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Recombinant p42^{IP4}, a brain-specific 42-kDa high-affinity Ins(1,3,4,5)P_4 receptor protein, specifically interacts with lipid membranes containing Ptd-Ins(3,4,5)P_3

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We have recently cloned the cDNA of p42^{IP4}, a membrane-associated and cytosolic inositol (1,3,4,5)tetrakisphosphate receptor protein [Stricker, R., Hülser, E., Fischer, J., Jarchau, T., Walter, U., Lottspeich, F. & Reiser, G. (1997) *FEBS Lett.* **405**, 229–236.] p42^{IP4} is a protein of 374 amino acids with M_r of 42 kDa. The p42^{IP4} protein has a zinc finger motif at its N-terminus, followed by two pleckstrin homology domains. To characterize further the biochemical and functional properties of p42^{IP4}, it was expressed as a glutathione-S-transferase fusion protein in Sf9 cells using a recombinant baculovirus vector. The protein was affinity adsorbed on glutathione beads, cleaved from glutathione-S-transferase with the protease factor-Xa and purified on heparin agarose. The recombinant purified protein is active because it shows binding affinities similar to those of the native p42^{IP4}, purified from pig cerebellum or rat brain (K_i for inositol(1,3,4,5)P_4 of 4.1 nM and 2.2 nM, respectively). Moreover the ligand specificity of the recombinant protein for various inositol polyphosphates is similar to that of the native protein purified from brain. Importantly, we show here that p42^{IP4} binds phosphatidylinositol(3,4,5)P_3 specifically, as the recombinant protein can associate with lipid membranes (vesicles) containing phosphatidylinositol(3,4,5)P_3; this binding occurs in a concentration-dependent manner and is blocked by inositol(1,3,4,5)P_4. This specific association and the possibility that endogenous p42^{IP4} can be converted from a membrane-associated state to a soluble state support the hypothesis that p42^{IP4} might be redistributed between cellular compartments upon hormonal stimulation.

Keywords: baculovirus; inositolP_4; membrane interaction; pleckstrin homology domain; phosphatidylinositolP_3; receptor; vesicle association.

Two second messengers, inositol 1,4,5-trisphosphate [Ins(1,4,5)P_3] and diacylglycerol, are produced from phosphatidylinositol 4,5-bisphosphate [Ptd-Ins(4,5)P_2]. The generation of Ptd-Ins(4,5)P_2 and its cleavage by phospholipase C (PLC) are tightly regulated signal transduction pathways [1]. Ins(1,4,5)P_3 mobilizes Ca^{2+} from intracellular stores whereas diacylglycerol activates protein kinase C [2]. During the last few years, the putative second messengers Ins(1,3,4,5)P_4 and Ptd-Ins(3,4,5)P_3, which are generated by phosphorylating the D3 position of the inositol ring, have attracted increasing interest. The functions of Ins(1,3,4,5)P_4 and Ptd-Ins(3,4,5)P_3 are much less clear than that of Ins(1,4,5)P_3 [2]. Ptd-Ins(3,4,5)P_3 is the product of class I phosphoinositide kinases. Phosphoinositide-3-kinase (PI3-kinase) α is regulated by membrane-bound receptor tyrosine kinases [3] whereas PI3-kinase γ is activated by heterotrimeric G-proteins [4]. Ins(1,4,5)P_3 can be phosphorylated to Ins(1,3,4,5)P_4 by Ins(1,4,5)P_3-3-kinase [5].

The physiological role of Ins(1,3,4,5)P_4 is still widely disputed [6]. Ptd-Ins(3,4,5)P_3 has been demonstrated to be an important regulator of membrane ruffling, membrane trafficking, protein sorting, cytoskeleton arrangement [7], cell growth and cell survival [8]. Several targets binding Ptd-Ins(3,4,5)P_3 have been identified such as Bruton's tyrosine kinase (Btk), T-lymphoma invasion and metastasis protein-1, β-adrenergic receptor kinase, and β-spectrin [9], cytohesin-1 [10], synaptotagmin [11], general receptor for phosphoinositides-1 (GRP-1) [10], and protein kinase B [12]. It was shown for protein kinase B-kinase [13], Btk [14], GRP-1-catalyzed guanosine nucleotide exchange on ADP-ribosylation factor [15], and PLC-γ [16] that their activity is regulated by Ptd-Ins(3,4,5)P_3. Most of the Ptd-Ins(3,4,5)P_3 dependent proteins have as a common feature one or two pleckstrin homology (PH) domains.

The p42^{IP4} protein, which we have identified previously, binds Ins(1,3,4,5)P_4 specifically. p42^{IP4} is expressed mainly in brain [17,18]; these proteins displaying two PH domains comprise the porcine p42^{IP4} [17], the rat p42^{IP4} [18], and the bovine Ptd-Ins(3,4,5)P_3-binding protein (PIP3BP) [19]. The homologous protein rat centaurin-α [20] differs from the p42^{IP4} proteins by disruption of the N-terminal PH domain and by a 45 amino acid extension at the C-terminus [18]. However, reverse

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Abbreviations: GST, glutathione-S-transferase; PI3-kinase, phosphoinositide-3-kinase; Ins, inositol; Ptd-Ins, phosphatidylinositol; PH, pleckstrin homology; PLC, phospholipase C; SH2 domain, src homology-2 domain; Btk, Bruton's tyrosine kinase; diC16, dipalmitoyl-L-α; diC8, dioctanly-L-α; GroPIns(3, 4,5)P_3, glycerophosphoinositol 3,4,5-trisphosphate; PIP3BP, Ptd-Ins(3,4,5)P_3-binding protein; Sf9, *Spodoptera frugiperda.*

Note: Theodor Hanck and Rolf Stricker contributed equally to this work.

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transcription/PCR analysis of rat brain detected cDNA of p42^{IP4} but not of centaurin-α [18].

The PH domain is a protein module of ≈100 amino acid residues, initially found in pleckstrin, the major substrate of protein kinase C in platelets [21]. This sequence motif is present in a large number of proteins that are supposed to be involved in signal transduction, cellular architecture and cell movement [22]. The ability of some PH domains to bind Ptd-Ins(4,5)P_2 or Ins(1,4,5)P_3 has been shown for several proteins [9,23,24]. Some of the PH domain-containing proteins that bind Ptd-Ins(3,4,5)P_3 also bind inositols, phosphorylated at position D3. For p42^{IP4} which binds Ins(1,3,4,5)P_4 with a K_d in the nM range [17], the role of the PH domain in determining ligand specificity is not yet clear; this is also the case for centaurin-α and PIP3BP [19,20]. The structure of the PH domain of Btk [9] which binds Ins(1,3,4,5)P_4 and Ptd-InsP_3, was resolved by X-ray crystallography [23]. It is noteworthy that there are several other proteins such as synoptotagmin, the Ptd-Ins transfer protein or the Ins(1,4,5)P_3 receptor that bind inositol polyphosphates and phosphatidyl inositols but do not have a PH domain. For PLC-γ [16] and p85 [25] it was shown that the SH2 domain is essential for activation by Ptd-Ins(3,4,5)P_3.

In the present study we show that the p42^{IP4} protein, purified from Sf9 cells infected with recombinant baculovirus is functional as it displays the same affinity to various inositol phosphate ligands as native p42^{IP4} purified from brain. Moreover, recombinant p42^{IP4} allowed us to determine that p42^{IP4} associates specifically with Ptd-Ins(3,4,5)P_3 in lipid vesicles.

MATERIALS AND METHODS

Construction of recombinant baculovirus

The cDNA of p42^{IP4} [17] was digested with *Mlu*NI and *Eco*RI, recessing ends were then filled with Klenow fragment of DNA polymerase I in the presence of 25 μM dNTPs. The resulting DNA fragment (1.2 kb) was prepared from low melting agarose and ligated to pAcG3X transfer vector [26] to produce the transfer vector pAcG3X-p42^{IP4}. The resulting plasmid was sequenced in both directions on an ABI310 automated sequencer with the cycle sequencing kit (Perkin Elmer) according to the manufacturer's instructions.

To construct recombinant baculovirus the following transfection mixture was prepared: 2 μg transfer vector-DNA with 2.5 μg BaculoGold-DNA (PharMingen), in a final volume of 16 μL, was mixed with 8 μL lipofectin (Gibco/BRL). The transfection mixture was applied to 3×10^6 Sf9 cells in 5 mL serum free IPL41-medium (Gibco/BRL). Recombinant baculovirus was amplified and tested for production of recombinant protein in Sf9 cells.

Production and purification of recombinant protein

Sf9 cells [4.5×10^7 in 20 mL IPL41-medium (Gibco/BRL) with 10% fetal calf serum] were seeded in tissue culture-flasks (175 cm^2; Greiner) and inoculated with recombinant baculovirus at a multiplicity of infection of 2–3 infectious units·cell^{-1}. After transfection with virus the Sf9 cells were incubated at 27 °C for 60–72 h. Cells were harvested by centrifugation at 1000 ***g*** for 10 min at room temperature. The cell pellet was resuspended in 3 mL ice-cold lysis buffer (1% Triton X-100, 50 mM Tris/HCl pH 7.5, 150 mM NaCl, 5 mM EDTA, 5 mM NaF, 1 μg·mL^{-1} aprotinin, 0.5 μg·mL^{-1} leupeptin, 2 mM phenylmethanesulfonyl fluoride, 10 mM benzamidine) and the cells were lysed by sonification (Branson Sonifier, microtip) or by passing for six times through a 23 gauge needle. After centrifugation (18 000 ***g***, 4 °C, 30 min) the cleared lysate was transferred to a fresh tube, 100 μL of glutathione beads (Pharmacia Biotech) were added and it was incubated for 1–2 h at 4 °C on a rotation wheel. The beads were sedimented and washed four times with ice-cold lysis buffer. The glutathione beads were resuspended in 3 mL of lysis buffer. For higher amounts of infected cells everything was scaled-up linearly. The protein solutions were stored at 4 °C.

The purification process for p42^{IP4} is summarized in Fig. 1A. From a cleared lysate from infected Sf9 cells (Fig. 1A, lane 3) the glutathione-S-transferase (GST)-p42^{IP4} fusion protein (M_r = 70 kDa) was affinity purified in a one-step purification procedure using glutathione beads (Fig. 1A, lane 4). The glutathione beads with immobilized fusion protein were washed three times with protease buffer (50 mM Tris/HCl pH 8.0, 100 mM NaCl, 1 mM $CaCl_2$, 0.5% lubrol-PX) without protease. The beads were resuspended in protease buffer to give a final concentration of the fusion protein of ≈50 μg·mL^{-1}. Then 1 U factor Xa protease (Pharmacia Biotech) per 0.1 mg fusion protein was added and the suspension was incubated for 16 h at room temperature. The fusion protein was cleaved with factor-Xa protease to more than 80%. The protein p42^{IP4} was released into the supernatant (Fig. 1A lane 5). To inactivate the protease, benzamidine was added to a final concentration of 10 mM.

The p42^{IP4} protein, cleaved off the GST tag was further purified on heparin agarose. 2 mL (bed volume) heparin agarose (BioRad) was equilibrated with protease buffer. The protein solution with p42^{IP4} was loaded on to the column at 0.5 mL·min^{-1}. The column was washed first with 15 bed volumes of protease buffer, then with 15 bed volumes C2B (20 mM cacodylate, 200 mM NaCl, 1 mM EDTA, pH 6.0) and with 15 bed volumes C4B (20 mM cacodylate, 400 mM NaCl, 1 mM EDTA, pH 6.0). The p42^{IP4} was eluted with five bed volumes C8B (20 mM cacodylate, 800 mM NaCl, 1 mM EDTA, pH 6.0) (Fig. 1A, lane 6). The protein solution of p42^{IP4} adjusted to a concentration of 100 μg·mL^{-1} was stable over several weeks at 4 °C.

Binding assays

Binding of Ins(1,3,4,5)P_4 to the recombinant protein was determined as described previously for the purified receptor protein from pig cerebellum [27]. In brief, purified recombinant protein (70–500 ng) was incubated for 20 min on ice in a total volume of 280 μL of 25 mM sodium acetate/25 mM potassium phosphate, pH 5.0, 0.05% Brij58, 1 mM EDTA supplemented with ≈1 nM [^{3}H]Ins(1,3,4,5)P_4 and varying concentrations of unlabelled D-Ins(1,3,4,5)P_4 or other inositol phosphates or phosphoinositides. Bound ligand was separated from free ligand by centrifugation through columns, containing 1.2 mL packed resin of BioGel P-4 equilibrated with binding buffer. Nonspecific binding was determined in the presence of 1 μM D-Ins(1,3,4,5)P_4. Bound radioactivity was measured by liquid scintillation counting. All determinations made in duplicate or triplicate showed less than 15% deviations.

For the analysis of the binding data, K_d values and the number of binding sites were estimated by using the RADLIG data-analysis computer program (Biosoft) as described previously [28]. In competition assays the data obtained with the displacing inositol phosphate were fitted to the data obtained for the displacement of [^{3}H]Ins(1,3,4,5)P_4 by D-Ins(1,3,4,5)P_4 within the same set of experiments, i.e. K_d, B_{max} and nonspecific binding were fixed to the values obtained from the homologous displacement curve.

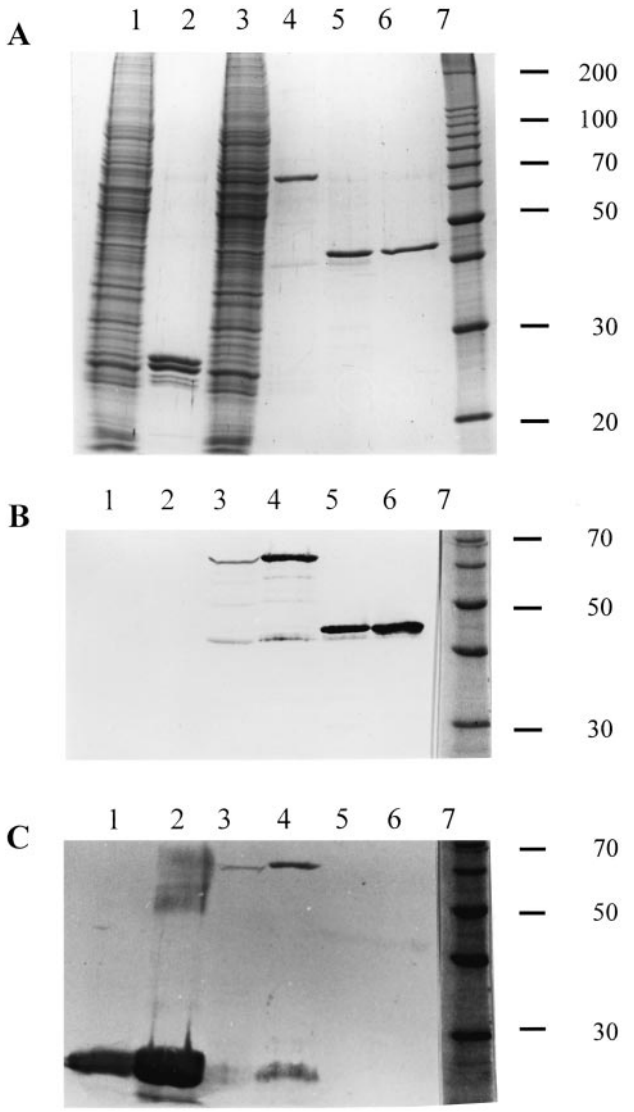

Fig. 1. Purification of p42^{IP4} protein expressed in Sf9 cells infected with recombinant baculoviruses. (A) Proteins stained with Coomassie R250. Lane 1, cleared lysate of Sf9 cells infected with recombinant baculovirus for expression of GST; lane 2, affinity purified GST; lane 3, cleared lysate of Sf9 cells infected with recombinant baculovirus for expression of GST-p42^{IP4}; lane 4, affinity purified GST-p42^{IP4}; lane 5, purified GST-p42^{IP4} after cleavage with protease factor Xa; lane 6, recombinant p42^{IP4} after purification on heparin agarose; lane 7, 10 kDa protein ladder. The samples were analysed on a 12.5% polyacrylamide gel. (B) Western blot with anti-p42^{IP4} antibody testing the same samples as in (A). The samples were analysed on a 12.5% polyacrylamide gel, transferred onto a poly(vinylidene difluoride) membrane and probed with rabbit anti-p42^{IP4} antibodies as primary antibody and goat anti-rabbit horse radish peroxidase-conjugated secondary antibody. (C) Western blot with anti-GST antibody testing the same samples as in (A). The experiment was carried out identically to that in (B) except that the transferred proteins were probed with rabbit anti-GST primary antibody. Similar results were seen in at least four experiments. The numbers besides the blots give the $M_r \times 10^{-3}$ of the molecular mass marker proteins.

Protein–lipid vesicle binding assay

Lipid vesicles were prepared according to the method of Bromann *et al*. [29]: 290 nmol phosphatidylethanolamine and 290 nmol phosphatidylcholine were mixed with different amounts of dipalmitoyl-L-α (diC16)Ptd-Ins(3,4,5)P_3 (0–10 nmol) and sonicated. The lipid mixtures were lyophilized and the dried thin lipid film was stored at −20 °C. Before use 1 mL binding buffer (50 mM Tris/HCl, pH 7.5, 100 mM KCl, 20 mM NaCl, 1 mM EDTA, 1 mM 2-mercaptoethanol) was added and the mixture was sonicated. Aliquots of 190 μL were centrifuged (40 000 ***g***, 10 min, 4 °C) and the resulting pellets were used for the protein-lipid vesicle binding assay.

Recombinant p42^{IP4} (0.3–0.5 μg of protein per assay) was incubated in a total volume of 250 μL binding buffer supplemented with 200 μg·mL^{-1} BSA and, as indicated in the figures, different inositol phosphates or water soluble dioctanly-L-α (diC8)Ptd-Ins phosphates (usually 10 μM final concentration) for 60 min on ice. The samples were then centrifuged (40 000 ***g***, 40 min, 4 °C), the pellets were rinsed carefully with binding buffer and then solubilized with 50 μL 2% SDS. Solubilized protein was precipitated by methanol/acetone precipitation and boiled in Laemmli sample buffer.

Likewise, association of cytosolic p42^{IP4} was assayed as described above. The cytosol was prepared from pig cerebellum as described previously by Stricker *et al*. [17]. In the test ≈1 mg of cytosolic protein from pig cerebellum was used, but BSA was omitted from the assay.

In some experiments the relative amount of p42^{IP4} bound to the vesicles was quantified. Blots were digitalized using a flatbed desktop scanner (ScanJet 6100C/T; Hewlett Packard) and the pixel densities were integrated by using the EAGLESIGHT 3.0 Image capture and analysis software (Stratagene). The p42^{IP4} signals were normalized by dividing the pixel densities of each individual band by the density obtained for the amount of p42^{IP4} used in the same experiment (control lane).

SDS/PAGE and Western blotting

The proteins were analysed on 12.5% polyacrylamide gels according to Laemmli [30], blotted on to a poly(vinylidene difluoride)-membrane and probed with rabbit anti-p42^{IP4}-antibodies (19-amino-acid peptide comprising amino acid 353–371 of p42^{IP4} as described [17,31]), or rabbit anti-GST (sc-459; Santa Cruz Biotechnology) as primary antibody and goat anti-rabbit horse radish peroxidase-conjugate (Dianova) as secondary antibody. The blots were stained with 4-chloro-1-naphthol (0.5 mg·mL^{-1}) and 0.01% H_2O_2. Alternatively proteins were stained with Coomassie R250.

Inositol phosphates and lipids

L-α-Phosphatidylethanolamine and L-α-phosphatidylcholine, both synthetic dipalmitoyl derivatives, were from Sigma, D-Ins(1,3,4,5)P_4 was from Boehringer, D-Ins(1,4,5)P_3 was from Biomol and D-[^{3}H]Ins(1,3,4,5)P_4 (777 GBq·mmol^{-1}) was from NEN. Regioisomers of InsP_4, InsP_3 and InsP_5 were synthesized by Chung *et al.* (references in [27]).

Glycerophosphoinositol 3,4,5-trisphosphate [GroPIns(3,4,5)P_3], diC8Ptd-Ins(3,4)P_2, diC8Ptd-Ins(3,4,5)P_3, diC16Ptd-Ins(3,4,5)P_3 were synthesized as described [32,33].

RESULTS

Previously we cloned the cDNA of p42^{IP4} a 42-kDa receptor protein for Ins(1,3,4,5)P_4 from pig cerebellum [17] and from rat brain [18]. Porcine and rat p42^{IP4} have 374 amino acid residues. PIP3BP reported by Tanaka *et al.* [19] is the corresponding bovine homologue with one amino-acid deletion. Sequence homology analysis reveals that p42^{IP4} has a zinc finger domain at its N-terminus followed by two PH-domains.

Attempts to express p42^{IP4} in *Escherichia coli* yielded protein that could not be solubilized and purified in a functionally active form. To overcome this problem we cloned the cDNA for p42^{IP4} into the transfer vector pAcG3X [26] to generate recombinant baculovirus to express p42^{IP4} in Sf9 insect cells. The baculovirus/Sf9 system is a powerful tool for the expression of functionally active proteins from eukaryotic origin. In eukaryotic cells the folding of the nascent protein chain is more complex than in bacteria and proteins can be modified post-translationally [34]. Recombinant baculovirus, generated with the pAcG3X transfer vectors, express proteins as an N-terminal fusion protein with GST. By this method p42^{IP4} purified to homogeneity was obtained (Fig. 1). The amount of homogeneous protein retrieved was 5 mg·L^{-1} cell culture or 5 mg per 2×10^9 Sf9 cells. We were able to concentrate the protein to at least 5 mg·mL^{-1}, but the concentrated protein showed a tendency to aggregate irreversibly.

The recombinant GST–p42^{IP4} fusion protein, purified from Sf9 cells and the cleavage product were detectable by a rabbit polyclonal anti-peptide antiserum directed against the native p42^{IP4} protein (Fig. 1B). Recognition by this antiserum confirms unequivocally the identity of the recombinant protein as we have demonstrated previously that the antiserum stains p42^{IP4} specifically in Western blots [31] and also in immunohistochemistry of brain sections [35]. The recombinant GST–p42^{IP4} fusion protein was also detectable by a rabbit polyclonal anti-GST antiserum (Fig. 1C). Lysates from nontransfected Sf9 cells (data not shown) or Sf9 cells transfected with baculovirus-expressing recombinant GST protein, contained no detectable amount of p42^{IP4} (Fig. 1B).

The p42^{IP4} protein purified from brain tissue binds Ins(1,3,4,5)P_4 and its analogue GroPIns(3,4,5)P_3 with similar affinity [17]. It was important to demonstrate that the recombinant p42^{IP4} had retained the characteristics of this binding site. Using recombinant p42^{IP4} we corroborated the affinity and selectivity profile of the InsP_4 binding site. Secondly, we used this protein for functional studies demonstrating the ability of the protein to interact specifically with Ptd-Ins(3,4,5)P_3 in membranes.

To elucidate the possible functional role of p42^{IP4} we investigated whether the recombinant p42^{IP4} was able to bind to lipid vesicles supplemented with diC16Ptd-Ins(3,4,5)P_3. Vesicles were prepared with equal amounts of diC16-phosphatidylcholine and diC16ethanolamine (290 nmol·mL^{-1} each) and varying concentrations of diC16Ptd-Ins(3,4,5)P_3 (0–10 nmol·mL^{-1}) according to methods already used successfully by Bromann *et al*. [29] and Fukuda and Mikoshiba [36]. The final concentrations of the lipids in the incubations were 220 μM for both phosphatidylethanolamine and phosphatidylcholine and 0–7.6 μM for diC16Ptd-Ins(3,4,5)P_3. It should be noted that this calculation gives the theoretical upper limit assuming that diC16Ptd-Ins(3,4,5)P_3 was completely inserted in the vesicles and that all of this lipid was accessible to the binding protein. Therefore the true concentration of diC16Ptd-Ins(3,4,5)P_3 in the binding assay was certainly much lower than that indicated in Fig. 2.

Figure 2A shows the results from an experiment in which different concentrations of diC16Ptd-Ins(3,4,5)P_3 were used to prepare the vesicles. The recombinant p42^{IP4} protein (0.5 μg of protein, lane 1) was incubated with the vesicles for 1 h on ice. Bound protein was then separated from free protein by centrifugation after which the pellets were washed, solubilized and then analysed by SDS/PAGE and Western blot to determine the presence of p42^{IP4}. No p42^{IP4} was detected when vesicles were omitted (lane 2) or when vesicles were prepared without diC16Ptd-Ins(3,4,5)P_3 (lane 3). Increasing concentrations of diC16Ptd-Ins(3,4,5)P_3 in the vesicles (lane 4–7) showed that p42^{IP4} can be detected associated with vesicles with at least 76 nM diC16Ptd-Ins(3,4,5)P_3 (lanes 5–7). In lane 8 the incubation was carried out using vesicles containing 7.6 μM diC16Ptd-Ins(3,4,5)P_3 as in lane 7 but with the addition of 10 μM D-Ins(1,3,4,5)P_4. To quantitate the amount of p42^{IP4} bound to the lipid vesicles prepared with different concentrations of Ptd-Ins(3,4,5)P_3, blots comparable to those shown in Fig. 2A were digitalized and the pixel densities of the p42^{IP4} protein bands were integrated; Fig. 2B shows the mean ratios of the p42^{IP4} signal and the pixel densities of the reference 42-kDa band obtained within the respective assay. The maximally obtained ratio shown in lane 7 has to be regarded with some caution due to saturation of reference signal in lane 1 (Fig. 2A). In summary, the data in Fig. 2A and B demonstrate that recombinant p42^{IP4} can bind to lipid vesicles containing diC16Ptd-Ins(3,4,5)P_3 in a Ptd-InsP_3 concentration-dependent manner and that this binding is reduced in the presence of Ins(1,3,4,5)P_4.

In further experiments we tested whether other inositol phosphates and water soluble diC8Ptd-Ins phosphates could also inhibit the association of p42^{IP4} with such vesicles. Figure 2C shows the results of one such experiment: recombinant protein binds to lipid vesicles prepared in the presence of 10 μM diC16Ptd-Ins(3,4,5)P_3 (lane 4) but not to vesicles prepared in the absence of this compound (lane 3) and binding is reduced by coincubation with 10 μM D-Ins(1,3,4,5)P_4 (lane 5) by about 80%, as also shown in Fig. 2B. Among the other compounds tested (all at 10 μM), diC8Ptd-Ins(3,4,5)P_3 (lane 8) showed a significant inhibitory effect whereas D-Ins(1,4,5)P_3 (lane 6) and diC8Ptd-Ins(3,4)P_2 (lane 9) did not affect the association of the protein with the vesicles. These results are consistent with the potencies of these compounds found in the displacement experiments described below (Table 1). Ins(1,3,4,5,6)P_5 inhibited the binding of the protein to the Ptd-Ins(3,4,5)P_3-containing vesicles only weakly (lane 7).

Fig. 2D shows data from an experiment in which cytosol from pig cerebellum was incubated with vesicles without (lane 3) or supplemented with diC16Ptd-Ins(3,4,5)P_3 (lanes 4 and 5). Thus, not only recombinant p42^{IP4} but also the native p42^{IP4} protein from cerebellar cytosol could bind to diC16Ptd-Ins(3,4,5)P_3-containing vesicles (Fig. 2D, lane 4) and binding was inhibited by 10 μM Ins(1,3,4,5)P_4 (lane 5). p42^{IP4} from brain exists in a soluble cytosolic and in a membrane associated form [17]. Preliminary results from experiments where we tested whether p42^{IP4} protein can be released from the cerebellar membranes by incubation in the presence of D-Ins(1,3,4,5)P_4 demonstrate that the protein was released (data not shown).

Properties of the recombinant protein were compared to the data obtained in earlier studies in which protein purified from pig cerebellar membranes [27] or from rat brain was used [18]. The displacement of [^{3}H]Ins(1,3,4,5)P_4 by different inositol-trisphosphate, tetrakisphosphate and pentakisphosphate regioisomers was measured. Binding assays were carried out using protein samples similar to that shown in Fig. 1A (lane 6). Binding of [^{3}H]Ins(1,3,4,5)P_4 to the recombinant protein was specific for D-Ins(1,3,4,5)P_4. The K_d of the recombinant protein for D-Ins(1,3,4,5)P_4 was 4.1 nM. The B_{max} of 9.1 nmol·mg^{-1} of protein (Table 1 and Fig. 3) represents the lower limit according to the estimate of the amount of protein derived from the intensity of the gel band. This B_{max} value is comparable to data obtained in our earlier studies with the p42^{IP4} protein purified from pig cerebellum [27,37]. The racemic mixture D/L-Ins(1,3,4,5)P_4 inhibited binding of [^{3}H]Ins(1,3,4,5)P_4 with a

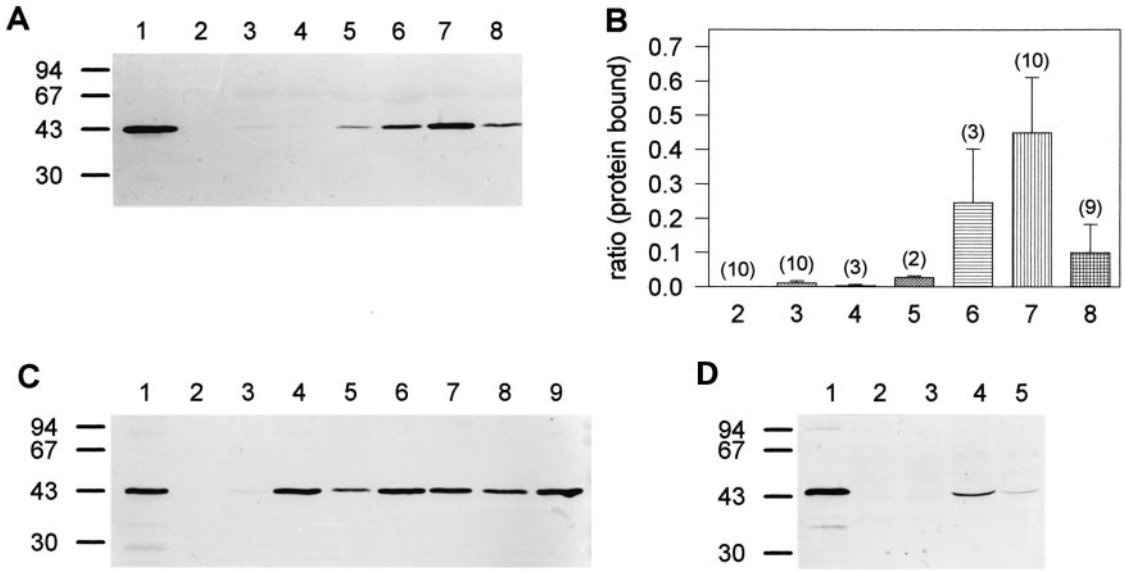

Fig. 2. Specific binding of p42^{IP4} to lipid vesicles containing Ptd-Ins(3,4,5)P_3. (A) p42^{IP4} binds in a concentration-dependent manner to Ptd-Ins(3,4,5)P_3-containing lipid vesicles. Purified recombinant p42^{IP4} (0.5 μg, lane 1) was incubated with either buffer as a control (lane 2) or vesicles of phosphatidylcholine/phosphatidylethanolamine prepared in the presence of 0 (lane 3), 10 nM (lane 4), 100 nM (lane 5), 1 μM (lane 6) or 10 μM (lane 7 and 8) diC16Ptd-Ins(3,4,5)P_3. In lane 8 the binding incubation was performed in the additional presence of 10 μM Ins(1,3,4,5)P_4. The Western blot of the gel is shown. (B) Blots from different experiments were digitalized and the pixel densities of the signals of the bands were determined. The ratio of the amount of p42^{IP4} protein bound to lipids was calculated by referring to a reference band, as in lane 1 (A), as 1. Shown are the mean ratios of p42^{IP4} protein bound; bars represent the standard deviations and the number of experiments is given in parentheses; the numbers below the bars are the numbers of the corresponding lanes shown in the blot in (A). (C) Influence of different inositol phosphates and phosphoinositides on the association of p42^{IP4} with Ptd-Ins(3,4,5)P_3-containing lipid vesicles. Protein–lipid vesicle binding assays were performed with recombinant p42^{IP4} (0.5 μg per assay, as exemplified in lane 1) in buffer alone (control in lane 2), or with vesicles prepared without (lane 3) or with of 10 μM diC16Ptd-Ins(3,4,5)P_3 (lanes 4–9). In lanes 4–9 different inositol phosphates or phosphoinositides (final concentration 10 μM) were present in the binding assay, as follows: lane 4, buffer; lane 5, D-Ins(1,3,4,5)P_4; lane 6, D-Ins(1,4,5)P_3; lane 7, Ins(1,3,4,5,6)P_5; lane 8, diC8Ptd-Ins(3,4,5)P_3, lane 9, diC8Ptd-Ins(3,4)P_2. (D) p42^{IP4} from pig cerebellum binds to Ptd-Ins(3,4,5)P_3-containing lipid vesicles. Cytosolic protein from pig cerebellum (0.94 mg of protein) was incubated with buffer (control, lane 2), or vesicles prepared without (lane 3) or with 10 μM diC16Ptd-Ins(3,4,5)P_3 (lanes 4 and 5). The incubation shown in lane 5 was done in the presence of 10 μM D-Ins(1,3,4,5)P_4. In lane 1, 0.3 μg recombinant p42^{IP4} was applied to the gel as a control. The numbers besides the blots give the $M_r \times 10^{-3}$ of the molecular mass marker proteins. In addition to those blots used for numerical analysis (B) the blots shown are representative of at least four experiments yielding comparable results using two different preparations of recombinant protein (A, B, C) or cytosol (D).

K_i value of 15.6 nM (Table 1) indicating that the L-isomer of this compound binds only weakly to the binding site. The two other inositoltetrakisphosphate regioisomers D/L-Ins(3,4,5,6)P_4 and Ins(1,3,5,6)P_4 were 15 and 80-fold less potent in displacing [^{3}H]Ins(1,3,4,5)P_4 than D-Ins(1,3,4,5)P_4 (Table 1). The inhibitory constant (K_i) for D/L-Ins(3,4,5,6)P_4 was virtually identical to the value found with the protein purified from pig cerebellum [27] whereas the affinity obtained with Ins(1,3,5,6)P_4 was approximately five times lower.

For both inositoltrisphosphate compounds, D-Ins(1,4,5)P_3 and D/L-Ins(1,5,6)P_3, the affinities found with the recombinant protein were in close agreement with the data described for the protein purified from cerebellum. The physiologically important metabolite D-Ins(1,4,5)P_3 inhibits Ins(1,3,4,5)P_4 binding with a K_i value $\geq$ 10 μM thus clearly distinguishing p42^{IP4} from Ins(1,4,5)P_3 binding proteins. D/L-Ins(1,5,6)P_3 displaced [^{3}H]Ins(1,3,4,5)P_4 with a more than 10-fold higher affinity than all of the other InsP_3 regioisomers tested from the recombinant p42^{IP4} (Table 1) and from the receptor protein purified from porcine cerebellum [27]. In our previous studies we found that the affinity of the protein for Ins(1,3,4,5,6)P_5 in membranes was more than 10 times lower than that for D-Ins(1,3,4,5)P_4. However, it was increased 10-fold after detergent solubilization of the receptor protein from the membrane fraction [37]. The same high affinity of p42^{IP4} for Ins(1,3,4,5,6)P_5 was detected with the recombinant protein assayed in detergent-containing buffer (Table 1).

It is obvious from Table 1 that the potency of unlabelled D-Ins(1,3,4,5)P_4 to displace [^{3}H]Ins(1,3,4,5)P_4 is equal to that of GroPIns(3,4,5)P_3. The inositol phosphate head group of the latter compound is part of the structure of D-Ins(1,3,4,5)P_4 and Ptd-Ins(3,4,5)P_3, suggesting that Ptd-Ins(3,4,5)P_3 is a physiological ligand for the InsP_4 receptor protein p42^{IP4}. This assumption was verified by our finding of specific interaction (binding) of recombinant p42^{IP4} with Ptd-Ins(3,4,5)P_3-containing lipid membranes (see above). Furthermore, we investigated the potencies of the water soluble phosphatidylinositols diC8Ptd-Ins(3,4)P_2, diC8Ptd-Ins(4,5)P_2, and diC8Ptd-Ins (3,4,5)P_3 (which posses short octanoyl fatty acid side chains) and diC16Ptd-Ins(3,4,5)P_3 (which contains two palmitoyl side chains) to compete with [^{3}H]Ins(1,3,4,5)P_4 for binding to the recombinant p42^{IP4}. DiC8Ptd-Ins(3,4,5)P_3 was equipotent with D-Ins(1,3,4,5)P_4 in competing for the binding site whereas the other two dioctanoyl compounds were approximately three orders of magnitude less effective (Table 1 and Fig. 3). Potencies similar to those described here were found for diC8Ptd-Ins(3,4)P_2 and diC8Ptd-Ins(3,4,5)P_3 either with membranes from pig cerebellum or with the purified protein, whereas the affinity for diC8Ptd-Ins(3,4,5)P_3 was slightly lower with the purified protein (K_i of 20 nM). When we tested diC16Ptd-

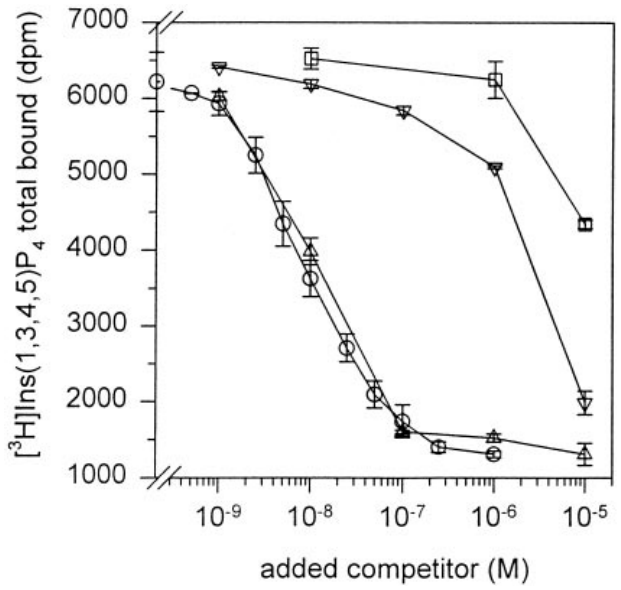

Fig. 3. Displacement of D-[^{3}H]Ins(1,3,4,5)P_4 binding to the purified recombinant p42^{IP4} protein. Binding assays were performed with 63 ng recombinant p42^{IP4} and 0.95 nM D-[^{3}H]Ins(1,3,4,5)P_4: D-Ins(1,3,4,5)P_4 (○); the water soluble phosphatidylinositol phosphates diC8Ptd-Ins(3,4,5)P_3 (△), diC8Ptd-Ins(3,4)P_2 (▽) and diC8Ptd-Ins(4,5)P_2 (□). The number of independent experiments was at least three (in some cases up to eight; see Table 1).

Ins(3,4,5)P_3 with the recombinant protein we detected that this compound was also a potent displacer of [^{3}H]Ins(1,3,4,5)P_4. In contrast with the lipids with short side chains the displacement curves obtained with diC16Ptd-Ins(3,4,5)P_3 showed a slope factor of ≈3 in all cases (Table 1). Therefore the IC_{50} value (120 nM) for this compound is given. Interestingly with membranes we found a slope factor of ≈1 and K_i of 300–500 nM for this compound.

Table 1. Specificity of ligand binding of recombinant p42^{IP4}. Displacement of D-[^{3}H]Ins(1,3,4,5)P_4 binding to purified recombinant p42^{IP4} protein by different inositol phosphates and phosphatidylinositol phosphates. The average B_{max} for three different p42^{IP4} batches used in the experiments was 9.1 ± 4.2 nmol·mg^{-1} of protein (n = 8). Nonspecific binding was determined in the presence of 1 μM D-Ins(1,3,4,5)P_4. In each experiment the K_i value was obtained from a displacement curve testing the compound at four to eight different concentrations. The mean values for K_i shown were derived from the number of experiments indicated in parentheses. With diC16Ptd-Ins(3,4,5)P_3 the displacement curves showed a slope significantly larger than 1 in all cases and so IC_{50} and slope are given in this case.

Displacer	K_i ± SD (n)
D-Ins(1,3,4,5)P_4 = L-Ins(1,3,5,6)P_4	4.10 ± 1.44 nM (8)
D/L-Ins(1,3,4,5)P_4 = L/D-Ins(1,3,5,6)P_4	15.6 ± 8.5 nM (3)
D/L-Ins(3,4,5,6)P_4 = L/D-Ins(1,4,5,6)P_4	74.6 ± 25.7 nM (3)
Ins(1,3,4,6)P_4	347 ± 120 nM (3)
D-Ins(1,4,5)P_3 = L-Ins(3,5,6)P_3	> 10 μM (3)
D/L-Ins(1,5,6)P_3 = L/D-Ins(3,4,5)P_3	262 nM (1)
Ins(1,3,4,5,6)P_5	3.2 ± 1.7 nM (3)
Glycero-PhosphoIns(3,4,5)P_3	6.0 ± 2.0 nM (7)
DiC8-Ptd-Ins(3,4,5)P_3	3.5 ± 1.5 nM (5)
DiC8-Ptd-Ins(3,4)P_2	1.5 ± 0.4 μM (4)
DiC8-Ptd-Ins(4,5)P_2	> 10 μM (2)
DiC16-Ptd-Ins(3,4,5)P_3: IC_{50}/slope	121 ± 69 nM/2.9 ± 0.9 (6)

DISCUSSION

Recombinant p42^{IP4} recognizes Ptd-Ins(3,4,5)P_3 in membranes

We were able to express the p42^{IP4} protein in Sf9 cells using a recombinant baculovirus. The protein expressed in Sf9 cells could be purified to homogeneity (Fig. 1A), with an amount of purified recombinant p42^{IP4} of 5 mg·L^{-1} cell culture. The ligand affinity for Ins(1,3,4,5)P_4 with a K_i of 4.1 nM of the recombinant p42^{IP4} was similar to that of the native p42^{IP4} protein (K_i of 2.2 nM) purified from pig cerebellum. Earlier experiments [17] gave K_i values for Ins(1,3,4,5)P_4 of 1.65 nM for the membrane fraction and 3.82 nM for the cytosolic fraction. Thus the K_i value of the recombinant protein which is nearly identical to that for the cytosolic fraction of the native p42^{IP4} protein shows that recombinant p42^{IP4} is functional.

The endogenous p42^{IP4} protein in brain can be found either in the cytosolic fraction or associated with the membrane [17]. Fig. 2 shows clearly that the recombinant p42^{IP4} protein binds specifically to Ptd-Ins(3,4,5)P_3 in lipid membranes in a concentration-dependent manner and can be inhibited by Ins(1,3,4,5)P_4. This association supports the idea that the p42^{IP4} protein is recruited to the cell membrane or the membrane of vesicles upon agonist stimulation of cells with subsequent production of Ptd-Ins(3,4,5)P_3. Shin *et al*. [38] described a high-level expression for PI3-kinases in brain. This is in line with our findings that the p42^{IP4} protein is highly expressed in brain [35].

Ligand specificity of recombinant p42^{IP4}

The heterologously expressed protein allowed further characterization of the ligand specificity of the p42^{IP4} protein. Different trisphosphate, tetrakisphosphate and pentakisphosphate regioisomers of *myo*-inositol or the water-soluble phosphatidylinositol analogues GroPIns(3,4,5)P_3, diC8Ptd-Ins(3,4)P_2, diC8Ptd-Ins(4,5)P_2, and diC8Ptd-Ins(3,4,5)P_3 and the lipid diC16Ptd-Ins(3,4,5)P_3 were tested. The ligand affinities for Ins(1,3,4,5)P_4 compared to the water-soluble analogues of Ptd-Ins(3,4,5)P_3 show K_i values in the same range, 6.0 nM for GroPIns(3,4,5)P_3, 3.5 nM for DiC8-Ptd-Ins(3,4,5)P_3. These findings are consistent with our previous results using native p42^{IP4} protein [17,30,31]. For rat centaurin-α [20] or the bovine PIP3BP [19], which are highly homologous to the pig p42^{IP4} protein, the ligand affinity for Ins(1,3,4,5)P_4 was reported to be lower than for Ptd-Ins(3,4,5)P_3. Those experiments, however, were not performed using an equilibrium binding assay. Due to the high homology between the bovine PIP3BP, the rat p42^{IP4} [18] and the porcine p42^{IP4} protein, there should be no difference in ligand affinities between the three proteins using the same experimental approach. So far, for other Ptd-Ins(3,4,5)P_3-binding proteins there are very few data available concerning the affinity to Ins(1,3,4,5)P_4.

For high-affinity ligand binding a phosphate group at position D3, D4 and D5 of the inositol ring is necessary [27]. Structural data for the PH domain of Btk [23], which binds specifically the D3 phosphorylated inositols, suggest that positively charged areas of the PH domain are the binding sites for Ins(1,3,4,5)P_4 as found for the binding of Ins(1,4,5)P_3 to the PH domain of PLCδ$_1$ or β-spectrin. For the PH domain of Btk the residues important for the discrimination of Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 were not yet identified. Hyvönen *et al*. [23] propose that residues outside the PH domain might contribute to ligand binding.

p42^{IP4} has two PH domains and possibly both PH domains contribute to ligand binding [19]. This could mean that p42^{IP4} binds the ligand in a horse-shoe-like manner, as proposed for the structure of p110γ [39].

Possible functional role of p42^{IP4}

Whether Ptd-Ins(3,4,5)P_3 *in vivo* is sufficient for recruitment of the p42^{IP4} protein to the membrane is still unclear. It is possible that the additional interaction with other proteins is necessary for this step. The zinc finger motif at the N-terminus of the p42^{IP4} protein might also be involved in this interaction. It still has to be investigated whether the p42^{IP4} protein interacts with other proteins involved in cellular signal transduction pathways, vesicle transport, membrane trafficking or effects regulated by Ptd-Ins(3,4,5)P_3.

The recruitment of p42^{IP4} protein to the membrane and its possible translocation to the cytosol upon rising Ins(1,3,4,5)P_4 levels fit a model of a regulation circle involving inositol polyphosphates: stimulation of cells by different agonists either results in increasing levels of Ins(1,4,5)P_3 after cleavage of Ptd-Ins(4,5)P_2 or the further phosphorylation of Ptd-Ins(4,5)P_2 to Ptd-Ins(3,4,5)P_3 by PI3-kinases; Ins(1,4,5)P_3 can be phosphorylated to Ins(1,3,4,5)P_4 by Ins(1,4,5)P_3-3-kinase. The rise in Ins(1,3,4,5)P_4 concentration can switch off reactions turned on by Ins(1,4,5)P_3 and/or has different, more long-lasting effects on cells. For *Xenopus laevis* oocytes it was shown that Ins(1,4,5)P_3 is preferentially phosphorylated to Ins(1,3,4,5)P_4, which is more stable and results in sustained effects [40].

The p42^{IP4} bound to Ptd-Ins(3,4,5)P_3 could protect Ptd-Ins(3,4,5)P_3 from dephosphorylation to Ptd-Ins(3,4)P_2 by SHIP (Src homology 2 domain containing inositol phosphatase) [41]. Ptd-Ins(3,4)P_2 can activate the Akt proto-oncogene product [42]. A recent publication from Eves *et al*. [43] has shown results indicating that Akt can transduce a survival signal for differentiating neuronal cells. On the other hand it was found, that Ins(1,3,4,5)P_4 is a mediator of neuronal cell death in ischaemic hippocampus [44]. Thus, p42^{IP4}, which can recognize either Ptd-Ins(3,4,5)P_3 or Ins(1,3,4,5)P_4 might be acting downstream of PI3-kinase regulated pathways such as cell survival. The results described here for p42^{IP4} link this receptor protein to important aspects of signal transduction pathways in normal and pathological situations.

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