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Binding of phospholipase C-related but catalytically inactive protein to phosphatidylinositol 4,5-bisphosphate *via* the PH domain

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ABSTRACT

A well-known protein module regulating molecular interactions is the pleckstrin homology (PH) domain whose best-characterised ligand is phosphoinositide. In the present study, we analysed the PH domain from PRIP (phospholipase C-related but catalytically inactive protein, comprising types 1 and 2) regarding phosphatidylinositol 4,5-bisphosphate [PtdIns(4,5)P_2] binding employing a variety of binding assays. The PH domains prepared from PRIP-1 and -2 showed similar binding profiles to soluble ligands *in vitro* and showed similar plasma membrane localisation to that of PLC-δ1; however, the PH domain with the N-terminal extension of PRIP-1 but not PRIP-2 showed even distribution throughout the cytoplasm, indicating that the N-terminal extension of PRIP-1 inhibited binding to PtdIns(4,5)P_2 present in the plasma membrane. A chimeric molecule of PLC-δ1 PH domain with the N-terminal extension of PRIP-1 exhibited similar localisation to PRIP-1 PH domain with the N-terminal extension. Binding assay to liposomes containing various concentrations of PtdIns(4,5)P_2 revealed that the PH domain of PLC-δ1 bound steeply to the maximum, even at a concentration of 1.2 mol%, whereas the PH domains from PRIP-1 and -2 bound depending on the concentration up to 5 mol%. We also performed binding experiments using saponin-permeabilised PC12 cells. PH domains from PRIP increased the binding to cells preincubated with the brain cytosol extract in the presence of ATP, during which PtdIns(4,5)P_2 were probably synthesised. The binding of PH domain with the following EF hand motifs showed Ca^{2+}-dependent binding. These results indicate that the PH domain of PRIP binds to PtdIns(4,5)P_2 present in the plasma membrane, depending on the concentrations of the lipid ligand and Ca^{2+}, suggesting that PRIP might play physiological roles in events involved in the changes of these parameters, probably including Ins(1,4,5)P_3.

1. Introduction

Phospholipase C-related, but catalytically inactive protein (PRIP-1) was first identified in the brain cytosol fraction as a novel D-*myo*-inositol 1,4,5-trisphosphate [Ins(1,4,5)P_3] binding protein and was tentatively named p130 based on molecular size [1]. Subsequent molecular cloning studies revealed that the molecule is similar to phospholipase C-δ1 but catalytically inactive, which is the reason for the revised name [2–5]. In an attempt to explore the biological function of PRIP-1 in relation to the binding to Ins(1,4,5)P_3 *via* its pleckstrin homology (PH) domain [6,7], we performed serial experiments, first using COS-1 cells stably over-expressing PRIP-1 [8] and then cultured neurons prepared from PRIP-1 knock-out mice [9]. The results showed that both cells over-expressing and little-expressing PRIP-1 produced a reduced Ins(1,4,5)P_3-mediated Ca^{2+} increase in cells, probably for different reasons [8,9], indicating that the presence of an appropriate amount of PRIP-1 is needed to produce right Ins(1,4,5)P_3-mediated Ca^{2+} signalling.

PRIP-1 was also isolated from the membrane fraction of the brain with much the same molecular size as that from the cytosol fraction [5], indicating no lipid modification for localisation in the membrane fraction, but association to the membrane constituents. The most feasible constituent of molecules bearing PH domains would be phosphatidylinositol 4,5-bisphosphate [PtdIns(4,5)P_2], which is a minor but key phospholipid mainly on the cytoplasmic leaflet of the plasma membrane. We then performed cellular experiments using COS-1 cells transfected with genes for a variety of deletion mutants of PRIP-1 to examine whether the PH domain is implicated in membrane localisation by binding to PtdIns(4,5)P_2, and drew the tentative conclusion that the membrane association of PRIP-1 indeed occurred, but the association *via* the binding of the PH domain to PtdIns(4,5)P_2 was partial [10]. Subsequently, *in vitro* experiments using the liposomes containing PtdIns(4,5)P_2 clearly indicated that the PH domain of PRIP-1 binds PtdIns(4,5)P_2, and further Ca^{2+} at physiological concentration enhances the binding when the recombinant PH domain molecule used contains EF hand motifs, like those of PLC-δ [11,12].

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We also observed that full-length PRIP-1 was mainly localised in the cytoplasm [6,8], but the isolated PH domain (amino acid residues 82–298) was mainly seen at the surface membrane [8,13]. We further examined the recombinant molecule comprising N-terminal extension (residues 24–81) plus the PH domain regarding localisation, resulting in the main location in the cytoplasm. These results indicate that the N-terminal region of PRIP-1 preceding the PH domain would prohibit it from associating with the surface membrane. Furthermore, an isoform of PRIP-1 with relatively broad tissue distribution, including the brain, was also reported [14,15], indicating that PRIP comprises types 1 and 2.

PtdIns(4,5)P_2 has been receiving constant attention because it regulates a wide variety of processes, including exocytic and endocytic membrane traffic [16–20], ion channel and transporter function [21], enzyme activation [22], and protein recruitment [23–26]. In order to extend our researches in exploring the biological function of PRIP, probably through competing for PtdIns(4,5)P_2 with other molecules involved in the functions described above, it would be important to reevaluate whether the PH domain is involved in the membrane association of PRIP *via* binding to PtdIns(4,5)P_2.

In the present study, we examined the cellular localisation and binding to PtdIns(4,5)P_2-containing liposome of the PH domains derived from PRIP-2 as well as PRIP-1, and further evaluated the effect of N-terminal extension on the localisation of the PH domain. Finally, we examined whether the association is indeed regulated by PtdIns(4,5)P_2 *via* binding by the PH domain, employing semi-intact cellular experiments.

2. Materials and methods

2.1. Materials

[^{3}H]Ins(1,4,5)P_3 (specific radioactivity: 851.0 GBq/mmol) was purchased from PerkinElmer (Waltham, MA). Ins(1,4,5)P_3 and short-chain (C8, water-soluble) PtdIns(4,5)P_2 and natural PtdIns(4,5)P_2 were obtained from Cell Signal Inc. (Lexington, KY). Glutathione-Sepharose 4B and pGEX vectors were from GE Healthcare (Uppsala, Sweden). pEGFP-N1 vector was from Clontech (Palo Alto, CA). Phosphatidylcholine (PC) was purchased from Sigma (St. Louis, MO). Monoclonal antibody against GST was from Santa Cruz Biotechnology (Santa Cruz, CA). Other reagents used were of the highest grade available.

2.2. DNA constructs, recombinant protein expression and purification

The PH domain of human PLC-δ1 (residues 1–140), and its mutant R40L was described previously [27,28]. The long version of PH domain (1–298) of PRIP-1 (1PHL) was amplified from full-length PRIP-1 construct using the primers 5′-TAGAATTCCACCATGGCTGAGGGCGCGG-3′ and 5′-ATGTCGACCGGGTAGTTAGTTTTTCTTTGC-3′. The short version (1PHS, 74–298) was amplified using the primers 5′-TAGAATTCC-ACCATGCCCCGGCGCACGAGCATC-3′ and 5′-ATGTCGACCGGGTAGT-TAGTTTTTCTTTGC-3′. The long version of the PH domain (1–325) of PRIP-2 (2PHL) was amplified from full-length PRIP-2 using the primers 5′-TAGAATTCCACCATGGCGGAGTGCGGCC-3′ and 5′-ATGTCGACTCGG-TACCAGCTTTGTCCTTTG-3′. The short version (2PHS, 102–325) was amplified using the primers 5′-TAGAATTCCACCATGCCCCGCCGGAGCAG-CATC-3′ and 5′-ATGTCGACTCGGTACCAGCTTTGTCCTTTG-3′. After the digestion with EcoRI and SalI, the product was ligated into the pEGFP-N1 plasmid (CLONTECH) and pGEX-4T3 plasmid digested with the same enzymes. The chimeric molecule of the PLC-δ1-PH domain (1N-δPH) was created by connecting the N-terminal PRIP-1 (1–81 residues) with PLC-δ1PH (1–142 residues). The mutant form of 1PHS (1PHSm) was also generated in plasmids pGEX-4T3 and pEGFP-N1 by site-directed mutagenesis (Quick Change; Stratagene) using the primers 5′-CTCT-CGCATCTACAACCAATTTTTCACCCTAGACAC-3′ and 5′-GTGTCTAG-GGTGAAAAATTGGTTGTAGATGCGAGAG-3′ for R134Q.

For purification of recombinant PH domains conjugated with GST, *Escherichia coli* BL-21(DE3) was transformed with pGEX-4T3 constructs. The bacterial cells were grown up to 0.4 of absorbance at 600 nm at 37 °C and then with 250 μM isopropyl -β-D(−)-thiogalactopyranoside (IPTG) at 18–20 °C for an additional 12–14 h. Bacterial lysate was prepared by sonication in a lysis buffer [50 mM Tris–HCl (pH 8.0), 300 mM NaCl, 1 mM EDTA, 1 mM DTT and protease inhibitor cocktail containing 5 μg/ml pepstatin A, 10 μM leupeptin, 1.7 μg/ml aprotinin, and 50 μM 4-amidinophenylmethanesulfonyl fluoride hydrochloride], followed by rotation after the addition of 1% Triton X-100 for 20 min. Purification was achieved using Glutathione-Sepharose 4B beads. After extensive washing with a lysis buffer without protease inhibitor cocktail, the proteins were eluted with 20 mM reduced glutathione in a lysis buffer without protease inhibitor cocktail, but containing 1 mM DTT. Purity was checked by staining with Coomassie Brilliant Blue (CBB) in SDS-polyacrylamide gel electrophoresis (SDS-PAGE). The recombinant molecules of interest were dialyzed against the solution for assay for more than 6 h, followed by centrifugation at 100,000 *g* for 30 min before use.

2.3. Binding assay of recombinant proteins to [^{3}H]Ins(1,4,5)P_3

The binding to [^{3}H]Ins(1,4,5)P_3 (0.9 nM; 0.37 kBq radioactivity) of each recombinant protein (8 pmol) was performed in a reaction mixture (0.45 ml) containing 50 mM Tris–HCl (pH 8.3), 0.2% Triton X-100, and 2 mM EGTA. The reaction mixture was incubated on ice for 15 min, followed by the addition of 50 μl of 10 mg/ml bovine g-globulin and 0.5 ml of 30% (w/v) polyethyleneglycol 6000. The precipitate formed after centrifugation at 15,000 rpm for 5 min was dissolved in 0.5 ml of 0.1 N NaOH and then counted for radioactivity as an emulsion with 5 ml scintillation cocktail. Non-specific binding in the presence of 100 μM unlabelled Ins(1,4,5)P_3 was also measured (approximately 200–250 dpm) and subtracted from the values measured in its absence (range 3500–5800 dpm), enabling calculation of the specific binding.

2.4. Binding assay of recombinant proteins to liposomes

Liposomes composed of phosphatidylcholine (PC) or with PtdIns(4,5)P_2 were made according to the method described [29]. Briefly, PC alone or with PtdIns(4,5)P_2 at different concentrations dissolved in chloroform solution was dried under nitrogen to make a lipid film in a glass tube. A solution (buffer A) containing 25 mM Hepes/NaOH buffer (pH 7.4), 100 mM NaCl and 1 mM DTT was added to make a final lipid concentration of 20 mM and the mixture was sonicated twice until uniform turbidity was achieved so that small unilamellar vesicles were formed. Stock liposomes thus made were kept in a refrigerator until use (for approximately a month). The phospholipid vesicles (final conc. of total lipid was 1 mM) were mixed with purified recombinant proteins in 50 μl buffer B [20 mM Tris–HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA and 1 mM DTT]. The mixture was incubated on ice for 10 min, followed by centrifugation at 100,000 *g* for 30 min. The supernatant was carefully removed, and mixed with 5× concentrated sample buffer for SDS-PAGE to a final volume of 50 μl. The pellet was dissolved in SDS buffer up to the same volume (50 μl). Both the supernatant and the pellet were analysed by SDS-PAGE, followed by staining with CBB. Densitometric analyses were performed on the stained gel with an NIH image analyser. Bovine serum albumin was used as the standard protein to assess the linearity of protein concentration after staining with "Quick CBB" (Wako Pure Chemical Co. Ltd., Tokyo, Japan) for 30 min.

2.5. Binding assay of recombinant proteins to permeabilised PC12 cells

The binding assay of recombinant proteins to permeabilised PC12 cells was performed as described [30]. PC12 cells were permeabilised