCCGAGGACGCCTCTGCCCGAGTGATGTCTCCTCTCTACAAAGTTGCGGCCGCCGCTGCAGCCGCCGTCGCGCGCTGAAA 360
CAAAGTCTAGCGGCGTTTCCTTCTGGTGCCTGAGCGCACCCTTACTCAGGGCTGGACGCCGCTGGCGGTCGTGCTGTCTCGCCGGGAGCCTAGAACGTGCTTGGCCATGGCTGAGGGCGC 480
                                                                                                                  M  A  E  G  A   5
GGCTAGCAGGGAGGCTCCGGCGCCCTCTCGACGTTGCGGGGGGCGAAGACGACCCTCGAGCCGGCGCCGATGCTGCCTCCGGGGACGCTGCACCCGAGGCCTCGGGGGGCCGGATGAGGGA 600
A  S  R  E  A  P  A  P  L  D  V  A  G  G  E  D  D  P  R  A  G  A  D  A  A  S  G  D  A  A  P  E  A  S  G  G  R  M  R  D   45
CCGTCGCAGCGGGGTTGCGCTGCCGGGCAATGCGGGGGTCCCCGCGGATAGCGAGGCTGGCCTCCTGGAGGCGGCACGAGCGACCCCCCGGCGCACGAGCATCATCAAGGATCCTTCAAA 720
R  R  S  G  V  A  L  P  G  N  A  G  V  P  A  D  S  E  A  G  L  L  E  A  A  R  A  T  P  R  R  T  S  I  I  K  D  P  S  N   85
CCAAAAATGTGGTGGCAGAAAGAAAACAGTGTCTTTCAGCAGCATGCCATCGGAGAAGAAGATTAGCAGTGCCCATGACTGCATTAGCTTCATGCAGGCTGGCTGCGAGCTGAAGAAAGT 840
Q  K  C  G  G  R  K  K  T  V  S  F  S  S  M  P  S  E  K  K  I  S  S  A  E  D  C  I  S  F  M  Q  A  G  C  E  L  K  K  V   125
GCGACCGAACTCTCGCATCTACAACCGATTTTTCACCCTAGACACTGACCTCCAGGCTCTACGCTGGGAACCTTCCAAGAAAGACCTCGAAAAAGCCAAGCTGGACATTTCTGCCATCAA 960
R  P  N  S  R  I  Y  N  R  F  F  T  L  D  T  D  L  Q  A  L  R  W  E  P  S  K  K  D  L  E  K  K  L  D  I  S  A  I  K   165
AGAGATCAGGCTGGGGAAGAACACGGAGACATTCAGAAACAATGGCCTTGCTGACCAGATCTGTGAAGACTGTGCCTTCTCCATACTCCATGGGGAAAATTACGAGTCTCTGGACCTAGT 1080
E  I  R  L  G  K  N  T  E  T  F  R  N  N  G  L  A  D  Q  I  C  E  D  C  A  F  S  I  L  H  G  E  N  Y  E  S  L  D  L  V   205
                P-22
TGCCAATTCAGCAGATGTAGCAAACATCTGGGTGTCGGGGTTACGGTACCTGGTCTCTCGCAGTAAGCAACCACTTGACTTTATGGAGGGCAACCAGAACACACCACGGTTCATGTGGCT 1200
A  N  S  A  D  V  A  N  I  W  V  S  G  L  R  Y  L  V  S  R  S  K  Q  P  L  D  F  M  E  G  N  Q  N  T  P  R  F  M  W  L   245
                                                                  P-14
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K  T  V  F  E  A  A  D  V  D  G  N  G  I  M  L  E  D  T  S  V  E  L  I  K  Q  L  N  P  T  L  K  E  S  K  I  R  L  K  F   285
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K  E  I  Q  K  S  K  E  K  L  T  T  R  V  T  E  E  E  F  C  E  A  F  C  E  L  C  T  R  P  E  V  Y  F  L  L  V  Q  I  S   325
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K  N  K  E  Y  L  D  A  N  D  L  M  L  F  L  E  V  E  Q  G  V  T  H  V  T  E  D  M  C  L  D  I  I  R  R  Y  E  L  S  E   365
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D  G  R  Q  K  G  F  L  A  I  D  G  F  T  Q  Y  L  L  S  P  E  C  D  I  F  D  P  E  Q  K  K  V  A  Q  D  M  T  Q  P  L   405
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S  H  Y  Y  I  N  A  S  H  N  T  Y  L  I  E  D  Q  F  R  G  P  A  D  I  N  G  Y  V  R  A  L  K  M  G  C  R  S  I  E  L   445
TGATGTAAGTGACGGTCCAGATAATGAACCCATCCTGTGCAATAGGAACAACATGGCCATGCTCCTTTCCTTTCGAAGTGTTCTGGAGGTGATAAATAAGTTCGCCTTCGTCGCTTCAGA 1920
D  V  S  D  G  P  D  N  E  P  I  L  C  N  R  N  N  M  A  M  L  L  S  F  R  S  V  L  E  V  I  N  K  F  A  F  V  A  S  E   485
GTACCCACTCATTCTCTGCTTGGGGAATCACTGTTCCCTGCCACAGCAGAGGGTCATGGTTCAGCAGATGAAAAAGGTGTTTGGCAATAAACTTTATACAGAAGCACCTCTGTCTTCAGA 2040
Y  P  L  I  L  C  L  G  N  H  C  S  L  P  Q  Q  R  V  M  V  Q  Q  M  K  K  V  F  G  N  K  L  Y  T  E  A  P  L  S  S  E   525
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S  R  R  V  S  G  D  Y  N  G  E  Q  K  H  I  W  L  C  R  E  L  S  D  L  V  S  I  C  K  S  V  Q  Y  R  D  F  E  L  S  M   605
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S  A  M  R  I  D  S  S  N  L  N  P  Q  D  F  W  N  C  G  C  Q  I  V  A  M  N  F  Q  T  P  G  P  M  M  D  L  H  T  G  W   685
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F  L  Q  N  G  G  C  G  Y  V  L  R  P  S  I  M  R  D  E  V  S  Y  F  S  A  N  T  K  G  I  V  P  G  V  S  P  L  L  L  H   725
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G  D  E  F  I  G  Q  Y  T  I  P  F  E  C  L  Q  P  G  Y  R  H  V  P  L  R  S  F  V  G  D  I  M  E  H  V  T  L  F  V  H   845
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D  I  F  K  I  A  V  H  P  L  R  E  A  I  D  M  R  E  N  M  Q  N  A  I  V  S  V  K  E  L  C  G  L  P  P  I  A  S  L  K   925
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Q  C  L  L  T  L  S  S  R  L  I  T  S  D  S  T  P  S  V  S  L  V  M  K  D  C  F  P  Y  L  E  P  L  G  T  I  P  D  V  Q   965
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K  R  M  L  A  A  Y  D  L  M  I  Q  E  S  R  V  L  I  E  M  A  D  T  V  Q  E  K  I  V  Q  C  Q  K  A  G  M  E  F  H  E  1005
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                                                    P-13
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E  K  E  S  S  E  E  N  G  K  L  *                                                                                        1096
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TTATTTCTGACTGTTCTTAATACTACTCTGTTCTTGTCGCACATTACCAAAAGAGTAAAGATACTGAAAACAG                                                5233
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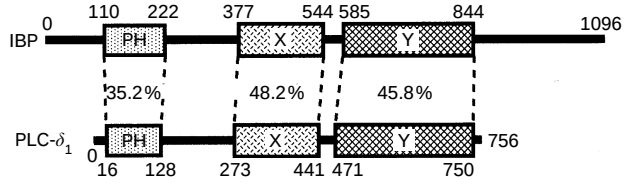
(b)



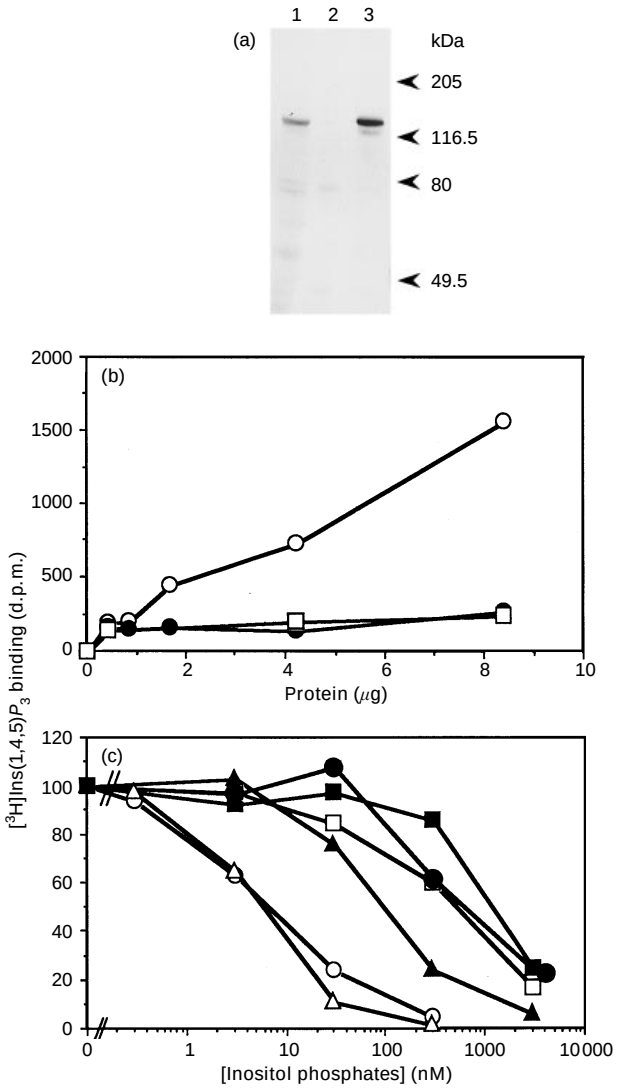


Figure 3 Expression of 130 kDa protein gene in COS-1 cells

(**a**) Western blot analysis: lane 1, rat brain cytosol (50 μg); lane 2, control cell extract (5 μg); lane 3, experimental cell extract (5 μg). (**b**) [^{3}H]Ins(1,4,5)P_3 binding activity. ○, Experimental cell extract; ●, plus 1 μM unlabelled Ins(1,4,5)P_3; □, control cell extract. Each point represents the mean of duplicate determinations. Four other experiments with different samples of extract gave essentially the same results. (**c**) Binding specificity. [^{3}H]Ins(1,4,5)P_3 binding to experimental cell extract (10 μg) was assayed in the presence of various concentrations of inositol polyphosphates. In the present study, the pure D-enantiomer of each inositol phopshate was used, whereas the racemic mixtures were used in the previous report [11]. ○, Ins(1,4,5)P_3; △, Ins(1,4,5,6)P_4; ▲, Ins(3,4,5,6)P_4; ■, Ins(1,4,6)P_3; □, Ins(1,3,4,6)P_4; ●, PtdIns(4,5)P_2. Each point represents the mean of duplicate determinations. Three other experiments with different samples of extract gave similar patterns of inhibition.

antibody against a purified sample of the 130 kDa protein; and (iii) the cytosol fraction of COS-1 cells transfected with the cDNA exhibited binding of Ins(1,4,5)P_3 with similar affinity and specificity to those seen with a purified sample of the 130 kDa protein.

Analysis of sequences of both the gene and the deduced amino acid with several databases revealed that the 95–850 amino acid sequence of the 130 kDa Ins(1,4,5)P_3 binding protein resembled the whole PLC-δ_1 with an overall homology of 38.2%. The protein sequences of all the intracellular PLC isoenzymes including PLC-δ_1 contain two highly conserved regions, designated X and Y, which are believed to be a catalytic region [22,23]. Although the overall similarity of the 130 kDa protein to PLC-δ_1 is 38.2%, the similarity, compared with the X and Y domains alone, increases to about 50%. Therefore one would expect that the 130 kDa protein has intrinsic PLC activity. However, the recombinant as well as a biochemically purified sample of the 130 kDa protein showed no PLC activity [11,12]. In addition, we examined whether the 130 kDa protein exerts a catalytic activity on synthetic unlabelled PtdIns(3,4,5)P_3 at 50 μM as a substrate, by measuring liberated organic phosphates; there was no activity, although the assay sensitivity [hydrolysis of more than 40% of 50 μM PtdIns(3,4,5)P_3 was required] was about one-fortieth of that with an isotope. It is possible that the 130 kDa protein might not recognize inositol phospholipids as substrates in the catalytic domains, because one amino acid replacement in the putative PtdIns(4,5)P_2 binding motif suggested by Yu et al. [24] occurs in the 130 kDa molecule, i.e. the corresponding region of the 130 kDa protein was *M*XXXK(R)XK(R)K(R), whereas the putative PtdIns(4,5)P_2 binding motif, located at the C-terminus of the X domain of all the PLC isoenzymes, is *K*(*R*)XXXK(R)XK(R)K(R) [24]. Recently, Ellis et al. [25] reported that a point mutation of a serine residue at 309 of PLC-δ_1 to an alanine residue reduced the enzyme activity to less than half of the original. In the 130 kDa protein, the corresponding amino acid is an alanine residue at 412. Thus the mutation of one or two amino acids in the conserved regions, which are critical for enzymic activity, may occur in the 130 kDa protein. Recently, Homma and Emori [26] found an activator protein for PLC-δ_1 and Rho-GTPase activating protein. This 130 kDa protein may require such an activator protein for catalysis. Alternatively, extra regions of the 130 kDa protein over PLC-δ_1, i.e. the N-terminal residues 1–94 and/or the C-terminal 851–1096 may have an inhibitory action on the catalytic activity exerted by the region similar to PLC-δ_1. However, the fact that there is no inhibition of exogenous PLC-δ_1 activity seems to make this suggestion unlikely.

The biological function of the 130 kDa protein is currently unknown, but there are several possibilities. For instance, the protein may play a role in compartmentalization of cellular Ins(1,4,5)P_3, i.e. the cellular level of Ins(1,4,5)P_3, under unstimulated conditions, seems to be high enough to evoke the release of Ca^{2+} [27]. Thus the protein could bind Ins(1,4,5)P_3 to reduce the available Ins(1,4,5)P_3 for releasing Ca^{2+} from the endoplasmic reticulum in unstimulated cells. To confirm the physiological significance of this notion, further quantitative analyses are required. Another possibility is related to the regulatory role of PLC activity. The N-terminal non-catalytic region of PLC-δ_1, the region responsible for binding Ins(1,4,5)P_3 and its parent phospholipid PtdIns(4,5)P_2 [13,14,28], allows the enzyme to anchor to PtdIns(4,5)P_2 in the surface of the

Figure 2 Nucleotide and deduced amino acid sequences of 130 kDa Ins(1,4,5)P_3 binding protein and its comparison with PLC-δ_1

(**a**) Nucleotide and deduced amino acid sequences. The nucleotides and deduced amino acids are numbered on the right. Amino acid sequences determined from tryptic peptides isolated from purified sample are underlined. The stop codons limiting the open reading frame are indicated by asterisks. (**b**) Comparison of domain structures of the 130 kDa protein (top, IBP) and PLC-δ_1 (bottom). The nucleotide sequence data reported in this paper will appear in the GSDB, DDBJ, EMBL and NCBI nucleotide sequence databases with the accession number D45920.

plasma membrane in intact cells, thus enhancing the probability that the enzyme will acquire neighbouring PtdIns(4,5)P_2 as a substrate, as first suggested by Rebecchi and colleagues [29,30]. According to this proposal, it would still be possible that the 130 kDa protein acts as an inhibitor of PLC-δ_1 inside the cells, because the 130 kDa protein competes for the membrane PtdIns(4,5)P_2 with PLC-δ_1. However, in experiments *in vitro*, cell extracts containing the 130 kDa protein showed no inhibitory effects on exogenous PLC-δ_1. Alternatively, the protein could be involved in the unknown function of Ins(1,4,5,6)P_4, because the protein seems to bind it with a high affinity, although a definite conclusion awaits the direct evaluation with [^{3}H]Ins(1,4,5,6)P_4. The cellular level of Ins(1,4,5,6)P_4 was reported to be increased, without any relation to metabolic flux of increased Ins(1,4,5)P_3, on stimulation of rat mammary cells with vasopressin [31].

PLC-δ_1 is known as one of the proteins that carry a PH domain in its N-terminal region [19,32]. The PH domain is defined by six weakly conserved sequence blocks (six subdomains) and within subdomain 6, a single tryptophan residue is conserved in all proteins carrying a PH domain [19,33]. Furthermore, within subdomain 1, 3, 5 or 6, one or two hydrophobic residues are well conserved in many proteins with a PH domain, including PLC-δ_1 [19,32]. These residues are implicated in the formation of a hydrophobic core that is assumed to stabilize the three-dimensional structure of the PH domain [33]. The common tryptophan residue is located at position 215 in the 130 kDa protein, and other common residues are: leucine at position 122 in subdomain 1; leucine at 145 in subdomain 3; phenylalanine at 191 and isoleucine at 193 in subdomain 5; and leucine at 202 and 204, valine at 205 and 216, and leucine at 219 in subdomain 6. Therefore the 130 kDa protein would qualify as having a PH domain.

The domain of PLC-δ_1 responsible for the binding of Ins(1,4,5)P_3 was identified as the region of residues 30–43 [13,14], which resides at the N-terminal (subdomain 1) of the PH domain. The binding affinities and specificities of PLC-δ_1 and the 130 kDa protein were essentially the same, except for their affinities for Ins(1,4,5,6)P_4. Furthermore the region of residues 124–137 of the 130 kDa protein, corresponding to residues 30–43 of PLC-δ_1, contains four basic amino acids, which are believed to be involved in the interaction with the phosphate moieties of Ins(1,4,5)P_3 [13,14]. In addition, the Fab fragment of antibodies against residues 30–43 of PLC-δ_1 caused a dose-dependent inhibition of [^{3}H]Ins(1,4,5)P_3 binding to the 130 kDa protein (M. Hirata and M. Yoshida, unpublished work). Therefore it is most likely that the region of residues 124–137 of the 130 kDa protein is involved in the binding. However, there is a difference in the affinity for Ins(1,4,5,6)P_4. Thus the possibility cannot completely be excluded that the regions other than residues 124–137, such as residues 75–93, 151–165 or 277–294, which also contain several basic amino acids, are involved in binding Ins(1,4,5)P_3. Further experiments of gene truncation, as done with the PLC-δ_1 gene [13], would be required to locate the binding domain in the 130 kDa protein.

Harlan et al. [34] reported that the N-terminus of the PH domain of spectrin seems to be involved in the interaction with phospholipid bearing inositol polyphosphate as a polar head. However, Gibson et al. [35] argued against this idea because there is no sufficient hydrophobic pocket and poor overall surface conservation for accepting the fatty acid moieties of phospholipids. They propose that a common ligand for the PH domain would be a phosphorylated serine/threonine residue. From our data, the N-terminus of the PH domain of PLC-δ_1 specifically binds to Ins(1,4,5)P_3, and if the corresponding region of the 130 kDa protein is really involved in the binding as discussed above, the ligand for the PH domain of the 130 kDa protein is Ins(1,4,5,6)P_4 as well as Ins(1,4,5)P_3. There are more than 20 types of inositol polyphosphate inside cells [36]. We propose that some of the PH domain may specifically bind a respective inositol polyphosphate.

This is the first indication that a molecule containing putative catalytic X and Y domains has no PLC catalytic activity, which may pave the way to a clarification of the minimal requirement for PLC catalytic activity and of the regulatory mechanisms of PLC-δ_1 activation, and also to a clarification of the biological function of the 130 kDa protein with special reference to the function of the PH domain.

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